SUMOylation Exerts Minimal Effects on the Splicing Regulatory Activity of the Influenza A Virus Non-Structural Protein NS1

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SUMOYLATION EXERTS MINIMAL EFFECTS ON THE SPLICING REGULATORY ACTIVITY OF THE INFLUENZA A VIRUS NON-STRUCTURAL PROTEIN NS1

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by

GRISELDA MELENDEZ, B.S.

THESIS

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Abstract

One of the greatest challenges facing the development of a universal treatment for influenza infection is the virus’s ability to mutate rapidly and produce new antigenic properties. Targeting the viral components of one strain does not ensure that the treatment will be effective with a newly developed strain. One alternative would be to develop therapies that target cellular factors the virus depends on for survivability. One of these factors is the cellular SUMOylation system. We have previously demonstrated that the Small Ubiquitin-like MOdifier (SUMO) interacts with the influenza virus by SUMOylating several viral proteins and by affecting the interferon blocking activity of its non-structural protein NS1. These interactions open the door to the potential development of a treatment that would not be constrained by virus specificity. Here, we further elucidate the ways in which the influenza virus is being affected by SUMOylation. Through confocal microscopy experiments where components of the cellular SUMOylation system were over-expressed through transfection, we were able to establish that with the over-expression of SUMO and the conjugating enzyme, Ubc9, cells were less likely to progress into the late stages of infection, characterized by the presence of the late viral M1 protein therefore suggesting a potential anti-influenza role for the cellular SUMOylation system. Furthermore, through RNP reconstitution assays and primer extension analyses, we discovered that the SUMOylation of NS1 plays a minor role in NS1’s ability to regulate the splicing of viral gene transcripts. Taken together, these studies provide a deeper insight into the interplay between the influenza virus and the cellular SUMOylation system.
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Chapter 1: Introduction

As an acute viral infection, influenza has been a long-running threat to public health (Ison and Lee; Taubenberger and Morens). Annual epidemics around the world will infect about 10% of the population causing anywhere from 250,000 to 500,000 deaths (WHO). Occasionally, the emergence of new viruses will lead to pandemics which will have a much higher mortality rate than that of seasonal influenza (McCaughey). Within the last century, several severe pandemics have occurred including the Spanish Influenza pandemic of 1918 that killed an estimated 50 million people worldwide, the Asian Flu pandemic of 1957 which had a death toll of roughly 1 million, and the Hong Kong flu of 1968 which was responsible for approximately 700,000 deaths (Rajagopal and Treanor 2007).

Currently, there are two predominant ways to treat influenza; through vaccination or antivirals. However, both approaches have their limitations. Present vaccines are able to produce a strong antibody response to surface viral glycoproteins, haemagglutinin and neuraminidase; however this protection relies on type, sub-type and strain specificity (Couch 2003; Hampson 2008). Antivirals, such as Zanamivir and Oseltamivir (Tamiflu), are both neuraminidase inhibitors, yet a single amino acid change in the active site of neuraminidase can render the virus resistant to the drug (von Itzstein 2007). Other antivirals include Rimantadine and Amantadine, both M2-ion channel protein inhibitors. While both were proven to be effective, they were known to cause substantial side effects in the central nervous system and many current transmitted strains are resistant to these drugs (von Itzstein 2007).

It is evident that influenza poses a serious risk to the population and that our current countermeasures are less than ideal. Therefore, it would be advantageous to develop a universal treatment for influenza that utilizes the host machinery to eliminate resistance due to the ever
changing virus. However, we first need to dissect the interplay between the virus and host cell mechanisms. One such mechanism is the cellular SUMOylation system. In this study, we aimed to determine how modulating the SUMOylation system affects the ability of the virus to infect new cells and how increasing the SUMOylation of a viral protein, NS1, affects its reported ability to regulate splicing during infection.

1.1 The Influenza A virus

Influenza A viruses are enveloped viruses that belong to the family Orthomyxoviridae (Cheung and Poon 2007; Samji 2009). They contain a single-stranded, segmented, negative sense RNA genome which consists of eight segments that encode for 10 to 11 viral proteins (Samji 2009). All eight viral RNA segments are found inside the virion bound to the nucleoprotein (NP) and the viral polymerase complex, made up of PB1, PB2 and PA, to form ribonucleoprotein (RNP) complexes (Cheung and Poon 2007).

The viral life cycle can be divided into several stages: entry into the cell, entry of vRNPs to the nucleus, transcription and replication of the genome, export of the vRNPs and assembly and budding at the plasma membrane (Samji 2009). During entry, haemagglutinin binds to sialic acid receptors on the host’s cell membrane (Skehel and Wiley 2000). Once bound, the virus enters the cell in an endosome through receptor mediated endocytosis (Samji 2009). Acidification of the viral particle caused by the opening of the M2 ion channel causes the fusion of the viral membrane with the endosomal membrane (Holsinger, Nichani et al. 1994). This fusion consequently causes the release of the vRNPs away from the viral matrix protein, M1, into the host cell’s cytoplasm (Samji 2009). Once released, the vRNPs enter the nucleus by utilizing a nuclear localization signal that is present in NP, PA and PB2 (Fodor and Smith 2004). Replication of the viral genome results in the generation of full length vRNA through a cRNA
intermediate. The vRNA segments are used as templates for transcription and further replication. Some vRNA transcripts are exported out into the cytoplasm as RNPs where they will be used for the formation of new viral particles (Nagata, Kawaguchi et al. 2008).

In transcription, mRNA is generated by directly copying the vRNA template; however, this requires the use of a primer. PB2 protein employs a “cap-snatching” mechanism were it is able to cleave cellular mRNA caps and use the stolen cap to prime viral transcription (Li, Rao et al. 2001). To achieve polyadenylation, the viral RNA dependent RNA polymerase, RdRp, will stutter over a stretch of U residues at the 5’ end of the template (Poon, Pritlove et al. 1999). Negative sense vRNAs are assembled into vRNPs and are exported from the nucleus where they will travel to the site of assembly and budding at the host’s cell plasma membrane (Samji 2009). Before viral particles are released, Neuraminidase, NA, cleaves sialic acid residues from glycoproteins (Palese, Tobita et al. 1974). Once released, the virus will go on to infect another cell.
Figure 1. Influenza virus life cycle. 1) Binding of HA to sialic acid receptors and entry via endocytosis. 2) Viral membrane fuses to endosomal membrane and releases vRNPs. 3) Replication and transcription of genome within nucleus. 4) Export of viral mRNAs and translation. 5) Trafficking of vRNPs to membrane leading to budding and release.
1.2 The NS1A and NEP Proteins

The NS1 protein of the Influenza A virus is a multifunctional protein that contributes many roles to the virus. The protein has two distinctive domains, the RNA binding domain at the N terminus and the effector domain at the C terminus (Wang, Basler et al. 2002; Chien, Xu et al. 2004). The RNA binding domain has been shown to bind \textit{in vitro} to RNA with low affinity in a sequence independent manner, although sequence-specific binding has also been reported (Marc, Barbachou et al.; Qian, Chien et al. 1995; Chien, Xu et al. 2004). The effector domain is believed to predominantly mediate the interaction with cellular proteins but it also stabilizes the RNA-binding domain (Wang, Basler et al. 2002). It is highly probable that the NS1 protein occurs as a homodimer with both domains taking part in the dimerization (Nemeroff, Qian et al. 1995).

It is widely regarded that the main function of NS1 is the neutralization of the host’s interferon response. Studies have shown that NS1 is able to prevent the activation of IRF-3, NFκB and c-Jun/ATF, transcription factors that are essential for the induction of IFN-β (Talon, Horvath et al. 2000; Wang, Li et al. 2000; Ludwig, Wang et al. 2002). Other studies have also suggested that NS1 is able to block the induction of IFN-β by creating a complex with RIG-I consequently preventing RIG-I from inducing IFN-β (Pichlmair, Schulz et al. 2006; Guo, Chen et al. 2007; Mibayashi, Martinez-Sobrido et al. 2007; Opitz, Rejaibi et al. 2007).

An additional role for NS1 is the regulation of viral RNA synthesis. One of the primary studies in this area used temperature sensitive mutants of NS vRNA and found decreased levels of all the vRNA segments in infected cells, but found no changes in mRNA or cRNA levels (Wolstenholme, Barrett et al. 1980). NS1 has also been reported to interact with the viral
polymerase complex and to have high affinity for dsRNA that contain panhandle structures like those found in vRNA (Hatada and Fukuda 1992; Marion, Zurcher et al. 1997).

Furthermore, it has also been reported that during infection with the influenza virus there is a selective translation of the viral mRNAs versus cellular mRNAs (Garfinkel and Katze 1993). Hatada et al., demonstrated that temperature sensitive influenza viruses with mutations in NS1 had a decrease in the synthesis of viral protein (Hatada, Hasegawa et al. 1990). Enami et al. further supported this finding by showing that while NS1 does not cause a change in viral mRNA transcription, it instead enhances the translation of viral mRNAs through a 5’-UTR-dependent manner (Enami, Sato et al. 1994).

The splice product of the NS gene segment generates the 121 amino acid protein, NS2, also known as the nuclear export protein, NEP (Lamb and Lai 1980). Although initially believed to not have a structural role within the virion, small amounts of NS2 were later discovered existing within the virion where it may interact with M1 (Inglis, Barrett et al. 1979; Yasuda, Nakada et al. 1993). NS2 was subsequently associated with mediating the export of vRNPs from the nucleus, which led to the protein’s renaming to NEP (Paterson and Fodor ; O’Neill, Talon et al. 1998). Recently, studies have suggested that NEP holds more than one duty within the virus. Some studies have exhibited data demonstrating that NEP may regulate the accumulation of vRNA, cRNA and mRNA which may lead to a switch from viral transcription to favor the generation of vRNPs (Paterson and Fodor ; Robb, Smith et al. 2009). Due to this function, recent studies have implicated NEP in playing an essential role for the avian H5N1 to adapt to replicate effectively in mammalian cells (Manz, Brunotte et al.).
1.3 The M1 and M2 proteins

The M1 protein is comprised of 252 amino acids and includes an N-terminal and C-terminal domain linked together by a protease-sensitive loop (Ito, Gorman et al. 1991). The M1 protein, encoded by segment 7, is the most plentiful structural protein found within the virion (Ruigrok, Calder et al. 1989; Fujiyoshi, Kume et al. 1994). Like NS1, M1 plays several functional roles throughout various stages of the viral life cycle (Zhang, Wang et al.). As the matrix protein, it forms the layer bounded by the lipid membrane and the viral ribonucleoproteins (vRNPs), guaranteeing the stability of the virion (Zhang, Wang et al.; Coloma, Valpuesta et al. 2009). M1 is also involved in the viral life cycle by interacting with RNA and vRNPs through its involvement in localization, inhibition of RNA transcription and regulating the import and export of newly generated vRNPs (Cros and Palese 2003; Liu, Sun et al. 2009). Its role as a key structural protein deals with assembly and budding of the virus. Through the interaction with other viral proteins, it can assemble viral like particles (VLPs), elicit viral elements to the site of budding, as well as host components to complete budding (Avalos, Yu et al. 1997; Ali, Avalos et al. 2000; Gomez-Puertas, Albo et al. 2000).

The M2 protein is the splice product of the segment 7 transcript and is comprised of 97 amino acids (Cross, Dong et al.). It is an integral membrane protein and exists as a homotetramer, which has ion channel activity thus allowing it to control the internal pH of the viral particle. M2 allows for the acidification of the internal area of the approaching viral particle, which is thought to be essential for effective viral infection as it allows the vRNPs to dissociate from M1 and be transported into the nucleus (Lamb, Zebedee et al. 1985; Wang, Lamb et al. 1994; Bui, Whittaker et al. 1996; Sakaguchi, Tu et al. 1997). It is also reported that M2 conserves a pH within Golgi vesicles that stabilizes the innate conformation of haemagglutinin while it is being transported for use in viral assembly (Takeuchi and Lamb 1994).
1.4 The cellular splicing machinery

In vertebrates, protein coding genes produce immature mRNA transcripts that contain both introns and exons. Where the intron areas of sequence are removed and the exons are ligated to form the mature mRNA (Jurica and Moore 2003). The maturation of the mRNA occurs in the nucleus where it is moved to the cytoplasm to be translated. Like many mechanisms within cells, splicing occurs in a series of steps. Excision and joining of the introns and exons occur at specific sequences known as splice sites. At the 5’ splice site, the consensus sequence is – AG|GUAAGU and at the 3’ splice site it is U(C)AG| (Jurica and Moore 2003). At the 3’ end, the splice site region has three conserved sequence elements: a branch point, a polypyrimidine tract and a terminal AG at the end of the intron (Black 2003).

The initial steps of splicing begin with the coating of naked pre-mRNA with hnRNPs (heterogenous ribonucleoprotein) proteins. Once this binding has occurred, the spliceosome begins to assemble. The spliceosome contains the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6, plus additional accessory proteins (Norton 1994; Jurica and Moore 2003). The earliest complex characterized during spliceosome assembly is the E (early) complex made up of U1 and the dimeric U2 auxiliary factor, U2AF. The A complex is formed when the U2 snRNP joins the E complex. Joining of the U4/U5/U6 tri-snRNP to the A complex creates the B complex. The B complex undergoes a substantial rearrangement and becomes the C complex. The C complex will then catalyze the two trans-esterification events that take place during splicing. The 5’ exon is cleaved from the intron and produces two reaction intermediates, a detached 5’ exon and an intron fragment. The intron fragment will be attacked by the detached exon. The ligation of the two exons will form the mature mRNA (Black 2003).

It is generally accepted that protein-RNA interactions are the main mechanisms by which splicing is regulated (Witten and Ule). The importance of protein-RNA interactions have been assessed through bioinformatics studies which analyzed RNA motifs to predict exon regulation.
by the SR, Fox and NOVA proteins, all of which are known as RNA binding proteins (RBPs) (Liu, Zhang et al. 1998).

Preliminary studies into splicing regulation revealed SR proteins as exonic splicing enhancers (ESEs) (Liu, Zhang et al. 1998). SR proteins are highly conserved serine/arginine rich RNA binding proteins that regulate splice site selection (Zahler, Neugebauer et al. 1993). SR proteins are active in the early stages of spliceosome assembly by stimulating the binding of U1 snRNP to the 5’ splice site (Kohtz, Jamison et al. 1994; Staknis and Reed 1994). Conversely, hnRNPs have been attributed to being exonic splicing silencers (ESSs) by binding to sites equivalent to those of SR binding proteins, thereby inhibiting their enhancing effect (Mayeda and Krainer 1992; Shin and Manley 2004). It has also been reported that the assembly of the spliceosome participates in determining which splice site will be used. The choice of splice site is thought to also be regulated in part by the binding of the initial factors to the pre-mRNA and the subsequent formation of early spliceosomal complexes (Black 2003). Multiple factors play a role in determining how splicing will occur. Any factors altering the activity of RBPs could potentially have an effect on the regulation of splicing. Such changes may include post-translational modifications (Witten and Ule). The mechanism by which splicing is regulated by these elements has not yet been fully characterized. It is suggestive that concentration or activity of these components may modify complex assembly and therefore mediate the final splicing outcome (Shin and Manley 2004). What is evident is that RBPs play a large role in splicing regulation (Witten and Ule).

1.5 Regulation of splicing by NS1

Apart from the many functions for NS1 already stated, there is still another that deserves mentioning: Its ability to regulate splicing. In eukaryotic cells, only the spliced mRNA products
are exported from the nucleus. Influenza virus partially avoids this control because both spliced and unspliced viral mRNAs encode proteins, therefore, some transcripts must be exported without having undergone any processing by the splicing machinery (Robb, Jackson et al.). During infection, splicing of NS1 and M1 is regulated so that approximately only 10% of the transcripts are splice products. Early work done had suggested that specific viral products, more specifically NS1, were able to regulate the production of spliced viral mRNA (Robb, Jackson et al.). Since then, several papers have attempted to characterize what effects NS1 plays on the splicing regulation of cellular and viral mRNAs. Many of the studies conducted agreed on the fact that NS1 plays a role in the regulation of cellular RNA splicing. Furthermore, the majority of the reports seemed to be in agreement that NS1 plays a role in the splicing of the M gene segment. In a study performed by the Fodor group, it was demonstrated that in transfection experiments where NS1 was expressed together with a plasmid encoding the M segment mRNA, the accumulation of spliced products was decreased (Robb and Fodor). As influenza expresses proteins from spliced and un-spliced mRNA transcripts, regulation of this mechanism would be essential for the proper ratio of expression of both proteins (Robb and Fodor).

However, one of the questions that was also addressed by the papers was whether NS1 plays a role in its own splicing. The data published regarding this issue has not reached a definitive answer. Two papers came to the conclusion that NS1 does have an inhibitory effect on the splicing of its own mRNA, while two other papers disagreed with these conclusions and found that NS1 did not have any effect on its own splicing. For example, one report by Garaigorta and another by Fortes, stated that their results showed that NS1 inhibits the splicing of the collinear transcript, but two additional papers by Lu and Robb reported that NS1 did not play any role in the splicing of its own transcript (Robb and Fodor; Fortes, Beloso et al. 1994;
Lu, Qian et al. 1994; Garaigorta and Ortin 2007). In Lu’s study, it was reported that the NS gene segment was resistant to inhibition by the NS1 protein while Robb reported that the NS1 protein had no effect on the accumulation of spliced NS mRNA (Robb, Jackson et al.; Lu, Qian et al. 1994). A possible explanation for the discrepancies found in these studies could be attributed to the different experimental techniques employed by each group. While all used transfection as their method of introducing the viral gene segments into cells, several different types of cells were used and the strain from which the gene segments were derived from also varied. Various processes of RNA detection were also utilized. Fortes used an RNA protection assay where a probe would anneal to an area of RNA that they were interested in, digested RNA would then be analyzed by electrophoresis (Fortes, Beloso et al. 1994). While nuclease protection assays are extremely sensitive, the plasmids used for this study did not provide a lot of confidence to their expression as shown by their immunoblots. Lu also employed this method for RNA detection but unlike Fortes, their plasmids were well tested and proven to work (Lu, Qian et al. 1994). The studies conducted by (Robb and Fodor; Robb, Jackson et al.) used primer extension analysis to detect their species of RNA. Primer extension analysis used a primer that binds at the 3’ end of a transcript where cDNA will then be synthesized through the use of a reverse transcriptase until the 5’ end is reached. Primer extension is also a sensitive and reliable method for the detection of RNA (Boorstein and Craig 1989). Although all methods are generally accepted as being trustworthy, there are other factors involved that may affect the outcome of the results.

1.6 The cellular SUMOylation system

The reversible, post-translational modification of proteins by the Small Ubiquitin-like Modifier (SUMO) protein is known as SUMOylation (Gareau and Lima; Geiss-Friedlander and Melchior 2007). Previous reports have shown SUMOylation to play a role in the regulation of
many cellular processes including cell cycle regulation, transcription, replication, DNA repair, cellular localization and protein degradation (Gareau and Lima; Hannoun, Greenhough et al.). SUMO is ubiquitously expressed in eukaryotic cells, including yeast, *C. elegans*, and *D. melanogaster* (Geiss-Friedlander and Melchior 2007). The human genome encodes for four different homologs of SUMO, SUMO1-4 (Melchior 2000; Hay 2005). SUMO1-3 are expressed ubiquitously within cells while SUMO4 is mainly expressed in kidneys, lymph nodes and the spleen (Guo, Li et al. 2004; Geiss-Friedlander and Melchior 2007). Furthermore, SUMO2 and SUMO3, largely referred to as SUMO2/3, share a 97% sequence identity, however they share only a 50% sequence identity to SUMO1 (Geiss-Friedlander and Melchior 2007). Except for SUMO1 and SUMO4, the other SUMO homologs are able to form SUMO chains, but a chain will be terminated if SUMO1 binds because of its lack of a SUMOylation site (Geiss-Friedlander and Melchior 2007). The ability of SUMO4 to be conjugated *in vivo* remains controversial and therefore SUMO4 is usually not given any further consideration in most SUMOylation studies (Owerbach, McKay et al. 2005).

SUMO is synthesized as a 101 amino acid protein with a size of about 12kDA and very closely resembles the three dimensional structure of ubiquitin. The major difference between SUMO and ubiquitin is due to a variable extension of 10-25 amino acids at SUMO’s N terminus (Desterro, Thomson et al. 1997; Hay 2005). Furthermore, SUMO proteins share less than a 20% sequence identity with ubiquitin and differ in the overall surface charge (Barry and Lock; Hay 2005; Geiss-Friedlander and Melchior 2007). Like ubiquitin and other ubiquitin like modifiers, SUMO is conjugated to a target protein through an enzymatic cascade that involves the activating enzymes (E1), the conjugating enzyme (E2) and a protein ligase (E3) (Desterro, Thomson et al. 1997; Hay 2005). The initial step of the cascade involves the processing of an
immature form of SUMO by the SUMO protease, Sentrin-Specific Protease, or SENP, to generate the mature form (Barry and Lock). The maturation process involves cleavage of the C-terminus of SUMO by the protease to reveal a diglycine motif where the activating enzyme, a heterodimer made up of SAE1/SAE2, can bind (Barry and Lock). Upon the formation of the mature protein, activation of SUMO is performed by the activating enzyme by forming a high energy thioester bond (Hannoun, Greenhough et al.). Following this step, SUMO is transferred from the activating enzyme to the E2 conjugating enzyme, Ubc9, which binds to SUMO through a thioester bond established with a cysteine residue on its active site (Geiss-Friedlander and Melchior 2007). Ubc9 has the unique characteristic of being able to recognize substrate proteins directly; therefore the Ubc9-SUMO thioester can catalyze the formation of an isopeptide bond between the C terminal glycine residue of SUMO and the lysine of the target protein SUMO and the substrate protein (Hay 2005). This final stage can be facilitated by a SUMO E3 ligase, which may provide specificity for one of the three SUMO modifiers and speed up the process of SUMO conjugation. Typically, SUMO will be conjugated at a SUMO consensus site in the target protein, which follows the sequence $\psi$KXE, where $\psi$ is any large hydrophobic residue and X is any amino acid (Hay 2005). SUMO conjugation is a reversible modification and can therefore be reversed through the action of SUMO proteases that function by cleaving the isopeptide bond between SUMO and the target protein (Hannoun, Greenhough et al.; Geiss-Friedlander and Melchior 2007). Most of the enzymes capable of processing the immature form of SUMO through a peptidase activity are also capable of de-conjugating SUMO from its targets through an isopeptide activity (Geiss-Friedlander and Melchior 2007).
Figure 2. The SUMOylation pathway. The immature precursor form is cleaved with SENP to reveal the C-terminal diglycine motif. The mature form is activated by an E1 conjugating enzyme in an ATP dependent manner. It is then transferred to an E2 conjugating enzyme, then to the lysine residue of the target protein, forming an isopeptide bond, with or without the use of an E3 ligase. SUMOylation is reversible when SENP cleaves the isopeptide bond.
1.7 SUMOylation and viruses

The interaction between SUMOylation and viruses has only recently begun to develop as a significant theme within the field of virology. Considering how remarkably well viruses have adapted to exploit their host’s cellular machinery, it was only a matter of time before viruses were discovered to manipulate the SUMOylation system of its host cells (Boggio and Chiocca 2006).

The first viral protein discovered to be modified by the SUMOylation system was the immediate early 1 protein (IE1) of human cytomegalovirus (HCMV). The 72kDa protein regulates early events in the life cycle of the virus and evidence suggested that while SUMOylation was not required for effective viral replication, it did contribute to the full activity of the protein (Muller and Dejean 1999). Subsequent studies demonstrated that SUMOylation of the IE1 is needed for expression of IE2, an 86 kDa immediate early protein that acts as the predominant initiator of the virus’ lytic life cycle (Nevels, Brune et al. 2004). The Epstein-Barr virus, a member of the herpesviridae family, was also reported to use the SUMOylation system in order to proliferate via the SUMOylation of the immediate-early proteins BZLF1 (Z) and BRLF1 (R) are SUMOylated (Adamson and Kenney 2001; Chang, Lee et al. 2004).

Among other viral proteins currently known to be SUMOylated is the E1 protein of the bovine and human papilloma viruses. Interestingly, the was SUMOylation of these proteins was found to be enhanced by the PIAS family of proteins, thus providing an example of the complex interactions established between viruses and the cellular SUMOylation system (Rosas-Acosta, Langereis et al. 2005). Although the initial reports of interactions between viruses and the SUMOylation system were limited to DNA viruses, more recent studies have demonstrated that RNA viruses also interact with this cellular post-translational modification. For instance, a more
recent analysis by Sun determined that the polymerase (P) protein of the parainfluenza virus 5 is SUMOylated. This report indicated that SUMOylation of the P protein could play a role in regulating gene expression as a mutation at a lysine residue thought to be a SUMOylation site affected viral RNA transcription (Sun, Xu et al.). An investigation into the *picornaviridae* family also reported that the activity of the 3C protease protein could be diminished when SUMOylated (Chen, Chang et al. 2011). Upon further research, it was determined that upon mutation of the SUMOylation site, apoptosis induced by the virus was down-regulated, causing reduced amounts of the virus (Chen, Chang et al. 2011).

More recently, our group was able to find a direct link between Influenza and SUMOylation (Pal, Rosas et al.). Data from these studies have shown that several viral proteins are modified by SUMO (Pal, Santos et al.). Furthermore, several studies have been conducted to analyze the importance of the SUMOylation of NS1 within the virus. Site-directed mutagenesis tests have mapped the SUMOylation sites to residues K70 and K219. Results have shown that the SUMOylation of NS1 affect its major function, the ability to neutralize interferon. It was further discovered that while it does not affect stability or localization, there seems to be an optimal level of SUMOylation of NS1 that is required to occur in order for the protein to function at its peak (Santos, Pal et al.)

### 1.8 The Artificial SUMO Ligase (ASL)

The Artificial SUMO Ligase (ASL) was designed by a previous member of the lab to specifically enhance the SUMOylation of NS1. This construct which codes for the amino acid residues 1-87 of NS1 fused to the full length amino acid sequence of Ubc9 was designed with the expectation that such a fusion protein of the N-terminal RNA binding domain of the PR8 NS1 with the SUMO conjugating enzyme, Ubc9, would increase the SUMOylation of NS1 by the
interaction between the N-terminal region of NS1 and the N-terminal region of the NS1-Ubc9 fusion. This contact was expected to place Ubc9 in the immediate vicinity of the C-terminal domain of NS1, consequently facilitating the SUMOylation of NS1 at position K219, the main SUMOylation acceptor site in NS1. Furthermore, as it had been previously shown that the RNA binding domain is responsible for the protein’s IFN blocking activity, a mutant form of the ASL was designed to include two mutations in the RNA binding domain, R38A and K41A, which prevent RNA binding. This RNA binding domain mutant ASL has been shown experimentally to intensify the SUMOylation of NS1 while not exhibiting any self-SUMOylation and not appearing to stimulate the SUMOylation of any other viral or cellular protein (Santos, Pal et al.).

1.9 The overall aim of this study

This study was aimed at achieving three main goals: 1) Determine whether an over active SUMOylation system affects influenza viral infection; 2) Determine whether the SUMOylation of NS1 plays a role in its ability to regulate the splicing of the M gene segment; and 3) Determine if the SUMOylation of NS1 plays a role in its ability to regulate the splicing of its own co-linear transcript. Through the execution of this study we have discovered that up-regulating the cellular SUMOylation system through transfection prevents the simultaneous expression of the late viral protein, M1. We also found that while influenza infection triggers a global interaction occurring between the influenza virus and the cellular SUMOylation system, the data collected indicates that the SUMOylation of NS1 is not critical for its splicing regulating activity. However, this is only one piece of the puzzle of how the SUMOylation system and the influenza virus are interacting, and while it may barely begin to scratch the surface of what is truly occurring between these two elements, this report will provide a closer look into their interaction and present an opportunity for further areas to be explored.
Chapter 2: Materials and Methods

2.1 Cells and viruses used: HEK293FT cells (Invitrogen Corp., Carlsbad, CA) were maintained in complete medium comprising of 1x Dulbecco’s Modified Essential Medium (DMEM) supplemented with high glucose, L-glutamine, sodium pyruvate and 10% fetal bovine serum. Geneticin (Invitrogen Corp.) was added at a final concentration of 500μg/mL. Hek293A cells (Invitrogen Corp.) were also maintained in complete media comprising of 1x Dulbecco’s Modified Essential Medium (DMEM) supplemented with high glucose, L-glutamine, sodium pyruvate and 10% fetal plex. Cells were maintained at 37°C and 5% CO₂. Influenza A/PR/8/34 (H1N1) was a gift from Dr. John M. Quarles (Dept. of Microbial and Molecular Pathogenesis, College of Medicine, Texas A&M Health Science Center). Viruses were used at an MOI of 10 for immunofluorescence assays.

2.2 Plasmids: The pcDNA3/PolII/T7T7NS-ΔSpl and the pcDNA3/PolII/T7T7 (K70AK219A)-ΔSpl were developed by inserting a mutation in the splicing acceptor site located within the NS gene segment through site-directed mutagenesis. The A/WSN/1933 pPol1/WSN/M plasmid was provided by Yoshihiro Kawaoka (Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin). The pPol1/WSN/T7T7NS(R38AK41A) plasmid and the Artificial SUMO Ligase (here referred to as ASL) were developed as previously reported (Santos, Pal et al.).

2.3 Transient Transfection: HEK293FT cells were seeded at a density of 1.8x10⁵ cells/well into a 24-well plate, 8.0x10⁵ cells/well into a 6-well plate, or 10,000 cells/well for a 96 well plate. Following the plating, 24 hours later, the cells were transfected by liposome-mediated
transfection using the desired combinations of the plasmids and TransIT-LT1 (Mirus Bio LLC, Madison, WI) according to the manufacturer’s instructions. For the 24-well plate, a total of 3 μg total of DNA and 6 μL of TransIT-LT1 reagent were used per well. Similarly, 12 μg of DNA and 24 μL of TransIT-LT1 were used per well for the 6-well plate and 100ng of DNA and 0.2μL of TransIT-LT1 per well for the 96-well plate. At the appropriate times post-transfection, total cell extracts for Western Blotting were collected by adding boiling 2x Sample Buffer (24mM Tris [pH 6.8], 5% Glycerol, 2% SDS, 0.01% Bromophenol Blue). For RNA purification, the cells were trypsinized with TrypLE Express (Life Technologies, Corp.), then spun down at 5,000 x RPMs for 5 minutes and then resuspended in sterile1x phosphate buffered saline (PBS).

2.4 Immunoblot Analyses: Cell extracts were passed numerous times through a 29 ½ gauge needle to break down genomic DNA and decrease the viscosity of the samples. Afterwards, β-mercaptoethanol was added to each sample to a final concentration of 10%. Samples were then boiled for three minutes. Hand-made 10% SDS-PAGE gels were used to resolve the samples. The proteins were then transferred onto Immobilon-FL (Millipore Corp., Bedford, MA) to be detected with IRDye-conjugated secondary antibodies (LI-COR Biosciences, Inc., Lincoln, NE) and infrared fluorescence imaging.

2.5 Infrared Fluorescence Imaging: The Immobilon-FL membranes were washed three times in 1x PBS, then blocked in Odyssey Blocking Buffer (OBB) (Li-COR Biosciences, Inc.) for an hour at room temperature. Primary antibody was diluted in OBB supplemented with 0.1% Tween 20 (TOBB) at 4°C overnight. Following incubation with primary antibody, the membranes were washed four times with 1x PBS supplemented with 0.1% Tween 20 (TPBS), then incubated for
one hour at room temperature with the highly cross-absorbed IRDye 800 CW and IRDye 680 LT conjugated secondary antibodies (Li-COR Biosciences Inc.) diluted in TOBB at a dilution of 1:20,000. The membranes were then washed 3 times with 1x TPBS and twice again with 1x PBS. Following the washes, the membranes were scanned on an Odyssey CLx infrared imaging system (Li-COR Biosciences Inc.). Quantification was done through the Odyssey Infrared Imaging System Application software version 3.0.29 (Li-COR Biosciences Inc.). Statistical analyses and graphics of the data were generated by using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc.).

The following dilutions were utilized for the various primary antibodies used for immunoblotting: Anti-T7 tag mouse monoclonal antibody (MAb)(Novagen, EMD Biosciences Inc., San Diego, CA) at a 1,2000 dilution, anti-Ubc9 rabbit MAb EP2938Y (Abcam, PLC, Cambridge, MA) at a 1:2500 dilution, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse MAb 2D4A7 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1:5000 dilution, anti-Influenza A M1 mouse monoclonal (MAb)(Santa Cruz, Biotechnology Inc., Santa Cruz, CA) at a 1:2500 dilution, anti-Influenza A M2 mouse monoclonal MAb (Pierce Antibodies, Thermo Scientific Inc., Rockford, IL) at a 1:5,000 dilution.

2.6 Immunofluorescence Analysis: Once transfected with the indicated plasmids, the cells were incubated at 37°C in 5% CO₂. At 24 hours post-transfection the cells were infected, and 18 hour post-infection the cells were fixed with 1x PBS plus 4% paraformaldehyde for 10 minutes at room temperature, permeabilized by incubation with 100% Methanol for 10 minutes at room temperature and washed twice with 1XPBS. The cells were incubated in blocking solution (1x PBS plus 1% Goat Serum) for an hour at room temperature, and cells were subsequently
incubated for two hours at room temperature with primary antibodies, all diluted at 1:500 in blocking solution. Primary antibodies used were anti-Ubc9 rabbit MAb EP2938Y (Abcam, PLC, Cambridge, MA), anti-Influenza A M1 mouse monoclonal (MAb)(Santa Cruz, Biotechnology Inc., Santa Cruz, CA) and anti-β-Galactosidase rabbit PAb (Pierce Antibodies, Thermo Scientific Inc., Rockford, IL). Primary antibody was removed and cells were washed three times with 1x PBS. Fluorescently labeled secondary antibodies Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (both from Molecular Probes, Life Technologies Corp., Carlsbad, CA) were added to the cells diluted 1:500 in blocking solution and incubated for two hours at room temperature. The cells were washed three times with 1x PBS then stained with 4’,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 5 minutes then washed once again three times in 1XPBS. Images were captured using an LSM 700 confocal microscope (Zeiss, New York, NY) with a 40x objective and three lasers at 405nm (DAPI), 488nm (Alexa Fluor 488), and 555nm (Alexa Fluor 594). Image acquisition was performed by using ZEN 2009 software (Zeiss, New York, NY). The ZEN 2009 software was used to add pseudocolor to the images since the microscope system outfitted with a monochromatic camera.

2.7 Primer Extension Analysis: RNA was purified using the RNeasy Mini Kit and the Qiashredder system (Qiagen, N.V.) following the manufacturer’s protocol. Total RNA was analyzed through a 1% agarose formaldehyde gel and quantified using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc.) and normalized to equal concentrations. Primer extension reactions were completed using the Primer Extension System- AMV Reverse Transcriptase (Promega Corp.), following the manufacturer’s protocol, and using the primers presented in figures 4 and 7. The sequencing reactions were performed using the same primers.
used for primer extension analyses, and the Sequenase Version 2.0 DNA sequencing Kit (USB Corporation, Affymatrix Inc.) following the manufacturer’s protocol. Transcription products were analyzed on 6% polyacrylamide gels containing 7 M urea (RapidGel™-XL-6% Liquid Acrylamide Ultrapure MB Grade, USB Corp., Affymatrix, Santa, CA) in TBE buffer (Tris-Borate-EDTA, Fisher Scientific International Inc.). The gel was scanned on an Odyssey CLx infrared imaging system (Li-COR Biosciences Inc.) and quantified using the Odyssey Infrared Imaging System Application software version 3.0.29 (LI-COR Biosciences Inc.). Statistical analyses and graphics of the data were generated by using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc.).
Chapter 3: Results and Discussion

3.1 Over-expression of the SUMOylation system prevents influenza from progressing to late stages of infection:

Previous work by our group demonstrated that influenza virus establishes a complex interaction with the cellular SUMOylation system during infection. However, the molecular intricacies and consequences of these interactions remain to be characterized. Among others, one important question that we considered of high relevance was to establish whether the influenza virus is affected when the cellular SUMOylation system is altered. To this end, we decided to evaluate whether over-expression of Ubc9 alone, or Ubc9 and SUMO1 simultaneously altered the likelihood of an infected cell being able to progress to the late stages of viral infection, characterized by the expression of the late viral protein M1, an indicator of the successful progression to the late stages of infection. To this end, HEK293A cells were transfected with various combinations of expression plasmids for β-galactosidase, an HA tagged form of Ubc9, and the dicistronic plasmids SUMO1/IRES/Ubc9 and SUMO3/IRES/Ubc9 (which code for SUMO1 and SUMO3 respectively). At 24 hours post-transfection, the cells were infected with A/PR8/34 H1N1 at an MOI of 10. At 14 hours post-infection, the cells were fixed and analyzed by immunofluorescence using a mouse monoclonal antibody against M1 to detect infected cells and a rabbit polyclonal antibody against Ubc9 to indentify cells over-expressing Ubc9. Alexa 488 Goat anti-mouse and Alexa 594 Goat anti-rabbit secondary antibodies were used. A rabbit polyclonal antibody against β-galactosidase was also used, and the cell nuclei were stained with DAPI. Representative images are shown in Figure 3, images are of cells stained with DAPI, the indicated primary antibody, and the merged images. N values indicate total number of cells counted. Cells were counted and tallied as belonging to one of the following four groups: cells expressing high levels of Ubc9 only, cells expressing M1 only, cells expressing both Ubc9 and M1 or cells that did not express either of these proteins. Because 10 wells within a 96 well plate were being used for each condition, it was not necessary to repeat each individual experiment.
Instead, what was needed was a large number of cells to be counted. A few thousand cells were counted by hand per condition to provide for a large sample size to be evaluated. A large sample size would diminish the probability that any effects that were noticed would be due to a random occurring event. Chi-square and p-values were calculated by inserting these values into a chi-square calculator.

<table>
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<tr>
<th>Plasmids</th>
<th>Influenza A/PR/8/1934 H1N1 MOI 10</th>
<th>N</th>
<th>$\sum \chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-gal</td>
<td>DAPI ant-M1 MAb anti-β-gal MAb Merge</td>
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<td>2.313</td>
<td>0.128</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>HA-Ubc9</th>
<th>DAPI ant-M1 MAb anti-Ubc9 MAb Merge</th>
<th>N</th>
<th>$\sum \chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMO1/</td>
<td>8143</td>
<td>1.377</td>
<td>0.241</td>
<td></td>
</tr>
<tr>
<td>IRES/Ubc9</td>
<td>SUMO3/</td>
<td>7295</td>
<td>6.805</td>
<td>0.009</td>
</tr>
<tr>
<td>IRES/Ubc9</td>
<td>SUMO3/</td>
<td>5002</td>
<td>26.611</td>
<td>2.5E-7</td>
</tr>
</tbody>
</table>

**Figure 3. Effects of artificially increasing the cellular SUMOylation system on viral infection.** HEK293A cells were transfected with or without PolIII driven plasmids expressing β-galactosidase, Ubc9, SUMO1/IRES/Ubc9 or SUMO3/IRES/Ubc9. 24 hours post-transfection, the cells were infected with PR8 at an MOI of 10 and then fixed and prepared for immunofluorescence 14 h.p.i. Nuclei were stained with DAPI (blue), infection was detected by expression of M1 (green) and over-expression of the SUMOylation system by expression of Ubc9 (red). A. Cells transfected with β-galactosidase and expressing M1. B. Cells transfected with HA-Ubc9, SUMO1/IRES/Ubc9, SUMO3/IRES/Ubc9 and expressing M1. Chi-square and p-values shown for each condition.
As shown in Figure 3B, the observed frequency of cells that simultaneously co-expressed M1 and high levels of Ubc9 and SUMO was significantly lower than expected if the two events were independent from each other, as evidenced by the chi-square and p values calculated. This effect was greater when SUMO3 was the SUMO isotype overexpressed. The high Chi-square values obtained and low p values indicate that the results obtained are not due to pure chance but instead indicate a strong co-dependency between the two characteristics observed (expression of the late viral protein M1 and high co-expression of Ubc9 and SUMO). Specifically, the data indicates that cells over-expressing Ubc9 and SUMO have a dramatically decreased likelihood of progressing to the late stages of infection (i.e. expression of M1). The relatively small p-value would imply that these changes are statistically significant. In contrast, over-expressing Ubc9 alone was not enough to confer this unique characteristic onto the cell, as the differences in Chi-square values and p-values do not differ from those in the β-galactosidase control.

It is evident that multiple elements of the complex SUMOylation machinery are engaging with the influenza virus during infection. This data suggests that over-expression of the two components of the SUMOylation system tested (SUMO and Ubc9), conveys to the cells a protective effect that prevents influenza from progressing into the late stages of infection. This is the first set of data suggesting a protective effect mediated by the SUMOylation system during influenza infection. Other confocal microscopy studies conducted in our lab have shown that during infection there is an overall increase in SUMO expression. However, the cells exhibiting this over-expression of SUMO are cells directly adjacent to cells that are express M1 (i.e. cells in late stages of infection), but no increase in SUMO has been observed in the cells that show M1 expression. In view of the data presented here, it is now possible to postulate that the global increase in cellular SUMOylation previously reported by our laboratory may therefore be playing
an antiviral, keeping the cell from allowing influenza virus infection to progress to the late stages of infection. This data complements our recently published data regarding the effects of SUMOylation on NS1’s ability to neutralize interferon. We reported that modifying the SUMOylation of NS1, either increasing it through the use of ASL, or decreasing it through the use of a non-SUMOylatable form of NS1, results in a decrease in NS1’s ability to neutralize the IFN response. Thus it is likely that an overactive SUMOylation system may exert numerous effects that contribute to an overall antiviral activity, including a SUMO-associated decrease in NS1’s anti-IFN activity. In summary, the data presented here suggests that there is an interplay between the SUMOylation system and influenza where simultaneous over-expression of Ubc9 and SUMO prevents the cell from progressing to the late stages of infection.
3.2 SUMOylation of NS1 exerts a minimal effect on the splicing regulation ability of NS1 on the M gene segment.

As mentioned earlier, NS1 was reported to regulate splicing of mRNA transcripts. As NS1 is one of the most highly SUMOylated influenza proteins, and SUMOylation is known to exert numerous effects on its targets, we wanted to determine whether SUMOylation affected NS1’s ability to regulate the splicing of the M gene segment. To this end, we performed quantitative infrared primer extension assays to analyze the amount of spliced and unspliced mRNA produced from the M gene segment in the presence of normal, increased and decreased levels of SUMOylated NS1. To detect spliced and unspliced M gene mRNA products, two 680 IRDye® Infrared Dye labeled primers were created, an M-174 primer and an M-900 primer, which together enabled the detection of the M1 and M2 mRNAs, respectively, as shown in Figure 4 (the unspliced co-linear mRNA produced from the M gene codes for the M1 whereas the spliced product codes for M2).

HEK293FT cells were co-transfected with various combinations of plasmids encoding the viral RdRp, a PolII driven plasmid encoding for the M gene segment, a PolIII driven plasmid that encode for a T7 tagged non-spliceable wild-type form of NS1 (T7T7NS1ΔSpl), or a non-
spliceable, non-SUMOylatable form of NS1 (T7T7NS1DMΔSpl), and the artificial SUMO ligase to specifically increase the SUMOylation of NS1. Total RNA was harvested 24 hours post-transfection using the Qiagen RNeasy mini kit. The total RNA purified was quantified by agarose formaldehyde gel. Upon quantification, all RNAs were diluted to equal amounts and then analyzed by primer extension analysis. Total cell extracts were also collected and analyzed by Western Blotting to check the presence of SUMOylated NS1. All data presented correspond to the quantitative values collected in two independent experiments. Figure 5A confirms that NS1 is being SUMOylated by the appearance of a band around 40 kDa that is not present in samples that do not contain the NS1 PolIII driven plasmid. The production of M1 and M2 are also confirmed by the appearance of bands around the 28kDa and 15kDa sizes. Successful transfection of the ASL is also shown by the band at 25kDa in the appropriate samples. Figure 5B demonstrates the percent of NS1 that is SUMOylated compared to total NS1. There is no SUMOylated NS1 within the first two samples as NS1 was not present. The amount of NS1 that is SUMOylated without any additional assistance was about 0.3% of the total. With the ASL, SUMOylation increased to almost 4%. The non-SUMOylatable NS1 showed about a 0.2% SUMOylation, which increased to 1.8% with the ASL. This is due to the existence of alternative SUMOylation sites within NS1 that allow for some residual NS1 SUMOylation to take place in the presence of the K70AK219A mutations. Figure 6A demonstrates that the primers designed are detecting the correct mRNA species. The correct mRNA species were determined by comparison with a sequencing ladder produced using the same primers used in the primer extension analyses (not shown). Moreover, the GAPDH product shows an approximately equal signal among all samples, thus indicating that very similar amounts of mRNA were used across all samples. Figure 6B presents the quantitative differences in spliced versus unspliced mRNA
obtained under the different NS1 conditions. While there is an increase in the total amount of splicing that is shown when NS1 is present, there does not seem to be any significant changes in the amount of splicing occurring when comparing the samples where SUMOylation of NS1 is increased or decreased. However, this data is contrary to what had been seen in an earlier report by Robb (Robb, Jackson et al.). Their study determined that NS1 inhibited the splicing of the M gene segment, as they found lesser amounts of M2 mRNA when NS1 was present, as compared to the amounts of M2 mRNA that did not have the addition of NS1 (Robb, Jackson et al.). In our studies we see an obvious increase in spliced products when NS1 is added to the system. While there does not seem to be a significant difference in the amount of splicing across all conditions, there seems to be a slight increase in the production of M2 effect NS1 is hyper-SUMOylated.
### Figure 5. NS1 SUMOylation profile in the samples used for the RNP reconstitution assay.

**A.** HEK293FT cells transfected with the indicated combinations of plasmids. Expression of proteins analyzed by immunoblot. **B.** Quantification of amount of SUMOylated NS1. The immunoblot presented in A is a representative sample from two independent experiments.
Figure 6. Splicing of the M gene segment in an RNP reconstitution assay. 
A. Primer extension analysis of the splicing of the M gene segment in an RNP reconstitution assay. B. Quantification of the M1 and M2 transcript products.
3.3 SUMOylation of NS1 does not play a role in the splicing regulation of the NS gene segment

The NS gene segment transcript of the influenza genome undergoes splicing as well. The collinear unspliced mRNA produced from the NS gene segment codes for the NS1 protein, while the spliced product codes for the NEP protein. As NS1 has been reported by some groups to have the ability to modulate the splicing of its own transcript, we considered it important to evaluate what role, if any, was played by the SUMOylation of NS1 on the regulation of the splicing of the NS transcript. Once again, we performed quantitative infrared primer extension assays to analyze the amount of spliced and unspliced mRNA produced from the NS gene segment in the presence of normal, increased or decreased levels of SUMOylated NS1. To detect these spliced and unspliced mRNA species, two primers were created, an NS-171 800 IRDye® Infrared Dye labeled primer for the detection of NS1 and an NS-658 680 IRDye® Infrared Dye labeled primer to detect NEP mRNA, as shown in Figure 6.

Using the same methodology as before, HEK293FT cells were co-transfected with plasmids encoding the viral RdRp, a PolI driven plasmid encoding for the NS gene segment, a PolIII driven plasmid that encodes for a T7 tagged non-spliceable wild-type form of NS1 (T7T7NS1ΔSpl), or a nonspliceable and non-SUMOylatable form of NS1 (T7T7NS1DMΔSpl),
and the artificial SUMO ligase to specifically increase the SUMOylation of NS1. Total RNA was harvested 24 hours post-transfection using the Qiagen RNeasy mini kit and analyzed by primer extension analysis. Total cell extracts were also collected and analyzed by western blotting to check the presence of SUMOylated NS1, however a suitable western blot could not be obtained at this time. The quantitative RNA data presented corresponds to two independent experiments.

Interestingly, when dealing with the NS gene segment, we found the opposite effects of occur when compared to the splicing of the M gene segment. Addition of NS1 seems to decrease the amount of NS mRNA splicing. When the NS gene segment is transfected alone, the percent of spliced product generated is about 10% of the unspliced transcript. This is the amount usually seen under wild-type conditions. The addition of the NS1 protein into the system causes a slight decrease in splicing. Introduction of a non-SUMOylatable form of NS1 causes only a minimal change in the amount of splicing that occurs. On the other hand, when NS1’s SUMOylation is artificially increased with the use of the ASL, the decrease in NS splicing becomes more pronounced.

This data is in contrast to the study by Robb et al. who found that there were no changes in the amount of splicing when NS1 was introduced into an RNP reconstitution assay (Robb, Jackson et al.). Here, there does seem to be a greater effect in the amount of spliced transcript when NS1 is hyper-SUMOylated. However, this change is opposite to what was seen with the M gene segment. Where the splicing of the M gene segment was increased, the splicing of the NS gene segment decreases overall. Mutation of the SUMOylation sites within NS1 does not seem to affect the amount of splicing, with or without the ASL.
Figure 8. Splicing of the NS gene segment in an RNP reconstitution assay. HEK 293FT cells transfected with described combinations. **A.** Primer extension analysis of the splicing of the NS gene segment in an RNP reconstitution assay. **B.** Quantification of the NS and NEP gene segments.
3.4 Discussion

The data obtained to date shows that SUMOylation of NS1 may play a minor role in NS1’s regulation of splicing. SUMOylation of NS1 may have a more dramatic role in the creation of the NEP spliced product as there was a more marked effect in the splicing of the NS gene segment. The explanation behind this phenomenon may relate back to how splicing is regulated.

It could be postulated that SUMOylation of NS1 prompts NS1 to become a splicing silencer. Many exon splicing silencers contain an RNA-binding domain, such as that present in NS1 (Wang and Burge 2008). Binding of NS1 to mRNA could be preventing the binding of other RBPs that facilitate splicing. SUMOylation has also been known to modify protein-protein interactions of its target protein (Geiss-Friedlander and Melchior 2007). Perhaps SUMOylation of NS1 allows it to establish an interaction with splicing receptors, such as those of the hnRNP class. PTB, an hnRNP can block the interactions between U1 and U2, thereby inhibiting the formation of the spliceosome and the subsequent splicing of a transcript (Wang and Burge 2008).

Moreover, as previously mentioned, protein-RNA interactions play a significant role in mediating how splicing occurs. It has already been determined that RBPs undergo several posttranslational modifications, including phosphorylation, arginine methylation and SUMOylation. hnRNP C, which plays a direct role in mRNA splicing, was shown to be modified by SUMO, and it was postulated that this modification could be facilitating a role involved in protein-protein and protein-RNA interactions (Vassileva and Matunis 2004). The same study determined that SUMOylation was able to significantly alter hnRNPs binding to nucleic acids. This effect would prevent hnRNPs from competing for binding with SR proteins, thereby causing an enhancement to splicing. It could be hypothesized that a similar effect would be occurring with NS1. SUMOylation of NS1 may be causing it to have an
inhibitory or enhancing effect on splicing which would be sequence dependent as we see two different effects for distinct transcripts. Furthermore, as it’s possible that NS1 may be present at the location where splicing is occurring and the addition of the ASL may be causing the SUMOylation of nearby RNA binding proteins, causing a change in splicing regulation.

SR proteins have also been established as associating with influenza. One study determined that over-expression of SR proteins inhibited H5N1 NS mRNA splicing. However, H1N1 segments did not appear to be affected by adjusted SR protein concentrations. This study suggested that regulation of splicing in influenza is regulated differently strain to strain (Backstrom Winquist, Abdurahman et al.). They determined different levels of NS1 mRNA across several different strains of virus. This could also be subtype specific as reported by our data. While both gene segments were from an H1N1 virus, the gene segments came from different subtypes. Furthermore, the NS gene segment of H1N1 is said to be inefficiently spliced causing a greater production in the amount of the NS1 protein which is linked to determining the pathogenic properties of a virus (Backstrom Winquist, Abdurahman et al.).

Because splicing itself has not been fully elucidated, it is difficult to say with absolute certainty what is occurring in this process when SUMO and NS1 are brought together. SUMOylation of NS1 may be stimulating NS1 as an exonic splicing silencer by intensifying its RNA binding ability and thereby preventing splicing enhancers from binding to RNA, consequently causing decreased amounts of splice products. Conversely, it could also be proposed that SUMOylation of NS1 is causing NS1 to act as an enhancer by boosting its protein-protein interaction. While it has not been shown, perhaps SUMOylated NS1 is able to bind to SR proteins thereby expediting the binding of SR proteins to RNA and therefore causing an increasing in the production of spliced products.
While it is evident that there are definitive changes occurring in the regulation of splicing when the SUMOylation of NS1 is modified, the mechanistic actions behind this occurrence are still unknown. It’s possible that NS1 represses its own splicing to allow enough NS1 production but then enhances M1 splicing to speed up the viral release during the late stages of infection. Further in depth studies into splicing regulation and NS1 SUMOylation would need to be conducted before an answer could be reached. However, the data presented here gave slight insight into how this cellular post-translational modification is interacting with the influenza virus.
Chapter 4: Summary, Conclusions and Future Directions

4.1 Summary and conclusions
Influenza infection continues to be a severe threat to the population with annual epidemics affecting about 10% of the world’s population. The emergence of rapidly changing lethal viruses makes the importance of developing a generalized therapeutic more critical. Our current treatments, antivirals and vaccinations have a very limited effective range in relation to the spectrum of existent viral strains because of this high dependence upon specific to viral proteins. Because of the error prone characteristic of the viral RdRp, many treatments become obsolete within a short period of time. A new vaccination is required every year to protect against newly emerging strains and many currently circulating strains are now resistant to the available antivirals. With influenza causing between 250,000 to 500,000 deaths per year, and the likelihood of a pandemic becoming more and more probable. There is an urgent need for a more effective wide spectrum treatment for this disease. It is evident that a novel treatment is necessary, and perhaps it is time to target a cellular system that the virus is dependent on to halt, or impair, its replication. As already mentioned, influenza has already been confirmed to interact with the SUMOylation system, and has been shown to modulate the interferon blocking activity of NS1 (Santos, Pal et al.). Here, we provide further insight into how the SUMOylation system may play a role in influenza infection. There is evidence presented that points towards SUMOylation offering cells a protective quality when SUMOylation components are increased. Under such conditions, infection could not progress to the late stages of infection; hence likely preventing virus is budding and spreading. Therefore, modulating the SUMOylation system may be able to limit the infection and turn it into a minor disease. Furthermore, we also determined that SUMOylation plays a minor role in affecting how NS1 regulates splicing. It seems that over-SUMOylation of NS1 causes an alteration in how much splicing occurs. This could have adverse
effects for the virus as the proportion of the different components needed to create an infective viral particle are relatively restricted, any change in this process could potentially have antagonistic effects on the virus and potentially increase the release of noninfectious viral particles.

**4.2 Future Directions**

With the new insights into how the SUMOylation system interacts with influenza virus, many new areas of study have been opened as a consequence. Analyzing the underlying mechanism for why increasing the levels of SUMO and Ubc9 prevents cells from expressing M1 will need to be studied with a closer look. The many other functions of NS1 will have to be examined to determine if SUMOylation plays any role in those functions. With every new study conducted, it will bring us closer and closer to an answer for what function SUMOylation serves within influenza infection. There are many other areas to look at, such as how SUMOylation is affecting other viral proteins that become SUMOylated. Perhaps one of these interactions will lead us to develop the elusive universal treatment for this disease.


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Vita

Griselda Melendez was born in El Paso's sister city, Juarez, Chihuahua, Mexico. She was the youngest daughter born to Cecilia and Salvador Melendez. After two brief years in Mexico, her family relocated to El Paso, Tx, permanently. She graduated third in her class from Parkland High School in the Spring of 2013. After graduation, she began to pursue her undergraduate career at The University of Texas at El Paso studying Microbiology. She was awarded the Presidential Scholarship as well as the Texas Grant throughout the four years of her undergraduate career. She was also a RISE Scholar during her last semester as an undergraduate. She began her laboratory experience in her final year as an undergraduate. After receiving her Bachelors of Science in Microbiology in the Spring of 2010, she was accepted into the Master's program in the Biology Department at the University of Texas at El Paso. She was chosen as a teaching assistant and taught the Molecular Cell Biology and Anatomy and Physiology laboratories for two years. She will publish her data as a first person author in Journal of Virology, and she be a supportive author in a separate paper.

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