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Effects of the Cellular SUMOylation System on the Influenza Nuclear Export Protein (NEP)

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Abstract

The high genetic compatibility between the 2009 H1N1 pandemic "swine flu" and the highly pathogenic H5N1 "bird flu" viruses indicates that the odds of a deadly bird flu strain mingling its genetic material with a human virus and becoming easily transmissible among humans are at an all-time high. This fact stresses the urgent need for new treatment and prevention methods to combat influenza. Our current anti-influenza weapons include vaccination and two type of antivirals that target specific viral components (the so-called M2 ion channel inhibitors and the neuraminidase inhibitors). However, the high genetic variability of the virus enables it to easily circumvent by favoring the development of mutations that either, change the antigenic makeup of the virus making it unrecognizable by the immune effectors stimulated by vaccination, or confer resistance against the antivirals indicated above. A promising new alternative for the development of broad-spectrum antivirals is to develop small molecules capable of either, decreasing the activity or abundance of cellular components essential for viral replication, or increasing the activity or abundance of cellular components endowed with antiviral activity. In this project we aim to further characterize the role played by the so-called cellular SUMOylation system during influenza virus infections, to determine whether it may provide new targets for the development of innovative anti-influenza therapies. Specifically, my work focuses on delineating the molecular effects mediated by the SUMOylation of the viral protein NEP, an essential viral factor required for the export of viral components from the cell nucleus.

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Chapter 1: Introduction

Influenza is an acute contagious respiratory disease characterized by recurrent annual epidemics with occasional but major worldwide pandemics. Despite all the efforts and current available anti-influenza therapies, it continues to represent a major threat to global human health. Influenza continues to cause severe illness to 3-5 million people and 250,000- 500,000 annual deaths worldwide, where 20,000-36,000 deaths account for the United States alone each year during epidemic seasons [1]. The seasonal influenza virus affects people from all ages, particularly the elderly from age 65 and older. The 2009 H1N1 influenza pandemic included a new high risk population by affecting younger children. People with certain health problems such as chronic heart, pulmonary diseases, liver and kidney damage, and metabolic diseases such as diabetes are at a higher risk of death due to the weakening of the body's ability to fight off the disease [1]. Society has experienced three major influenza pandemics worldwide during the last century, in 1918, 1957, 1968, and 2009. The 1918 Spanish flu pandemic killed 50 to100 million people worldwide, 28% of the total population at the time, making it one of the deadliest natural disasters in human history [2].

The most effective way to combat influenza is through vaccination. Currently, there are a few known anti-viral drugs against influenza, which target structural components of the virus; M2 and NA. Unfortunately, only one of them is effective since the M2 inhibitor, best known as amantadine, has shown resistance on 100% of seasonal H3N2 and 2009 H1N1viruses [3].This is possible due to the low fidelity of the viral polymerase which renders the virus prone to mutations and to develop resistance against available antiviral therapies. Recent findings in 2010 indicated the high genetic

compatibility between swine-origin H1N1 and the highly pathogenic avian H5N1 influenza viruses [4].Reassortment of the viruses containing HA from H5N1 and other genes from the 2009 H1N1 virus are better at replicating in human lung cells than any of the viruses alone, thus creating a dangerously improved human influenza strain. Even more recently in February 2013, the appearance of a new H7N9 influenza strain was reported in China with an on-going spread through Asia. According to reports from the World Health Organization, WHO, the new strain has infected 133 people in China and Taiwan, killing 43 of them. Even though, there have not been any cases reported in the United States yet, there is an urgent need for novel therapeutic and prophylactic methods to combat the disease. A viable alternative for the development of novel broad-spectrum tools against influenza virus is to target cellular components required for viral multiplication that will work irrespectively of the type, strain or antigen properties of the virus.

The Small Ubiquitin-like MOdifier, SUMO, is among the most dynamic post-translational regulation systems composed by four isoforms in humans (SUMO1-4). These proteins are conjugated to lysine residue(s) in their target protein by a common enzymatic pathway in a process known as SUMOylation, exerting hundreds of effects on cellular proteins and regulating numerous cellular activities. Many reports have shown crucial roles for SUMOylation in the life cycle of several viruses [5]. Moreover, previous data by our group indicated that influenza virus affects and is affected by the cellular SUMOylation system of the host [6], suggesting a critical role for the cellular SUMOylation system during influenza virus infection. Therefore, this post-translational

SUMOylation system may provide important targets for the development of innovative antiviral therapies.

The NS2 influenza viral protein exhibits characteristics that make it a likely SUMO target

The influenza viral segment 8 is an essential fragment coding a 121 amino acid polypeptide from a spliced form of the segment mRNA transcript. This not well characterized, and the second smallest influenza viral protein was originally thought to have no structural function within the virion, leading to its designation as non-structural protein 2 (NS2). Subsequently, it was noted that small amounts of NS2 protein were present in virions possibly interacting with the viral matrix protein M1. NS2 was later implicated in mediating the export of vRNPs from the host cell nucleus. This led to the proposal that the NS2 viral protein be renamed as the nuclear export protein (NEP).Previous studies done by our group have shown that several viral proteins are effectively SUMOylated. The NEP viral protein exhibits characteristics that make it a likely SUMO target [6]. We will emphasize this study on **the potential effect(s) of SUMOylation on NEP.**

In this project, we aim to explore the main interplay between the influenza A virus and the cellular SUMOylation system in which our previous studies have indicated that during influenza infection numerous viral proteins become SUMOylated. Therefore, to better characterize the role played by the cellular SUMOylation system during influenza infection, it is important to determine the specific effects exerted by SUMOylation on each of the viral proteins targeted by this post-translational modification. The studies presented here will focus on delineating the molecular effects mediated by the SUMOylation of the viral protein NEP, which is an essential viral factor required for the

export of viral components from the cell nucleus. In addition, these studies will allow us to determine whether NEP is a bona fide SUMO target during influenza infection to then determine its effects during infection. Finally, the data generated will provide critical insights on the post-translational regulation of NEP function and potentially lead to the identification of a novel target for the development of an anti-influenza therapy. Furthermore, these studies will expand our understanding of the interactions established between viruses and the cellular SUMOylation system.

1.1 Influenza A virus

Influenza virus is a well characterized linear single-stranded, negative- sense RNA virus with a total genome length of 10-15 Kb [5].This virus belongs to the *Orthomyxoviridae* family which includes influenza virus types A, B, and C*.* Among the most common *Orthomyxoviridae* viruses studied is the Influenza type A virus which is mainly responsible for most flu pandemics affecting a widespread of avian and mammalian species. Influenza type B is restricted to humans and influenza type C is known to infect both humans and pigs.

The enveloped influenza A virus consists of eight gene segments, varying in length between 890 and 2341 nucleotides. These eight RNA segments code for 10-11 different viral proteins. Most segments encode single viral proteins, with the exception of segments 7 and 8, and in some strains segment 2 as well. Most DNA viruses replicate in the nucleus using RNA polymerase II to create mRNA. On the other hand, RNA viruses cannot use cellular Pol II, so they replicate in the cytoplasm. Influenza virus is one of the few RNA viruses to replicate in the nucleus. This is due to the fact that the influenza virus requires a functioning nucleus and the host cell enzymatic activities to

supplement viral enzymes during the expression of the viral genome, therefore leading to an effective replication of the virus [7]. Each of the viral proteins encoded by the influenza virus is described in Table 1.

Table 1. RNA segments and encoded proteins of the Influenza A virus:

Segment	Nucleotide Length	Amino acid Size	Protein Encoded	Protein Function
1	2341	759	PB2	Protein basic 2; Binds to cap structure on cellular pre-mRNAs part of transcriptase complex
2	2341	757	PB1 PB1-N40	Protein Basic 1; RNA polymerase activity for transcription and replication
			PB1-F2	Induction of apoptosis
3	2233	716	PA PA-X	Polymerase A; Part of transcription and replication complexes; posses snipping (endonuclease) activity
4	1778	566	HA	Haemagglutinin; major surface glycoprotein; receptor- binding; mediates membrane fusion at low pH; antigenic determinant
5	1565	498	NP	Nucleocapsid protein; binds and encapsidates vRNA; control functions in RNA synthesis
6	1413	454	NA	Neuraminidase; major surface glycoprotein; receptor destruction;

				dissociation of virus aggregates; antigenic determinant
7	1027	252	M1	Matrix protein; interacts with envelope, nucleocapsids, and NEP
		97	M2	Integral membrane protein; ion channel activity essential for virus encoding and maturation
8	890	230	NS1	Nonstructural protein 1; down-regulation of host cell mRNA processing; sequesters dsRNA and reduces interferon responses
		121	NEP	Nuclear export protein; directs nuclear export viral nucleocapsids; interacts with M1; stimulates synthesis of viral cRNP replication; recruits F1Fo ATPase

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The two large surface glycoproteins are the main antigenic determinants, Haemagglutinin (HA) being the most abundant envelope protein covering 80%of the surface and Neuraminidase (NA) which makes up nearly 17% of the viral envelope proteins, both embedded in the lipid envelope [8].The Influenza A virus subtypes are based on these two glycoproteins of the virus: the (H) hemagglutinin and (N) neuraminidase. There are 16 H and 9 N subtypes. Influenza is a RNA virus with the unique ability to replicate in the nucleus of its host cell. During infection the virion attaches to sialic acid receptor on the surface of the cell via receptor binding site in the

glycoprotein HA to then enter the cell in an endosome through receptor mediated endocytosis. The endosomal vesicle and the inside of the viral particle become highly acidic due to the type III transmembrane protein M2, which serves as a proton-selective ion channel in a viral envelope to allow the influx of the H^+ ions. The acidity within the viral particle triggers a non-reversible conformational change in HA, disruption of protein-protein interactions between M1 and RNP causes an overall disassembly of the core of the virus and release of viral RNA (vRNA) and core proteins to the cytoplasm of the host cell. Since the transcription and replication occurs in the nucleus of the infected cell, the vRNP must migrate to the nucleus. PB1, PB2, PA, and NP form the viral polymerase complex (vRNP) which is translocated to the nucleus through Nuclear Localization Signals (NLS) already present in all of the vRNP forming complex. These viral proteins have the ability to bind to the cellular import machinery [8]. Once inside the nucleus, the viral RNA-dependent RNA-polymerase (vRdRp) composed of PB1, PB2, and PA initiate viral RNA synthesis to produce viral mRNA transcripts by a 'cap snatching' mechanism [8,9,10].This is done by PB2 binding to the 5' m^7G caps of the host mRNA which is then stolen by the N-terminus end of PA containing the endonuclease activity to then be used as the primer for viral transcriptase [8,9]. Cellular RNA polymerase II (Pol II) binds to DNA and start transcription. During transcription initiation, phosphorylation of the serine 5 on the C-terminal repeat domain (CTD) present on Pol II is then required to the activation of the cellular synthesis complex.

Then, M1 binds to a negative sense vRNPs through its C-terminal end and to NEP through its N-terminal end masking the nuclear import signal (NLS) of the protein. In turn, NEP binds to CRM1to export the vRNPs to the cytoplasm. After the vRNPs

have been exported out of the nucleus, the virus has to form viral particles by using the host's plasma membrane to leave the cell. These viral proteins, which are later transported to the apical plasma membrane, must contain the proteins within the viral lipid bilayer; HA, NA, and M2 [11].Packaging of the 8 genomic segments has two model hypotheses; random packaging and specific packaging model. The first one predicts that the viral genomic segments are randomly packaged into virions; whereas the specific packaging model predicts that there are specific signals within each of the viral segments dictating which segments will be packed in the virion. Most importantly, cleavage of sialic acid residues carried out by NA must occur before the viral particle can leave the plasma membrane and infect neighboring cells.

The NS gene segment is the last and smallest gene segment of the influenza virus encoding for two mRNA splice variants, NS1 and NEP. The non-structural protein 1 (NS1) is an important virulence factor expressed in high levels with a predominant localization in the nucleus. This protein is known to exert multiple roles during influenza infection, one of the most important functions is to antagonize host immune responses (interferon responses) by antagonizing or limiting IFN-α/β production, inhibiting apoptosis by PI3K activation, limiting the binding activity of PKR and OAS, preventing maturation and migration of dendritic cells, and antagonizing putative host defense by RNAi induction; all promoting viral pathogenicity. Several studies have indicated that the NS1 viral protein is responsible for the virulence of highly pathogenic human influenza viruses with the ability to cause viral pandemics [12].The second spliced product of the NS gene segment is mainly known as a Nuclear Export Protein (NEP). Recently, this

protein has been associated to a couple of different roles during influenza infection as described in the next section [13].

1.2 The role of the Nuclear Export Protein (NEP) in influenza A infection

Even though, the same segment (segment 8) encodes for the non-structural protein NS1 and the nuclear export protein, NEP, very little is known about this last one. NS1 is a well characterized protein known to exert multiple functions during infection to antagonize host immune responses and to promote viral pathogenicity. NEP is a less well characterized 14.5 kDa protein. Although NEP was initially designated as anon-structural protein, it was later found in purified viral particles. Also, it has been found in accumulation preferentially in the nuclei of infected eukaryotic cells. Previous studies have suggested a role for NEP in regulating viral RNA replication providing a

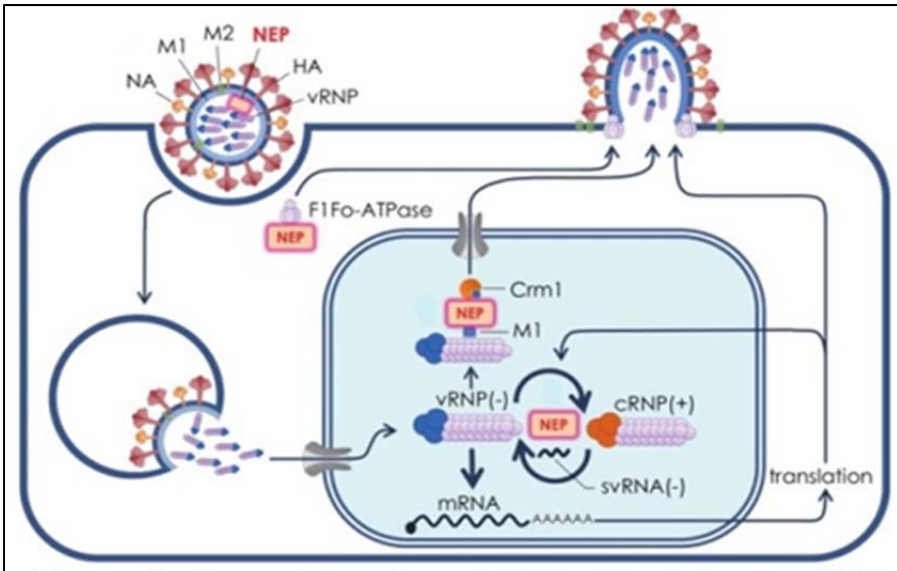

Figure 1. Emerging roles of NEP. The Influenza NEP protein is known to act as a nuclear exporter of vRNPs. In addition, it has been demonstrated that NEP contributes to viral budding through interaction with F1Fo- ATPase. Furthermore, NEP regulates the accumulation of mRNA, cRNA, and vRNA. PLoS Pathog. 2012;8(12):e1003019. doi: 10.1371/journal.ppat.1003019. Epub 2012 Dec 6.

possible explanation to its nuclear accumulation [14, 15]. For this reason, NS2 was renamed as the nuclear export protein (NEP). Exportins just like importins can directly recognize nuclear export signals by binding to a second exportin molecule to interact with the nuclear pore complex (NPC). Chromosome Region Maintenance Protein 1 (CRM1) is well known to mediate the export from the nucleus of many proteins, RNAs, and ribonucleoproteins complexes carrying a nuclear export signal (NES) [16].In order for CRM1 to leave the nucleus with the cargo, it needs to be associated to Ran and these needs to be associated to GTP to form RanGTP. As soon as the cargo/adaptor/CRM1/RanGTP leaves the nucleus; RanGAP stimulates the GTPase activity of Ran, thus allowing it to cleave GTP into GDP. In the presence of GDP, the ternary complex falls apart allowing the exportins and RanGDP to go back to the nucleus, but the cargo remains in the cytoplasm[17].Previous studies have demonstrated involvement of M1 in the nuclear export of vRNPs; these findings have suggested that M1 is required for vRNPs to get exported from the nucleus [18].

The matrix 1 protein is a two domain protein with a nuclear localization signal (NLS) on the N-terminal domain and affinity for RNPs on the C-terminal domain. In addition, M1 does not have the required NES motif to export RNPs. However, this motif is found in the N-terminal domain of NEP. It has been reported that the atomic structure of the C-terminal half of NEP, has one face that is extremely acidic containing an exposed tryptophan, W78, in the middle of glutamic acid side chains. These tryptophan and the glutamic acid chains play a significant role in the binding to M1 as the binding can be disassociated upon mutation of W78 to serine. Thus, the proposed model as illustrated in Fig. 1 is as follows; in the nucleus, M1 binds to RNPs through its C-terminal

domain, leaving its C-terminal domain with the NLS exposed so that this can associate with the N-terminal domain of NEP. Then, NEP will bind to M1 through its C-terminal domain leaving its N-terminal domain with the NES free for interactions with CRM1; lastly binding to RanGTP is needed so the complex can be exported[19,20].

Recent studies have suggested additional roles for the Influenza NEP protein. As mentioned above the main and most recognized role of this viral protein is to act as a nuclear export protein, hence its name. In addition to the main role of NEP, this protein has also been demonstrated to contribute to the viral budding process through interaction with a cellular ATPase known as the F1Fo-ATPase. TheF1Fo ATPase is a bifunctional enzyme complex that can be found mainly in the inner membrane of mitochondria and plasma membrane of mammalian cells. This enzyme has the ability to hydrolyze ATP as an AAA-type ATPase. As a bifunctional enzyme, it is composed of two parts. The F1 portion consists of five subunits, and the Fo portions consist of nine subunits. The ATPase activity of F1FoATPase is a critical factor for efficient influenza virus budding, but further studies are needed to fully understand the role of ATPase activity in virion budding [21].The role of NEP has not been identified yet, but it has been demonstrated that NEP is associated to one subunit of the F1 portion at the plasma membrane. Therefore, the interaction of NEP with the plasma membrane and the F1β subunit suggested a role for this F1 subunit during the late step of the influenza viral cycle [21]. Moreover, siRNA targeting F1β experiments revealed that M1 can induce VLP production and that NEP enhances M1-induced virus-like particle (VLP) production, which established a role for NEP in virion formation [21].

Another recent role for NEP is the ability to regulate the accumulation of mRNA, cRNA, and vRNA. Although the biochemical mechanism is not known yet, this ability has been linked to a potential switch from viral transcription during the early stages of viral replication to favor the production of genomic vRNPs [22].Interestingly, NEP plays an important role in the adaptation of some avian H5N1 influenza viruses to replicate in mammalian cells having a biological significance since most avian influenza viruses cannot replicate efficiently in mammalian cells [23]. Moreover, it was demonstrated that NEP has the ability to significantly inhibit reporter gene expression with concentrations that could physiologically be reached by infected cells. Further experiments indicated that high levels of NEP expression can inhibit the transcription as well as the replication of all viral RNAs [24]. Overall, NEP seems to perform different biologically important functions during influenza viral replication

Finally, NEP was reported to be SUMOylated *in vivo* when transiently expressed in HEK293 cells. Out of the 11 proteins encoded by the influenza virus, M2 and NEP exhibit some sequence variability among different viral strains. Even though NEP was proven to be a potential target modified by the SUMOylation system, it is still undetermined whether NEP would be SUMOylated under the normal levels of expression and activity displayed by the cellular SUMOylation machinery.

1.3 SUMOylation: a post-translational conjugation system of the Small Ubiquitin-like MOdifier (SUMO)

Initial studies done at the SUMO-Influenza laboratory for cellular and molecular virology, were based on the ability of the SUMO protein to regulate several viral

proteins. Initial studies were done by *in vitro* SUMOylation reactions revealing 7 of the viral proteins were SUMOylation with the exception of the two membrane viral proteins, HA and NA, and PB1-F2 and M2. Moreover, *in vitro* and *in vivo* studies showed that NS1 is a *bona- fide* SUMO target, making this the first report showing the relevance of SUMOylation on the influenza virus [6].The cellular SUMOylation system is known to affect a vast number of cellular proteins. As mentioned above, the SUMO protein has the ability to regulate cellular localization, protein stability, half-life, and protein activity [25].

As stated at the beginning of this project, SUMOylation is known to play a crucial role in a variety of biological processes such as modifying a target protein by altering its enzymatic activity, cellular localization, and serving as a docking site for other potential interacting molecules. Some cellular processes regulated by SUMOylation include

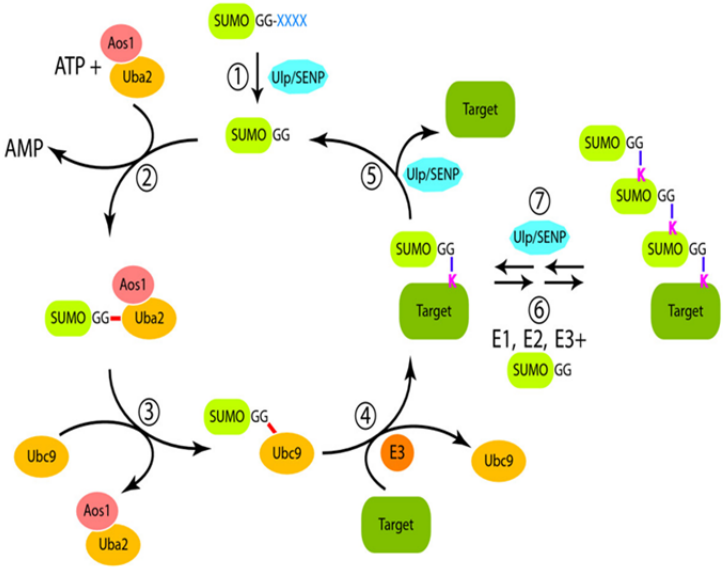

Figure 2. SUMO pathway.
SUMO proteins undergo post-translational maturation, catalyzed by Ulp/SENPs, to reveal a C-terminal di-glycine motif (Step 1). Mature SUMOs undergo ATP-dependent activation, resulting in a thiolester linkage between the C-terminal di-glycine and their activating enzyme, Uba2/Aos1 (Step 2). The thiolester is transferred to their conjugating enzyme, Ubc9 (Step 3). Ubc9 acts in concert with SUMO ligases/E3 enzymes to form an isopeptide linkage between the SUMO C-terminus and an ε-amino group of a lysine within the target protein (Step 4). SUMOs can be removed from conjugated species by the action of Ulp/SENPs (Step 5). In some cases, SUMO chains can be formed through linkage of additional SUMO moieties to previously conjugated SUMOs (Step 6). While it is possible that multiple Ulp/SENPs may disassemble SUMO chains (Step 7), members of the Ulp2 family appear to be specialized for this reaction.
Dasso *Cell Division* 2008 3:5 doi:10.1186/1747-1028-3-5

transcription, translation, protein degradation, and cell cycle regulation. The SUMO protein is ~11kDa in size and resembles the 3-D structure of ubiquitin, while sharing less than 20% amino acid sequence identity and 40% structure similarity to ubiquitin [26, 27].Another difference between these two post-translational modification systems is that the enzymes required for the SUMOylation cell cycle differ from the ones needed in the ubiquitination cycle. SUMO pathway requires the E1 and E2 enzymes for conjugation without the absolute need for the E3 ligase, while the ubiquitin pathway the E3 ligase is necessary for conjugation [28].A unique element of the SUMO protein is that it carries an unstructured stretch of 10-15 amino acids at the N-termini. There are four different SUMO proteins, SUMO1, SUMO2, SUMO3 and the most recently identified and least characterized; SUMO 4. SUMO 2 and 3 share 97% identity, so they are frequently referred as SUMO2/3, whereas they only share 50% identity with SUMO1, this being the most closely related homolog to ubiquitin. This post-translation modification system involves a multistep process. SUMO is synthesized as a precursor that is proteolytically cleaved near its C-terminal end by a SUMO protease (SENP) to expose its internal di-glycine motif required for conjugation. Next, a mature form of SUMO is triggered by the E1 activating enzyme in an energy consuming process. Then, SUMO is transferred to the E2 conjugations enzyme known as Ubc9, which conjugates it to a lysine residue of a target protein forming an isopeptide bond, which in some occasions be facilitated by E3 ligases. After the conjugation, SUMO specific proteases like SENP cleave the isopeptide bond between SUMO and its targeted protein, creating a reversible and dynamic process as presented in Figure 2. Also, SUMO is conjugated to the target protein via the isopeptide bond in the carboxyl group of its C-terminal

domain glycine residue and the lysine residue in the target protein. For the most part, the lysine residues are located within the SUMO consensus motif ΨKXE, Ψ being a hydrophobic residue, K is the lysine residue, X stands for any amino acid, and finally E is glutamic acid [29, 30, 31].

Furthermore, SUMO is emerging as a versatile modifier for a number of proteins in different pathways with a diverse range of roles in many cellular processes. The effects associated with SUMOylation are protein-specific, but the actual molecular mechanisms by which these effects are achieved are still unknown. One of the proteins regulated by the cellular SUMOylation system is the glucocorticoid receptor-interacting protein1 (GRIP1) and the viral protein IE2-p86 where conjugation of SUMO enhances their activity. Also, DNA binding activity of the heat shock factors HSF1 and HSF2 is stimulated with SUMO conjugation. On the contrary, SUMOylation of several transcription factors such as c-Jun, c-Myb, AP2, Sp3, and some nuclear receptors demonstrated downregulation of their transcriptional activity. In addition, SUMO conjugation is known to modify a large number of viral proteins by enhancing viral propagation or exerting antiviral activities [32, 33].

1.4 SUMOylation and viruses

SUMOylation is an important protein function modulator. A number of viruses interact with the cellular SUMOylation system either to secure the function of certain viral proteins or by enhancing the system for viral propagation. In contrast, the host may control the cellular system to prevent viral propagation. Although there are four isoforms of the SUMOylation system, the SUMO1 protein has the ability to modify a broad range of viral proteins from different viruses. Some of these viruses are the Human

Cytomegalovirus Immediate-early protein 1, HCMV-IE1, for which SUMO1 conjugation is required for efficient viral replication by activating the IE1 protein. Another virus modulated by the SUMO1 protein is the Bovine Papilloma virus E1 Protein; BPV-E1. SUMO modification is necessary to trigger the helicase activity of the BPV-E1 protein, thus allowing for intranuclear distribution and nuclear export [34].

On the other hand, there is evidence of SUMOylation exerting antiviral activities. For example, the Gam protein produced by an avian adenovirus with the ability to control the host's SUMOylation system by promoting the ubiquitination of the SUMO E1 enzyme, thus leading to protein degradation [35]. Some viruses such as Epstein-Barr virus and the Human Cytomegalovirus possess similar abilities to block the SUMO modification of proteins, such as PML, thus leading to the disruption of essential steps to induce viral infection [36, 37]. Also, the Ebola Zaire has the ability to hijack the host SUMOylation system to suppress the innate immune response by SUMOylating IRF-7[38].

Various siRNA screens in human lung epithelial (A549) cells and yeast two hybrid screens identified SUMOylation as one of the host machineries required by the influenza virus for effective viral replication [39, 40].More recent *in vitro* studies have demonstrated interactions between the cellular SUMOylation system with seven out of the eleven influenza viral proteins, predominantly with the non-structural protein NS1 [41]. Given that the influenza virus is an RNA virus not only with the ability to replicate in the nucleus of its host cell, but with conserved consensus SUMOylation motifs among different influenza virus strains, and the ability to interact with several viral proteins, it is possible that SUMOylation is playing an important role during influenza virus infection.

The interplay between influenza and the cellular SUMOylation system has gained substantial relevance over the past years as it modulates a variety of cellular processes affecting a number of essential cellular functions. For example, initial studies demonstrated NS1A as a bona fide SUMO target, thus implying that the cellular SUMOylation system may play important roles in during influenza infection. In addition to NS1, several other influenza viral proteins were identified as bona fide SUMO targets both *in vitro* and *in vivo* approaches, suggesting that there might be an active interplay between the virus and the host cellular system. Moreover, the presence of two high molecular weight bands, 70 and 52 kDa, are consistently present during viral infection as well as a global increase in cellular SUMOylation. Interferon (IFN) is the main anti-viral response mounted by the cell during viral infection. So, studies testing the effects of IFN during viral infection demonstrated an increase on viral replication and an increase on SUMOylation dependent on viral multiplication independently from IFN-stimulation.

In order to enhance the SUMOylation of NS1, a novel technique was developed referred to as the artificial SUMO ligase (ASL). This was created by a fusion of the N-terminal RNA binding domain (RBD) of the NS1 protein from PR8 with the SUMO conjugating enzyme Ubc9. The interaction between the N-terminal region of NS1 and the N-terminal region of NS1-Ubc9 positioned Ubc9 in relation to the N-terminal region of NS1. The unstructured linker region of NS1 enabled the movement of Ubc9, creating a fusion construct composed of amino acid residues 1-87 of NS1 and the full length amino acid sequence of Ubc9. Consequently, Ubc9 was brought in close proximity to the SUMOylation site of NS1, thus creating an increase in the SUMOylation of the

protein. The endogenous SUMOylation of the protein was increased to a remarkable level allowing for an extent functional analysis of the effects of SUMOylation on NS1 without increasing the global SUMOylation of the cell.

The development of the ASL leads to an extensive analysis on the effects of SUMOylation on NS1. Extensive studies demonstrated that the SUMOylation of NS1 does not affect its stability or cellular localization of the protein. In addition, several studies demonstrated that the abundance of SUMOylated NS1 is a major determinant of NS1's ability to neutralize IFN response. More importantly, our group has been the first one to test the effect of the interplay between the SUMOylation system and the Influenza virus in an animal model with the help of this novel tool [6, 28, 41].

1.5 Thesis goal

Our current research focus on the interactions between a post-translational modification system known as SUMOylation and the influenza A virus. This Small Ubiquitin-like Modifier (SUMO) has gained substantial relevance over the past years as it modulates a variety of cellular processes. Previous SUMOylation prediction analyses done using SUMOsp 2.0, revealed the presence of SUMO consensus motif(s) in most influenza viral proteins. Given that influenza is among the very few RNA viruses to replicate in the nucleus of host cells and that most of the viral proteins contain a conserved SUMOylation consensus motif, we concluded a possible role of SUMOylation during influenza infection. Several viral proteins, including NS1 and NEP, were shown to be SUMOylated upon viral infection. Currently available data indicated that the cellular SUMOylation system provides important functions for influenza upon infection, and therefore may provide novel targets for the development of antiviral therapies. NS2/NEP

has been well characterized as an adaptor allowing for the binding of viral RNPs and nucleoporins and most likely for the transport of RNPs through the NPC into the cytoplasm. **Thus, for the purpose of this project, we hypothesized that SUMOylation of Influenza NEP plays a crucial role in mediating the nuclear export of the vRNPs in infected cells**. Furthermore, we want to characterize the major role of SUMOylation on this viral protein, with the ultimate goal to identify new therapeutic approaches against influenza infections. To achieve this, we will pursue the following specific aims:

Specific Aim 1: Test whether NEP is a bona fide or true SUMO target during influenza viral infection.

Specific Aim 2: Characterize specific SUMOylation site(s) in NEP.

Specific Aim 3: Characterize the role(s) exerted by SUMOylation on NEP upon Influenza viral infection.

Our preliminary SUMOsp 2.0 analysis revealed the presence of conserved SUMO motifs (ΨKXD/E) on most influenza viral proteins including NEP. This protein contains a total of seven lysine residues with only one, K72, serving as a potential SUMO target. All influenza viral proteins were tested using an *in vitro* transcription-translation approach resulting in seven efficiently SUMOylated viral proteins. Additional immunoblots were performed using the total cell extracts of transfected mammalian cells revealing the presence of higher molecular weight form of NEP. The new form appeared to be a SUMOylated form of NEP due to the apparent high molecular weight of approximately 20 kDa larger than the unmodified wild-type protein. This change in

size corresponds to the molecular weight of His-S-SUMO1. Secondly, the higher molecular form of the protein was not visible in the presence of SENP1. As mentioned above, segment 8 codes for two viral proteins, NS1 and NEP. Extensive data has demonstrated that NS1A is the best SUMOylated protein on the influenza A virus; therefore it is easy to assume that NEP might also be modified by this cellular system. Due to these intriguing findings, it will be of high relevance to further characterize the SUMOylation of all viral proteins identified as potential SUMO targets. This study will allow us to identify the ground breaking role(s) of SUMOylation on NEP.

Chapter 2: Identification of the Nuclear Export Protein (NEP) as a target of the cellular SUMOylation system.

The Influenza virus is composed of 8 gene segments which can code for 10-12 viral proteins. Our previous studies have demonstrated that seven (PB2, PB1, PA, HA, NP, NA, M1, and NEP) out of the 11 viral proteins are effectively SUMOylated *in vitro* and at least five are SUMOylated *in vivo*. The Nuclear Export Protein has been shown as a possible candidate for interacting with the cellular SUMOylation system. Previous data provided by former graduate students in Dr. Rosas-Acosta's lab, has shown strong interactions between the cellular SUMOylation system and the influenza viral protein NEP through mass spectrometry and *in vivo* experiments. Despite these experiments, there is no direct data indicating that NEP is regulated by this cellular system and how. In this specific aim, we demonstrated the cellular SUMOylation system regulates the influenza viral protein NEP.

2.1 Materials and Methods

2.1.1 Immunoprecipitation

A549 cells were infected with WSN/T7T7NS1K701K219A~NS2 and MOCK at an MOI of 10. Then, cells were collected 12 h.p.i (hours post-infection) with SDS collecting buffer. Samples were then IPed either with anti-SUMO1 or anti-T7 and blotted against anti-NS2 and anti-SUMO, respectively.

2.1.2 Transduction and infection

To test the SUMOylation of NEP by transduction followed by infection, A549 cells were transduced with the Dual-SUMO1 (T95RQ94P) adenovirus at three different MOIs of 50,100 and 200. This plasmid up-regulates cellular SUMOylation due to the Q94P mutation preventing SUMO deconjugation from its target protein. The T95R mutation introduces a trypsin cleavage upstream the di-glycine motif of SUMO creating a signature tag and allowing for easy detection on any target protein containing a SUMOylation site. In order to test the effects of SUMOylation on NEP, we needed to separate NEP from NS1 and test them individually. The double mutant construct, WSN/T7T7[NS1K70AK219A~NS2], was initially created to test the effects of SUMOylation solely on NS1.Introduction of the K219A mutation, one of the main SUMO sites on NS1, into NS1 created a lethal N62H mutation on NEP. Therefore, we created a construct that could test the SUMOylation of NS1 in the absence of changes in the primary sequence of NEP by separating the open reading frame (ORF) for NS1 from NEP, while allowing NEP to still be produced as a splicing product. The splicing acceptor site on NS1 was inactivated by site-directed mutagenesis, then placing a copy of the second exon for NEP with a functional splicing acceptor site, downstream from the stop codon for NS1.Conclusively with this construct, cells were incubated for24 h.p.t. and infected with the double mutant influenza virus, and incubated for an additional 24 hours. Cells were transduced only, infected only, or transduced and infected. The cells were collected using 4X boiling sample buffer and the effects were evaluated by quantitative IR Western Blot analysis.

2.1.3 Transfection

To confirm the site of the SUMOylation on NEP, a point mutation bearing K to A substitution of the residue previously indicated at position 72 were generated. To this end, we generated the mutation using a previous plasmid with the NEP ORF shifted from the NS1K70AK219A~NS2 construct (from here on referred to as NS2[SUMO]). To analyze the effects of the point mutation on NS1K70AK219A~NS2K72A (from here on referred to as NS2[non-SUMO]), HEK293FT cells were be transiently transfected with the shifted NS2 constructs along with RNA-dependent RNA-polymerase, which include PA, PB1, PB2, and NP plasmids all driven by a Pol1 promoter only. At 24 hours post-transfection, the cells was collected using 4X boiling sample buffer and the SUMOylatability of the mutant NS1DM~NS2K72A was evaluated by quantitative IR Western Blot analysis.

2.2 Results

2.2.1 Purification of NEP by immunoprecipitation:

The molecular weight of NEP is approximately 14.5Kd. The addition of a T7 tag on our construct (molecular weight of 11kD) which form of the SUMO protein is ~23.5kD, which suggests a possible SUMOylation event on this protein when cells are transfected with the SUMO-deficient form of NS1. Interestingly, there is no previous record from other groups studying the interactions between NEP and the cellular SUMOylation system.

In order to confirm the identity of the shifted band obtained in co-transfections with the SUMO-deficient plasmid, we performed several co-IPs. Immunoprecipitations were used to isolate and concentrate the purified form of NEP. Therefore, we infected MDCK and A549 cells with a NS1-SUMO deficient influenza virus and a wild type shifted influenza virus. Additional controls consisted on MOCK infected and rabbit serum used for direct binding of the G beads. Despite the effort, we were unable to succeed on this approach. A number of experiments were performed testing for different conditions such as, different lysis buffers, hours post-infections, MOI's, higher density of cells, cell type, and transfection followed by infection. Unfortunately, we were never able to detect the presence of a higher molecular weight form of the protein. We have only being able to detect heavy and light chains on both IPed samples in most the experiments. In addition, supernatants were collected for every step of the protocol (e.g. TCEs before adding antibodies, after G bead pre-absorption, etc.) and analyzed by Western Blot, presenting a very faint band distinctive of

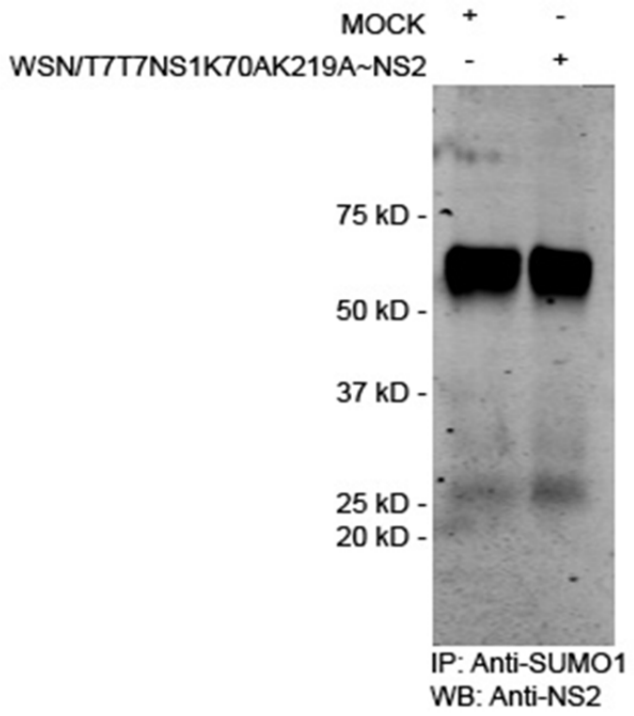

Figure 3. NEP immunoprecipitation. A549 cells were infected with either MOCK or WSN/T7T7NS1K70AK219A~NS2 at MOI of 10. 22 hours following infection, cells were collected with SDS lysis buffer for immunoprecipitation with G beads and anti-SUMO1 or anti-T7 antibodies. Analysis was done by SDS-PAGE and Western Blotting.

endogenous NEP. All attempts to this approach were inconclusive and the purification of the SUMOylated form of NEP remains elusive.

2.2.2 Testing the SUMOylation of NEP by Dual- SUMO1 transduction and infection with dual mutant Influenza virus

Previous experiments confirmed that co-transfection with lysine to alanine mutations at positions 70 and 219, identified as the two main SUMO sites on NS1, referred as the double mutant (DM) construct, revealed the appearance of a new band at ~38kD which coincides with the SUMOylated form of NEP. A straight-forward approach to determine whether NEP was modified by the cellular SUMOylation system was to add a His-tag to create a shift on the protein band of interest. The additional shift on the potential SUMOylated NEP band served as a confirmation of the SUMOylated form of the protein. We determined whether NEP was SUMOylated during infection with a DM influenza virus followed by overexpression of the dual-SUMO1 adenovirus transduction on A549 cells. This approach produced an additional shift of approximately 4kDa caused by the His-tag present in the dual-SUMO1 adenovirus, thus allowing further confirmation on the SUMOylated form of NEP.

The results presented the appearance of the ~38kD protein band in samples that were infected with the DM influenza virus. Intriguingly, as shown in Fig. 4 (lane 5), there is a new band at ~75kD present only when cells are transduced at an MOI of 100 followed by influenza infection with the DM virus. The identity of the band is still unknown. We discarded the possibility of a shifted version of a SUMOylated NEP,

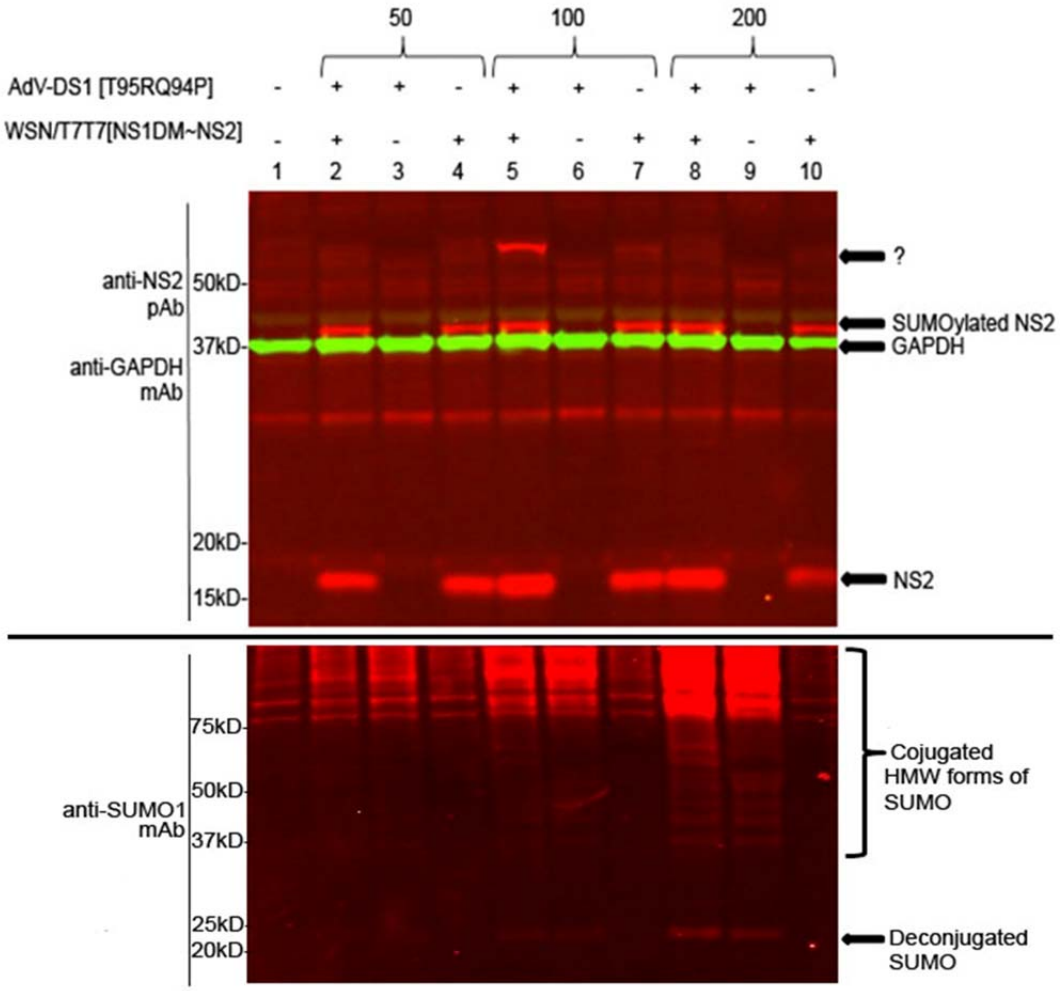

Fig 4. NEP SUMOylation in transduced cells followed by influenza infection. A549 cells were transduced at different MOIs as indicated, with AdV-DS1(T95RQ94P). 24 hours-post transduction, cells were infected with WSN/T7T7NS1K70AK219A~NS2 at MOI 1 for an additional 24 hours. Cell lysates were collected in 4X sample buffer and analyzed by SDS-PAGE and Western Blotting.

because the shift is more than 30kD higher than the expected shift of a SUMOylated form of NEP with the His-tag. Based on the data collected, we were able to reproduce the same band at ~38kD from transfection experiments when cells are transduced with a dual-SUMO1 adenovirus followed by infection with the DM influenza virus. Another possibility might be an alternative spliced form of the NS primary transcript that results

in a protein that shares NEP end, but with an extra sequence upstream of the 2nd exon of NEP.

2.2.3 Mapping the SUMOylation site(s) of the Nuclear Export Protein

The development of a non-SUMOylatable form of NEP is essential to assess the effects exerted by SUMOylation on this particular protein allowing us to evaluate the effects caused exclusively by the cellular SUMOylation system. Therefore, it is crucial to first assess possible SUMO consensus motifs (ΨKXE) by identifying lysine residue(s) responsible for the interaction between SUMO and NEP.

The tentative SUMOylation site(s) on NEP was predicted using the SUMOsp 2.0 software. Next, the predicted lysine residue(s) was mutated to an alanine residue using a site-directed mutagenesis approach. Finally, the site of SUMOylation was confirmed by evaluating the SUMOylatability of the mutant protein by transient transfections in HEK293FT cells.

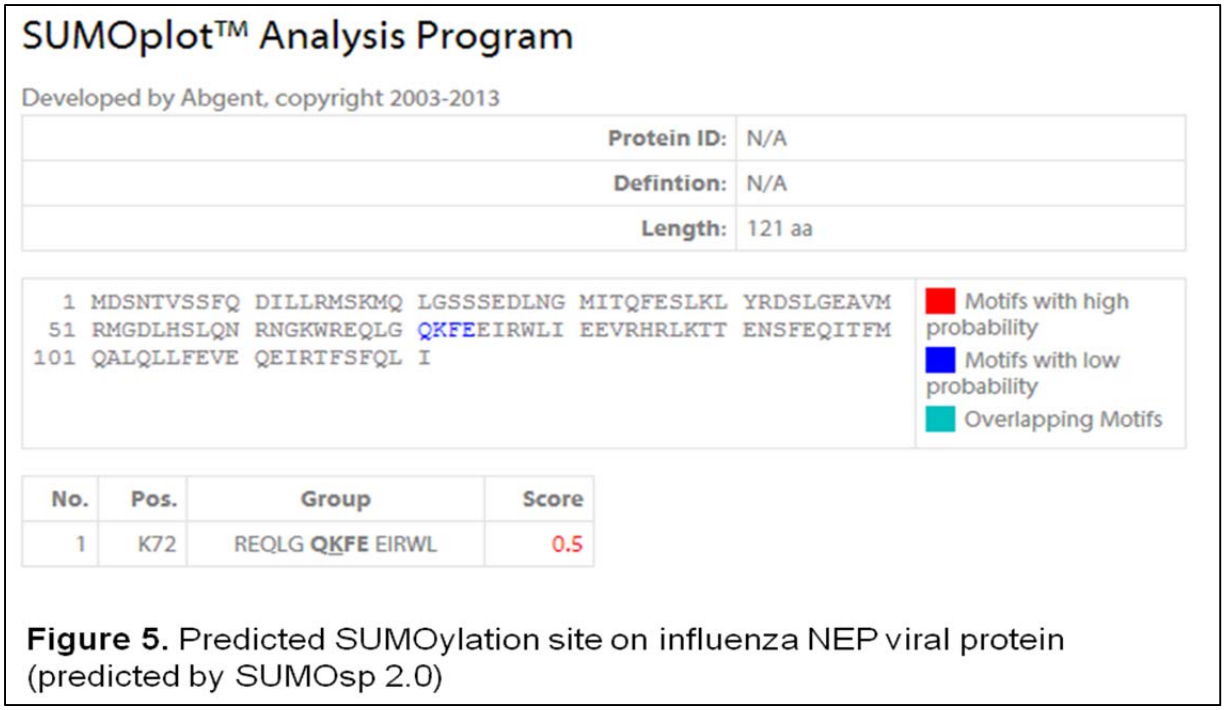

Figure 5. Predicted SUMOylation site on influenza NEP viral protein (predicted by SUMOsp 2.0)

Following the procedures described above for IR Western Blot analysis, we identified what seems to be the main SUMOylation site on NEP. As shown on Fig. 6, the appearance of a new higher molecular weight form of NEP at approximately 38kD is present with the addition of the NS2[non-SUMO]. The expression of the possible SUMOylated form of NEP was substantially decreased with the addition of the K72A mutant construct compared to the levels of SUMOylation observed with NS1DM~NS2, thus indicating that the mutation substantially decreases the SUMOylation of NEP. Western Blot analysis with anti-NS1 antibody reveals the presence of the NS1 viral protein in samples containing the NS2[non-SUMO] and NS2[SUMO] constructs, indicating that the NS1 sequence was not altered in the making of the constructs. In addition, the membrane was incubated with anti- GAPDH to serve as a loading control to indicate similar levels of protein loaded for all the different samples. An additional

experiment was done using EGFP as a transfection control. In Fig.7, the intensity of the EGFP is similar in all the samples co-transfected with EGFP, thus indicating that the decrease in the intensity of the NS2[SUMO] and the NS2[non-SUMO] is not due to a decrease in transfection efficiency. The data presented here identifies a potential SUMO site on NEP.

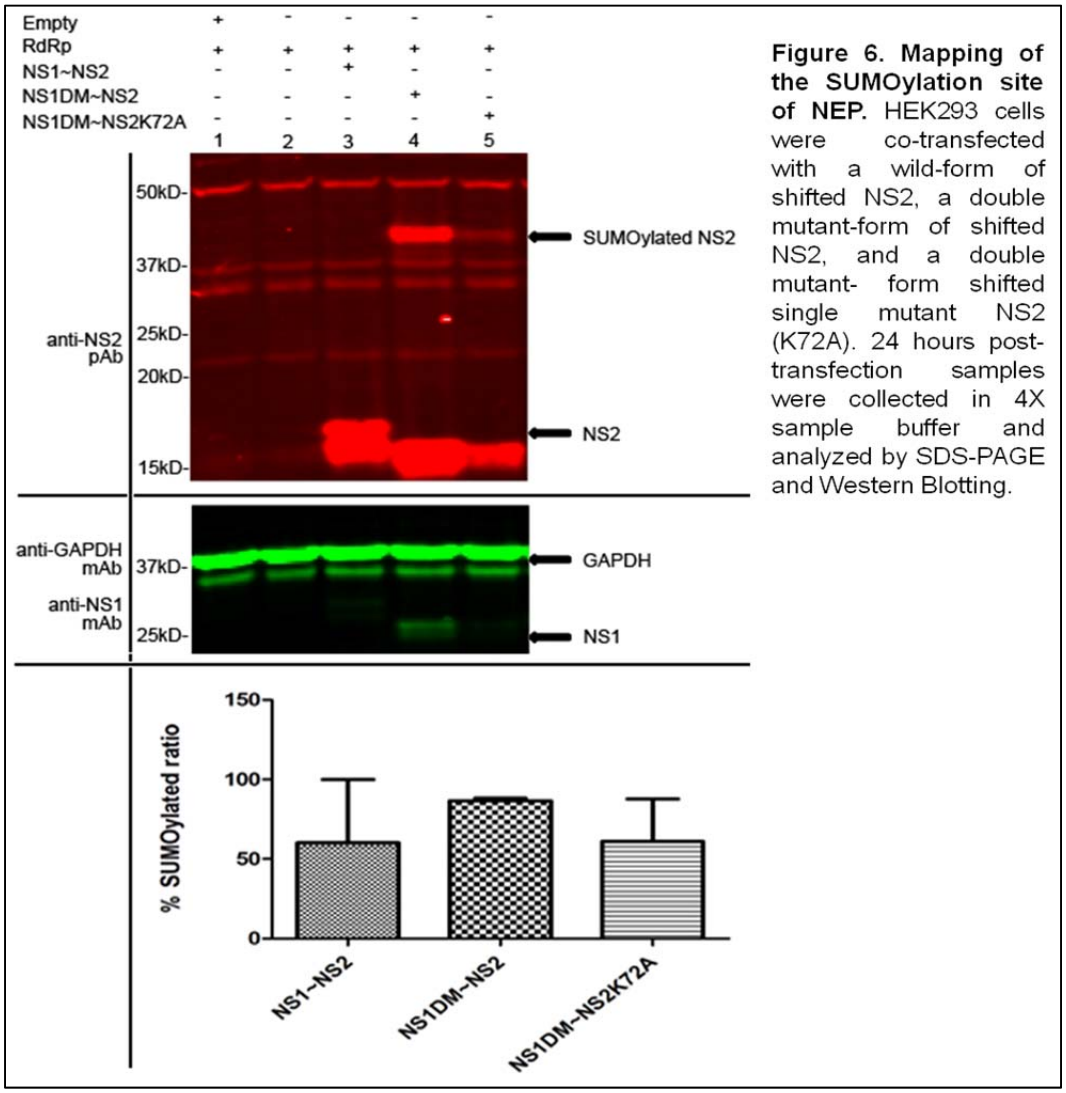

Figure 6. Mapping of the SUMOylation site of NEP. HEK293 cells were co-transfected with a wild-form of shifted NS2, a double mutant-form of shifted NS2, and a double mutant- form shifted single mutant NS2 (K72A). 24 hours post-transfection samples were collected in 4X sample buffer and analyzed by SDS-PAGE and Western Blotting.

2.3 Discussion

Studies looking at the interactions of the Influenza A virus and the cellular SUMOylation system have shown the Non-structural protein, NS1, as a *bona fide* SUMO target. Extensive analysis have been focused on the effects of SUMOylation on NS1 only. Previous data shown described by Pal et al. 2011, indicated that at least 7 out of the 11 viral proteins of the influenza virus can interact with the SUMO protein during *in vitro* analysis [6]. The nuclear export protein is one of the possible SUMO targets described in the analysis. The purpose of these studies is to determine whether NEP is a true SUMO target. Unfortunately, we were not able to purify a SUMOylated form of NEP nor detect a high molecular weight band of the protein during immunoprecipitations. Furthermore, we were unable to detect a shift in the high molecular weight form of NEP even with the addition of a His-tag during transduction. Despite our efforts to test whether NEP is a true SUMO target, we were able to map its main and possible only SUMOylation site. After SUMOplot analysis, we mutate the lysine residue at position 72 of the protein to an alanine residue in order to prevent the SUMOylation of NEP. As seen in Fig. 6, the appearance of a high molecular weight band is present when cells are transfected using a non-SUMOylated form of NS1 shifted construct. The same band is greatly diminish in the presence of the mutation, K72A, on NEP indicating that this is in fact lysine residue responsible for the interaction between SUMO and NEP. After extensive analysis NEP amino acid sequences, we are certain that the effects seen during the production of a higher molecular weight band are not due to an improper shift on the ORF of NS1 or NEP. Furthermore, the presence of

NS1 eliminated the possibility that the high molecular weight band is a fusion of NS1 and NEP.

Chapter 3: Characterization of the specific SUMOylation site(s) on the Nuclear Export Protein (NEP) and its effects exerted upon Influenza infection

The Influenza virus is unique among RNA viruses by replicating in the nucleus where SUMOylation appears to be most predominant. Among those SUMOylated proteins NEP was shown to be effectively SUMOylated *in vivo* when transiently over-expressed in mammalian cells. Along with these facts, most of the influenza viral proteins bear one or more predicted SUMO consensus motif(s), indicating that the influenza viral proteins may be targeted by the cellular SUMOylation system. In this aim we will seek to identify the SUMO target(s) present on NEP. To achieve this goal, we developed a lysine to alanine mutant at the one possible SUMO motif on NEP. Previous studies identified two main SUMO sites on the non-structural protein NS1A. Lysine residue 219 acts as the primary SUMO site followed by lysine residue 70. Lysine to alanine mutations at the two main SUMO sites on NS1 decreased the levels of SUMOylated NS1 even on the presence of an artificial SUMO ligase (ASL).

3.1 Materials and Methods

3.1.1 Transfection

To determine transfection efficiencies of the NS2[SUMO] construct,HEK293FT cells were transiently transfected with the shifted NEP constructs along with RNA-dependent RNA-polymerase, which include PA, PB1, PB2, and NP plasmids all driven by a Pol1 promoter only. EGFP was added to the co-transfection to serve as a control for transfection efficiencies. At 24 hours post- transfection, the cells was collected using

4X boiling sample buffer and the transfection efficiencies of the NS2[SUMO] plasmid were evaluated by quantitative IR Western Blot analysis.

3.1.2 Stability assay using cyclohexamide

To assess whether SUMOylation is having an effect on the stability of NEP, HEK293A cells were co-transfected with (+) or without (-) NS2[non-SUMO] and NS2[SUMO] constructs. At 24 post-transfection, cyclohexamide, CHX, was added to the cells to a final concentration of 4mM and incubated for an additional 4, 6, 12, and 24 hours post treatment. Cells were collected using 4X boiling sample buffer and the stability of SUMOylated NEP was evaluated by quantitative IR Western Blot analysis. Successive rounds of immunoblotting were done using anti-NS2 PAb, anti-NS1 MAb, as primary antibodies. Highly cross-absorbed secondary antibodies were used. Membranes were analyzed using the Odyssey CLx.

3.2 Results

3.2.1 Confirmation of transfection efficiencies of the NS2[SUMO] plasmid

Exact transfection of a plasmid is almost impossible to achieve. The addition of a plasmid to different wells of cells can create variations on the amount of plasmid added due to pipetting errors and cell density. Therefore, we decided to co-transfect the cells with EGFP as a transfection control and test the transfection efficiency throughout the experiment. This will allowed us to determine whether the effects previously seen in

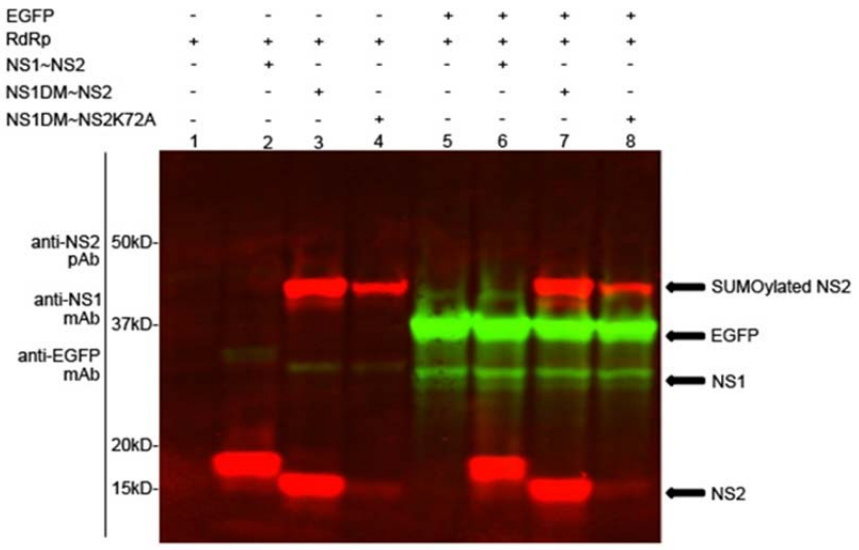

Fig 7. Transfection efficiency of NS2 mutant plasmid. HEK293 cells were co-transfected with the different shifted-forms of the NS2 construct, including the wild-form of the shifted NS2, DM~NS2, and DM~NS2 mutant. EGFP was used as a transfection control. 24 hours post- transfection samples were collected in 4X sample buffer and analyzed by SDS-PAGE and Western Blotting.

Fig.7 were due to transfection efficiencies or a real decrease of NEP when cells were transfected with NS2[SUMO] plasmid.

As stated above, the efficiency and volume of a transfected plasmid can differ from well to well. Therefore, we decided to determine whether the intensity of the ~38kD band decreased in cells co-transfected with the NS2[SUMO] plasmid were due to actual effects caused by the NEP K72A mutation or to transfection efficiencies. The data obtained revealed that the decrease in the band intensity of the NS2[SUMO] transfected cells was not due to problems or variability on the transfection technique. The expression of EGFP was approximately the same throughout the samples. Conclusively, the lysine to alanine mutation at residue 72 on the NEP Influenza viral protein is causing an effect by decreasing the expression of the ~38kD protein band. This leads to conclude that the K72A mutation could be the main SUMO site for NEP.

3.2.2 Testing the stability of the NS2[SUMO] using CHX treatment

To date, there is no evidence of any modification mediated by SUMOylation on the NEP viral protein. Previous studies have demonstrated that SUMOylation does not affect either the cellular localization or the stability/turnover of the PR8 NS1 [39].Since the NEP mRNA derived from the NS gene segment is a spliced product of the NS1 mRNA, we considered essential to determine whether SUMOylation affects the stability of this viral protein.

Under the conditions described above, both NS2[SUMO] and NS2[non-SUMO] accumulated to similar levels in the cells and appeared stable, not displaying significant decrease in cellular levels in any of the samples analyzed. Furthermore, the enhance SUMOylation observed upon the addition of the NS2[SUMO] construct did not seem to affect the stability of the protein, as similar profiles were observed with the addition of the NS2[non-SUMO] construct. Quantitative analysis performed by the Odyssey CLx infrared imaging system demonstrated little fluctuation in the cellular quantities of NEP throughout the experiment.

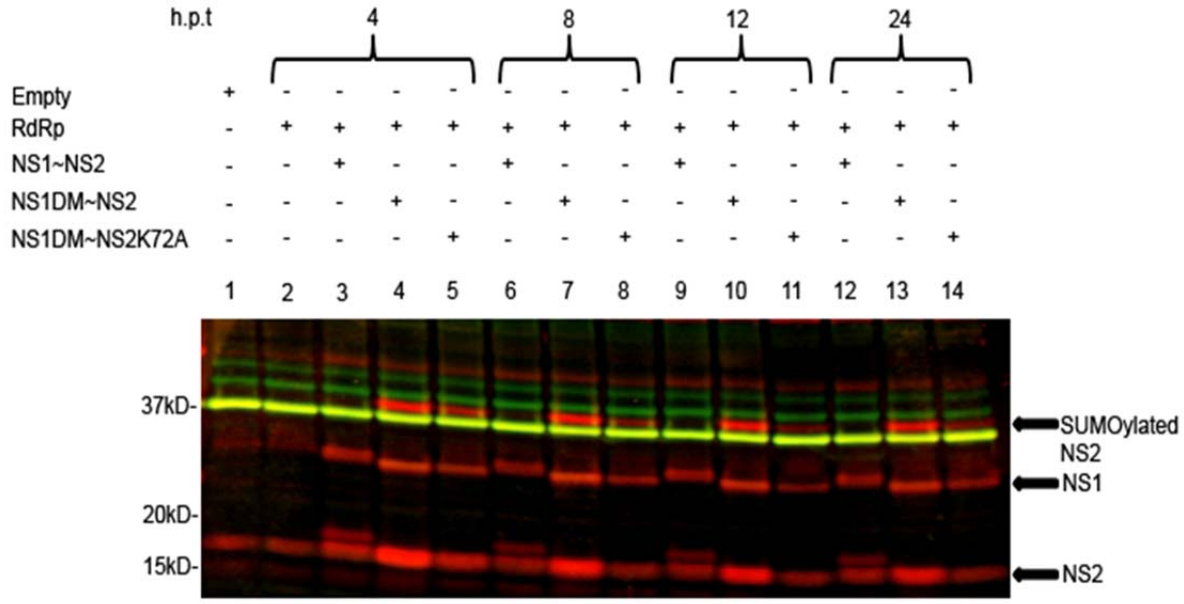

Fig 8. Testing the stability of NS2 mutant construct. HEK293FT cells were co-transfected with the different shifted-forms of the NS2 construct, including the wild-form of the shifted NS2, DM~NS2, and DM~NS2 mutant. CHX was added to the cells at a final concentration of 4mM and collected at 4, 8, 12, and 24 hours post-treatment. Cells were collected using 4X sample buffer and analyzed by SDS-PAGE and Western Blotting.

3.3 Discussion:

As described above, a mutation at lysine residue 72 decreases the SUMOylation of NEP. We were able to prove that the high molecular weight band of NEP in the presence of the non-SUMOylated form of NS1 shifted construct is not an artifact of transfection efficiencies. Also, we were able to prove that the SUMOylation of NEP does not have an effect on the stability of the viral protein.

Chapter 4: Characterized the role(s) exerted by SUMOylation on NEP upon Influenza viral infection

Previous studies done by our group presented NEP as a potential SUMO target. Based on preliminary data from specific aim 2, SUMOylation is binding to NEP through residue K72. Therefore, we will characterize the potential role of SUMOylation on the already known functions of the NEP viral protein as a nuclear export transporter. NEP is the second smallest viral protein in the Influenza A virus. In addition to the ability of NEP to act as a nuclear export of vRNPs, two more roles have been linked to this viral protein during influenza virus replication, i) the ability to contribute to viral budding through the interaction with a cellular ATPase referred as F1F0-ATPase, ii) the capability to regulate the accumulation of influenza virus mRNA, cRNA, and vRNA. As SUMOylation is known to affect the function of a number of proteins from a variety of viruses, the study of the role of SUMOylation on this nuclear export protein is likely to provide exciting insights to the development of novel anti-influenza therapies.

4.1 Materials and Methods

4.1.1 Confocal Microscopy

To monitor the localization of wild type NS2 and the possible SUMOylated form of NEP, MDCK cells will be seeded and infected for 6, 12, 18, and 24 hours with either WSN/T7T7NS1~NS2 and WSN/T7T7NS1K70AK219A~NS2 influenza virus at an MOI of 1. Cells were then fixed with 100ul of 4% paraformaldehyde and incubated for at least 30 min., the cells were fixed and analyzed by immunofluorescence using DAPI as a

DNA dye and anti-NS2 PAb as primary antibodies. We used highly cross- abundant anti-mouse and anti-rabbit secondary antibodies.

4.2 Results

4.2.1 Characterization of the potential effect of SUMOylation on the localization of the viral protein NEP

SUMOylation is known to modify several cellular processes, such as transcription regulation of transcriptional factors, apoptosis, protein stability, sub-cellular localization, and many more. In order to determine if SUMOylation plays a role in the sub-cellular localization of NEP, we will evaluate the effect of SUMOylation on the localization of NEP using a wild type shifted NS2 influenza virus and the double mutant shifted NS2 influenza virus, NS1~NS2 and NS1K70AK219A~NS2 respectively. Both viruses were done by inactivating the splicing the acceptor sites located on NS1 and placing a copy of the second exon with a functional splicing acceptor site for NEP, downstream from the stop codon for NS1. The NS1~NS2 is the wild-type form (no mutations) of the virus and the NS1K70AK219A~NS2 virus contains K to A mutations at positions 70 and 219. To achieve this goal, we will determine if there is any alteration in the localization of the double mutant virus, which exhibits the presence of a higher molecular weight band in the experiments described above and the wild type shifted virus.

4.3 Discussion

As shown in Fig.9, there was a significant change observed in the cellular localization of cells infected with the wild type NEP and the double mutant shifted NEP. As evidenced in the merge images, localization of NEP infected with the DM virus was altered compared to the localization seen in the wild type. In cells infected with the wild type virus, the localization was mainly nuclear, whereas in the cells infected with the DM virus, the localization was mainly homogenously distributed between the nucleus and the cytoplasm. These data confirmed that SUMOylation may be playing a main role affecting the subcellular localization in NEP.

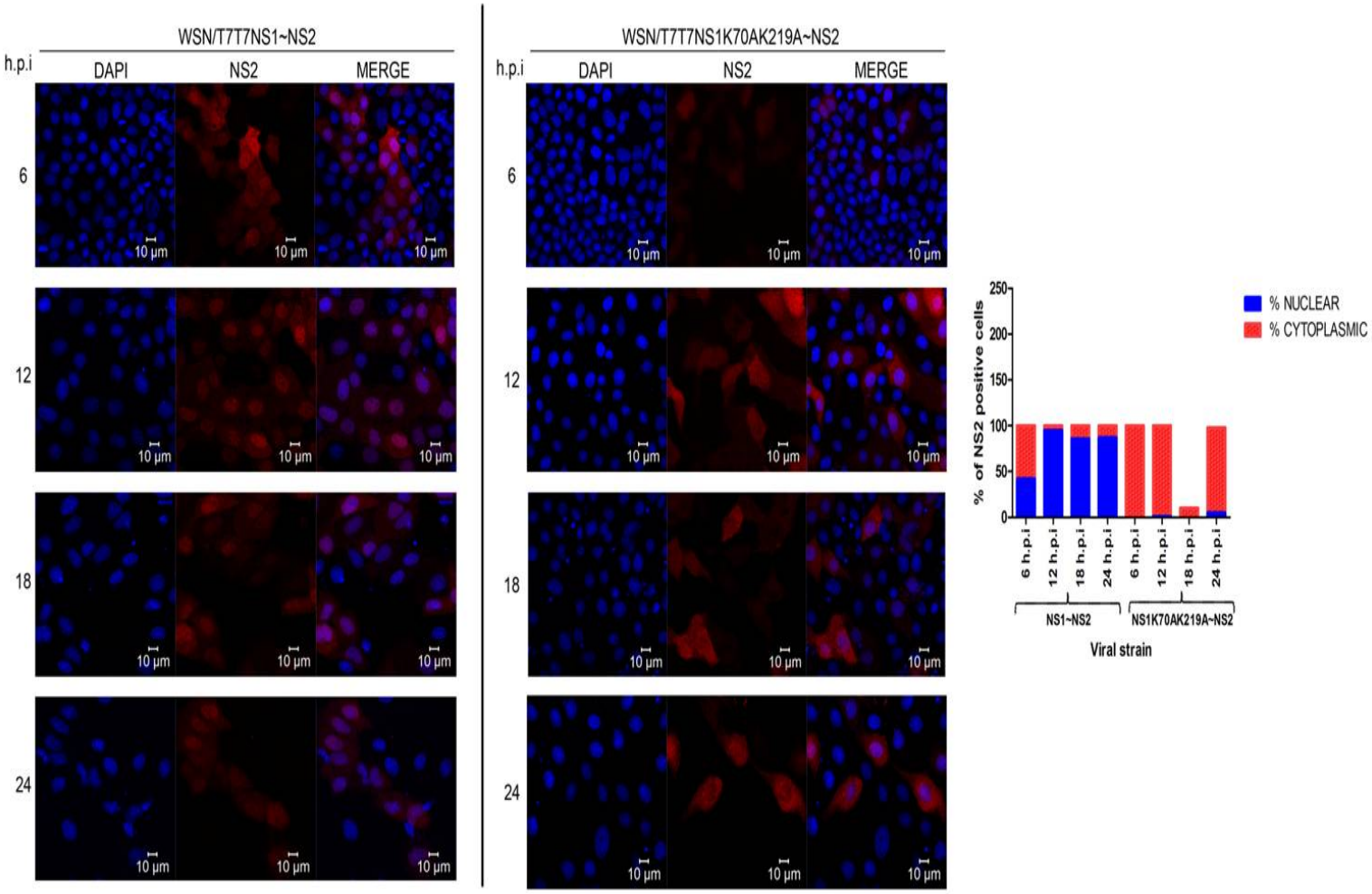

Fig 9. Effects exerted by the SUMOylation system on the localization of NEP upon infection. MDCK cells were infected with either WSN/T7T7NS1~NS2 or WSN/T7T7NS1K70AK219A~NS2 at MOI 1. cells were collected at 6, 12, 18, 24 hours post-infection (h.p.i) and treated with 4% paraformaldehyde. DAPI, anti-NS2 with anti-rabbit secondary antibody were used for detection. Bar, 10 micrometers.

Chapter 5: Summary and Discussion

At the moment, the most effective treatment against influenza virus is vaccination. However, there are a number of caveats with vaccination. First, effective vaccination requires antigenic match between the current viral type being transmitted among the population and the viral strain used during the development of vaccines or the strain present in current vaccines. Second, the high mutation rate of the virus due to the error prone of the RdRp introduces a high variation of the antigenic structure of the virus diminishing the effectiveness of current vaccines. The annual vaccination is targeted to the viral strains that closely resemble the changes occurring in the prevalent viral population. Generally, strain selection and vaccine production are time consuming procedures; vaccine development against a novel pandemic can easily take up to 6 months, thus making vaccination an inappropriate preventive weapon for viral infections during a viral spread.

Currently, there are two available anti-influenza drugs that have been proven to be helpful at preventing transmission of influenza. The first one is known as Adamantanes which target the viral M2 ion channel; and Neuraminidase inhibitors known to target the neuraminidase viral protein. There are two Adamantanes, Amantadine and Rimantadine, which are chemically derived from Adamantanes, and act by blocking the ion channel from the M2 transmembrane viral protein, therefore the acidification of the viral particle is prevented as well as the release into the cytoplasm of the host cell. Also, there are two available neuraminidase inhibitor drugs, Oseltamivir and Zanamivir, clinically referred as Tamiflu and Relenza correspondingly. These drugs

block viral release at the plasma membrane, thus preventing the spread into surrounding cells [45].

The resistance of Adamantanes was noted during its early development, but was not recognized as a real issue until the wide resistance was noticed, leading to the discontinuation of the drug two years later. Consequently, all circulating influenza strains are resistant to Adamantanes and the drug is no longer in use [46].

On the other hand, Oseltamivir was considered to be much more difficult to develop resistance because the mutations involved in providing the resistance to the drug were thought to reduce viral fitness and viral transmission [47]. However, resistance has been reported on the 2009 H1N1 influenza strain, thus making Oseltamivir likely to become resistant to circulating and future influenza viral strains. [48].

Evidently, there is an urgent need for an alternative weapon to combat future influenza viral pandemics. Current anti viral weapons such as vaccination and viral drugs are unlikely to play a major role in the control of highly pathogenic strains during a pandemic. The high mutation rate of the virus targeting viral components makes it difficult to develop a universal drug or vaccine against any strain of the virus. Available viral weapons have been proven to minimize the effect of seasonal influenza pandemics, but are very unlikely to eradicate the infectious disease.

One alternative to combat the virus is to target cellular factors necessary for the viral growth and multiplication of the virus, which are less prone to be affected by the emergence of resistant viral strains. Fortunately, targeting cellular systems of the virus will work independently of the strains and antigenic properties of the virus. This

approach will be of great advantage considering the broad array of subtypes of the virus. The only main drawback is that drugs targeting host cellular factors might be toxic to the host and could exert secondary effects. A wide variety of different antiviral approaches have been studied, leading to the targeting of cellular factors as the most promising approach [49].

Our lab has been the first group to identify several influenza viral proteins efficiently SUMOylated *in vitro* [41]. There is a substantial amount of knowledge on the basic functionality of the non-structural protein, NS1. Despite this, no one has looked at the potential interactions between the cellular system and NEP. In this project, a SUMOplot analysis identified the presence of a highly conserved SUMO motif at K72. As shown in the mutagenesis study, the presence of a high molecular weight, HMW, form of NEP was evident in cells transfected with a NS1DM~NS2 construct, the decrease of this HMW band was measured in samples transfected with the predicted K72 residue mutated in the shifted NS2 portion of the NS1DM~NS2 construct. These results demonstrated an interaction between the cellular SUMOylation system and the viral NEP. Considering the small size of the protein, K72 is likely to be the only SUMO motif present in NEP. Interestingly, the presence of the HMW form of NEP was not present in cells transfected with a non-shifted construct. This suggests a possible competitive biological mechanism between NS1 and NEP for SUMOylation.

Although the biological implications of SUMOylation for NEP still remain unknown, we were able to identify several roles of the protein upon SUMOylation. First, we were able to map what seems as the main SUMO site on NEP. These results were replicated many times throughout the completion of this project. Second, our group

demonstrated that SUMOylation does not affect the stability of PR8 NS1, so we tested for the stability of SUMOylated NEP with similar results. Third, the cellular localization of NEP is affected by the cellular SUMOylation system. The cellular localization of NEP was mainly nuclear in cells infected with a wild-type virus, whereas in the cells infected with the DM virus, the localization was mainly homogenously distributed between the nucleus and the cytoplasm. These data confirmed that SUMOylation is playing a role affecting the sub-cellular localization in NEP. Collectively, this study extended the knowledge of the interplay between the influenza virus and the cellular SUMOylation system, reporting NEP as a SUMO target and recognizing possible crucial roles of the protein upon SUMOylation for the first time.

5.1 Future Directions:

The influenza virus remains as one of the deadliest diseases in history. The increasing resistance of the current viral weapons creates likelihood for a viral outbreak. Analyzing how the influenza virus interacts with the SUMOylation system may provide new tools to develop a broad spectrum antiviral therapy independent of viral strain. In this study, NEP was identified as a new SUMO target. It will be intriguing to analyze the possible effects of the cellular system on the influenza NEP. In order to further understand the role of SUMOylated NEP, a virus coding for NS1DM~NS2K72A will be of great relevance to test the effects of cellular localization of NEP during infection with a non-SUMOylatable NEP. More importantly, studies need to fully demonstrate NEP as a bona fide SUMO target. Tough the precise role of SUMOylation on NEP is still

unknown; the accumulating evidence will provide insights on the biology of the virus and will further support the relevance of exploring SUMOylation as a potential therapeutic agent against the influenza virus.

Appendix A

SUMOylation does not affect either the cellular localization or the stability/turnover of PR8 NS1.

Importantly, for certain substrates, SUMOylation with SUMO2/3 is known to trigger the formation of long poly-SUMO chains that are recognized by ubiquitin ligases and lead to the poly-ubiquitinylation of the SUMOylated substrate, which in turns leads to their proteasomal degradation [50]. Data obtained during the execution of these experiments revealed the formation of poly- SUMOylated NS1 in the presence of wt-ASL and mut-ASL. As these occurred in the absence of exogenously added SUMO, the data indicated that NS1 was likely to be preferentially modified by SUMO2/3 in vivo and therefore suggested a potential role for SUMOylation in regulating the stability of NS1.

Furthermore, a previous report indicated that SUMOylation affects the function of the NS1 protein of a highly pathogenic avian influenza A virus strain by increasing its half-life [51]. Therefore, we considered it essential to determine whether SUMOylation affected PR8 NS1's stability. To measure the effect of SUMOylation on PR8 NS1's stability, we co-transfected HEK293FT cells with expression constructs for wt NS1 and NS1K70AK219A. To potentiate the effects mediated by SUMOylation, some cells were also transfected with mut-ASL.

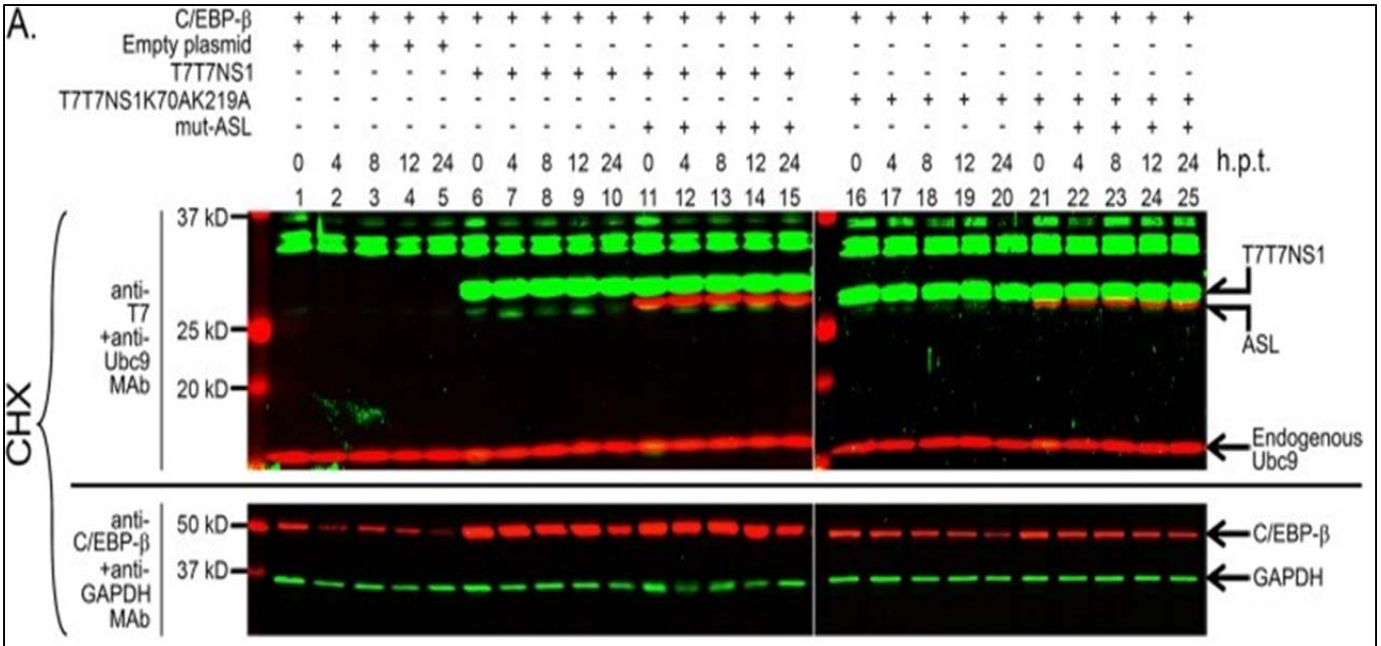

FIG 10. SUMOylation does not affect the stability of NS1. HEK293FT cells were co-transfected with (+) or (-) without the indicted expression constructs, including expression constructs for C/EBP-β and EGFP, which were used as controls. At 24 h posttransfection (h.p.t.), cycloheximide (CHX) was added to the cells at a final concentration of 4 mM, and the cells were collected 0, 4, 8, 12, and 24 h later in 2X sample buffer. The cell extractsgenerated were analyzed by SDS-PAGE and successive rounds of immunoblotting using anti-T7 MAb and anti-Ubc9 MAb, and anti-C/EBP-β MAb and anti-GAPDH MAb, as primary antibodies. Highly cross-absorbed IRDye 800 CW- and IRDye 680 LT- conjugated anti-mouse and anti-rabbit IgG antibodies were used as secondary antibodies. The immunoblots were read and analyzed by using an Odyssey CLx

Additionally, the cells were also co-transfected with C/EBP-β1, a transcriptional regulator that exhibits an intermediate half-life in the cell. Next, at 24 h post transfection, the cells were treated with cycloheximide, an inhibitor of protein synthesis, and samples were collected at 4-h intervals after cycloheximide treatment. Under these experimental conditions, both wt NS1 and NS1K70AK219A accumulated to similar levels in the cell and appeared very stable, not displaying any significant decrease in cellular levels during the time span analyzed (Fig. 11A, compare, for example, lanes 6 and 10 to lanes 16 and 20). Furthermore, the enhanced SUMOylation observed upon addition of mut-ASL did not seem to affect the stability of NS1, as very similar profiles were observed in the presence and absence of mut-ASL (Fig. 11A, compare lanes 11 and 15 to lanes 21

and 25). Quantitative analyses performed by using an Odyssey CLx infrared imaging system in combination with IRDye-conjugated secondary antibodies demonstrated little fluctuation in the cellular quantities of NS1 throughout the experiment (Fig. 11B). Similar analyses performed with the co-expressed protein C/EBP-β1 showed substantial changes in the cellular concentration of C/EBP-β1 throughout the

time frame analyzed, therefore demonstrating that the cycloheximide treatment had been effective (Fig. 11B). Altogether, our stability analysis performed by using cycloheximide treatment, indicated that the non-SUMOylatable form of PR8 NS1 exhibited stability similar to that of its wt form and that co-expression of mut-ASL did not affect their stability, therefore strongly indicating that SUMOylation does not affect the stability of PR8 NS1.

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Curriculum Vita

Karla Prieto was born in El Paso, Texas. The second daughter of Alfredo Prieto and Olga Yee, sister to Jonathan A. Prieto, and twin sister to Melissa Prieto. She graduated high school from Lydia Patterson Institute, El Paso, Texas, in December 2005 and entered El Paso Community College in January 2006. She completed an Associate's degree of Arts in 2008 and transferred to The University of Texas at El Paso, UTEP, in Spring 2009. While pursuing her bachelor's degree in Biological Sciences, she was awarded two Louis Strokes Alliance for Minority Participation, LSAMP, summer fellowships . She presented her research work in videoconferences, posters, and oral presentations. Also, she was selected by LSAMP for an oral presentation at UTEP. After receiving her bachelor's of science degree in Fall 2010, Karla was selected to represent UTEP at the undergraduate research at The Capitol in Austin, Texas. She was also awarded the Research Excellence Undergraduate Student Biology with a Biomedical Concentration award for the class of 2010 and served as the guest speaker for the career development class at Jefferson High School. In January 2011, she worked as a laboratory technician for Dr. Rosas-Acosta and later decided to continue her education by entering the Master's program at UTEP. She was awarded a teaching position for BIOL 1107 laboratory class. In Spring 2011, she was awarded the Bridge to the Doctorate fellowship. She was nominated for the Preparing Future Faculty workshop, and attended the NSF Join Annual Meeting both at Washington, DC. Furthermore, she was featured in the NSF Successful Stories national video. Karla participated in interviews for local news and science videos for elementary schools and

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