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INTERACTIONS OF THE CELLULAR SUMOYLATION SYSTEM WITH INFLUENZA A VIRUS AND ITS NON-STRUCTURAL PROTEIN NS1A (NS1A)

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ABSTRACT

The most important current anti-influenza weapons, vaccination and antiviral drugs, can be rapidly rendered fully ineffective thanks to the virus's high mutational rate, which produces viruses exhibiting new antigenic properties and structural proteins insensitive to the drug's mechanism of action. One attractive alternative is to develop drugs that modulate the activity of cellular systems either required for viral growth or able to neutralize viral growth. Here we demonstrate that the cellular SUMOylation system, a post-translational modification involving the conjugation of the Small Ubiquitin-like MOdifier (SUMO) to specific protein targets using a Ubiquitin-like enzymatic cascade, interacts closely with influenza virus during infection and therefore provides new targets for the development of anti-influenza therapeutics targeting cellular components. Briefly, in vitro SUMOylation assays performed using in vitro synthesized viral proteins demonstrated that most influenza viral proteins are readily SUMOylated in vitro and therefore constitute potential SUMO targets. Transfection experiments leading to the over-expression of specific viral proteins in the presence or absence of various recombinant DNA constructs designed to modulate the activity of the cellular SUMOylation system demonstrated that various viral proteins are also SUMOylated when over-expressed in mammalian cells. Furthermore, experiments performed using recombinant adenoviruses able to modulate the activity of the cellular SUMOylation system demonstrated the SUMOylation of specific viral proteins during influenza infection. Finally, this study characterizes the SUMOylation of the non-stuctural viral protein NS1A, the best SUMO target of all the viral proteins produced during infection, explores some of the potential effects exerted by SUMOylation on the

functions of this viral protein, and introduces the use of “artificial SUMO ligases” as an innovative method to increase the SUMOylation of specific targets in the cell and facilitate the characterization of the effects mediated by SUMOylation on protein function. Collectively, our studies provide new insights into the virus-host interactions established during influenza virus infections.

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CHAPTER 1: INTRODUCTION

Influenza is an acute contagious respiratory disease characterized by recurrent annual epidemics and occasional but major worldwide pandemics. Despite all the efforts and current available anti-influenza measures, influenza remains a global public health concern due to the significant morbidity and mortality associated with it. The most effective way to combat influenza is through vaccination, but this measure works efficiently only when the circulating strains of influenza virus match perfectly the vaccine strains. Natural mutations or antigenic drift, which occur frequently due to the highly variable nature of this virus, produce a severe drawback against the efficacy of annual vaccination, imposing the need to reformulate the vaccines every year depending on the predicted circulating viral strains [1]. The other current anti-influenza weapons are anti-viral drugs, which target structural components of the virus. To date there are only two types of successful anti-viral drugs against influenza, but the selective pressure and low fidelity of the viral polymerase render the virus prone to develop resistance against all available antiviral drugs. In this context, there is an urgent need to develop novel broad-spectrum tools against influenza virus that will work irrespective of the type, strain or antigenic properties of the virus. In recent studies, the multifunctional Non-Structural Protein 1 (NS1A) of influenza virus has emerged as an attractive target for antiviral drug development [2, 3]. In this study, we explored how influenza A virus and its Non-Structural Protein 1 (NS1A) interact with the cellular SUMOylation system, a post-translational modification system known to regulate diverse biological processes but previously unknown to have a link with influenza infection, with the ultimate goal of

evaluating whether this cellular system may provide new targets for the development of innovative therapies against influenza.

1.1 Influenza virus Biology:

Influenza virus is the prototype member of the family Orthomyxoviridae. It is an enveloped RNA virus with a single-stranded, negative sense segmented genome. The genome contains eight segments of viral RNA (vRNA) encoding 10-11 viral proteins, as listed in Table 1 [4, 5]. Most of the RNA segments encode single proteins with the exception of segments 7 and 8, and in some strains segment 2 as well, all of which code for two different proteins. Out of the three types of Influenza viruses, (A, B and C), Influenza A and B are important for human health. The antigenic determinants of the virus are two surface glycoproteins, Haemagglutinin (HA) and Neuraminidase (NA), which are embedded in the lipid membrane envelope. There are 16 HA and 9 NA subtypes of the virus, which are capable of generating over 100 viral subtypes [6].

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

RNA Segment	Protein formed	Probable function
1	PB2	Polymerase component; Required for host cap binding
2	PB1	Polymerase component; Catalytic sub-unit of polymerase
	PB1-F2	Induction of apoptosis
3	PA	Polymerase component; Active in genome replication; Required for endonuclease activity
4	HA	Antigenic determinant; Receptor binding and fusion with host cells
5	NP	Nucleoprotein; Viral assembly and RNA synthesis
6	NA	Antigenic determinant; Release of new viral particles
7	M1	Matrix protein; Viral assembly

	M2	Trans-membrane Ion channel; Release of vRNPs upon viral envelope fusion to endocytic vesicle
8	NS1A	Non-structural protein; Interferon antagonist; Inhibitor of host antiviral responses; Modulator of mRNA splicing and translation
	NS2	Nuclear export protein; Viral assembly

Influenza is unusual among RNA viruses by replicating its genome in the nucleus of the host cell. During influenza infection, a virion attaches to sialic acid receptors in the host cells via receptor binding sites in HA and enters the cells via receptor mediated endocytosis to an endosome. The endosomal vesicle becomes increasingly acidic due to the influx of H^+ ions carried by lysosomal vesicles. Simultaneously the inside of the viral particle also becomes more acidic, due to the transmembrane M2 protein, which forms an ion channel in the viral envelope, thus allowing the influx of H^+ ions. The acidic environment within the viral particle promotes disruption of protein-protein interactions, specifically the interaction of matrix protein M1 and nucleocapsids. In addition to that, low pH also triggers a non-reversible conformational change of the structure of HA. Altogether, these events lead to the fusion of the viral membrane with the endosomal membrane resulting in the uncoating of the virus and release of vRNPs in the host cytoplasm. From there the vRNPs translocate to the nucleus via nuclear pores. Once inside the nucleus, the three sub-units of the polymerase complex (the RNA dependent RNA Polymerase or RdRp), PB2, PB1 and PA, activate primary transcription leading to the production of viral mRNA transcripts by a 'cap snatching' mechanism. Following binding of PB2 subunit to the 5' m^7G caps of cellular mRNA transcripts, PA serves as the viral endonuclease that steals the 5' caps of cellular transcripts [7], which is then used as the primer for the viral transcriptase, PB1 to mediate viral mRNA transcription [8]. Transcription gives rise to eight primary transcripts, but the last two primary

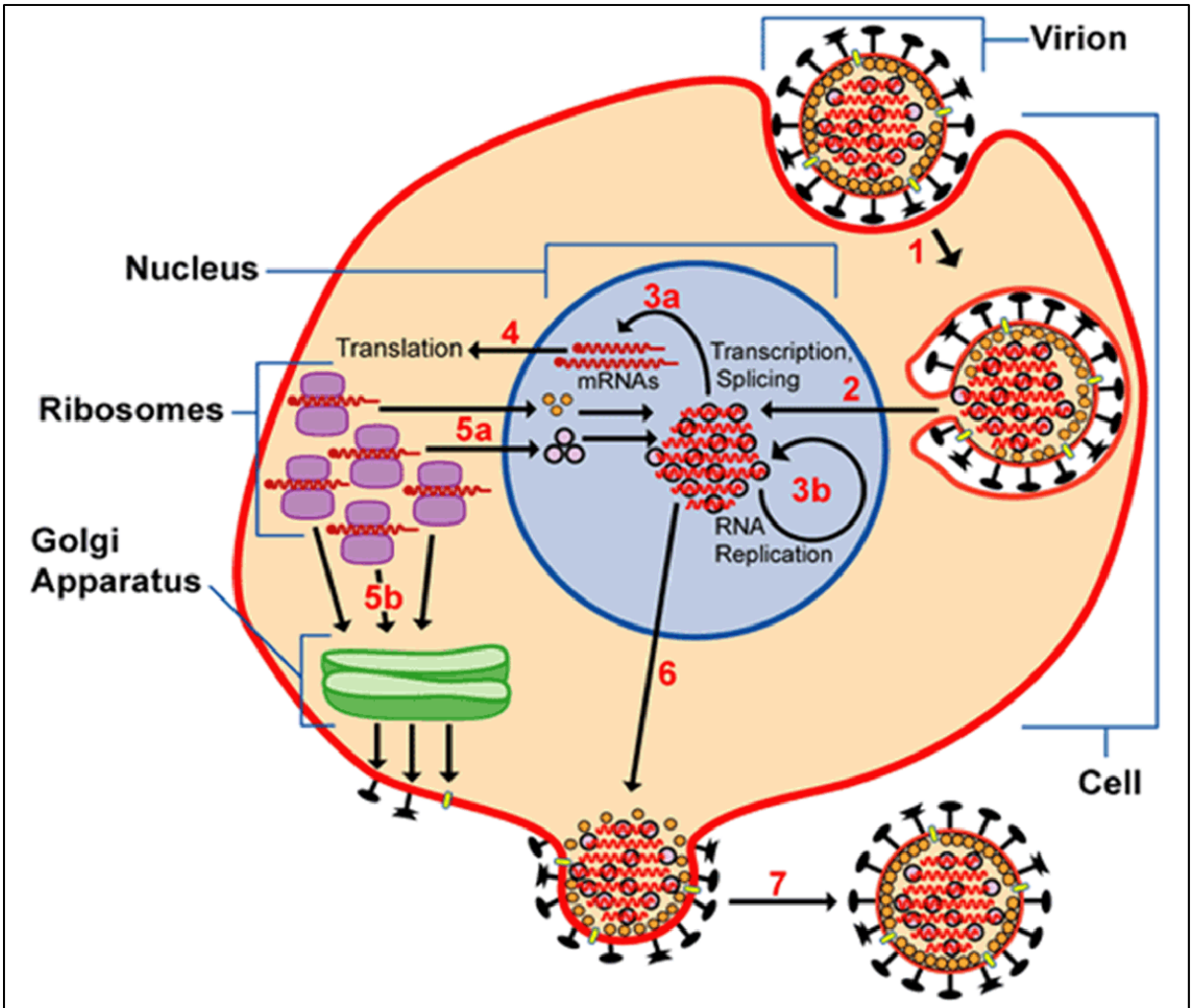

Figure 1. Schematic diagram of Influenza virus replication.
(1) Binding and entry of influenza virus to the host cell via endocytosis and fusion of viral membrane to endosomal membrane, (2) release of vRNPs and nuclear entry, (3a) Transcription of viral RNA within the nucleus and (3b) replication of viral RNA, (4) nuclear export and translation of viral mRNA, (5a) Nuclear and (5b) membrane targeting of influenza proteins, (6) nuclear export of viral ribonucleo protein complexes and viral assembly at plasma membrane, (7) viral budding. (http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/virusreplication4)

transcripts (M and NS) each generate two alternative transcripts via alternative splicing. The transcribed mRNAs migrate to the cytoplasm to be translated. After translation, some viral proteins follow the secretory pathway (HA, NA, M2) to travel towards the cell membrane while others are transported back to the nucleus (RdRp, NP, M1, NS1A, NS2). Once the initial proteins are translated, they stimulate viral genome replication to make new vRNAs, which happens later during infection as it requires encapsidation of

replication intermediates mediated by NP. The binding of M1 to the nucleocapsid formed by the tight association of NP and vRNA shuts down viral transcription and, with the help of Nuclear Export Protein (NS2/NEP), the nucleocapsid migrates towards the cell membrane. Meanwhile, the proteins following the secretory pathway (HA and NA) undergo different modifications (e.g. Glycosylation) and along with M2 become incorporated into the plasma membrane. M1 interacts both with nucleocapsids and membrane-inserted HA or NA, driving the budding of new virions from the cell and the release of new progeny virus upon sialic acid cleavage by NA [5, 6, 9, 10]. The schematic diagram of Figure 1 illustrates the life cycle of influenza virus, from its initial binding to the host cell to the ultimate release of infected virions from the cell.

1.2 Role of NS1A in Influenza A infection:

To counteract host defense mechanism viruses have evolved multiple strategies. NS1A is the most important virulence factor encoded by Influenza A virus, and it is known to exert multiple functions during infection to antagonize host immune responses and to promote viral pathogenicity. This 26 kDa non-structural protein is one of the major players during influenza infection as it antagonizes the interferon (IFN) response and a plethora of cellular antiviral responses mounted upon viral infection [11]. It was shown previously that mutant influenza viruses lacking NS1A gene can efficiently replicate only in an IFN deficient system [12]. Additionally, some studies indicated that NS1A serves as a major virulence factor for the highly pathogenic avian influenza virus [13] and also for highly pathogenic human influenza viruses with pandemic potential [14].

The NS gene (8th viral genome segment) is the smallest genome segment of influenza virus and it encodes for two mRNA splice variants, NS1A (NS1A) and NS2 (NEP). NS1A is expressed in very high levels and localized predominantly in the nucleus. It has a variable length in different influenza viruses ranging from 230 to 237 amino acids [11].

Functionally NS1A can be divided in two distinct functional domains, the N-terminal RNA binding domain (RBD) (amino acids 1-73) and C-terminal Effector domain (ED) (amino acids 74-230), which contains a disordered tail at its C-terminal end [11, 15]. The N-terminal RBD is mainly required for sequestration of dsRNA and direct blocking the activity of two antiviral cytoplasmic proteins, Protein Kinase R (PKR) and 2'-5- Oligoadenylate Synthetase (OAS). Both of these proteins mediate host antiviral response in a dsRNA dependent process. NS1A RBD outcompetes OAS for binding to dsRNA and helps the virus to overcome the OAS/RNAse L antiviral mechanism of the host, which is induced by IFN response. At the same time, RBD of NS1A also competes with PKR for binding to dsRNA, thus preventing the auto-activation of PKR following dsRNA binding that is required to shut off the host translational machinery and limit production of viral proteins. Additionally the RBD is also essential for viral pathogenesis due to its ability to suppress the RNAi response in the host cells [16]. The C-terminal ED of NS1A is important for binding to a number of host factors such as a cellular protein called Cleavage and Polyadenylation Specificity Factor (CPSF), which plays an essential role in the processing the 3' end of cellular mRNA transcripts. This interaction inhibits host mRNA production. ED also binds to the p85β subunit of phosphatidylinositol 3-kinase (PI3K), which results in activation of PI3K signaling

pathway and inhibition of apoptosis. Altogether the functions of NS1A lead to an efficient influenza virus infection by overcoming host antiviral pathways [2, 15]. Besides these key functions, NS1A is known to perform a plethora of other activities in influenza infected cells as outlined in Figure 2.

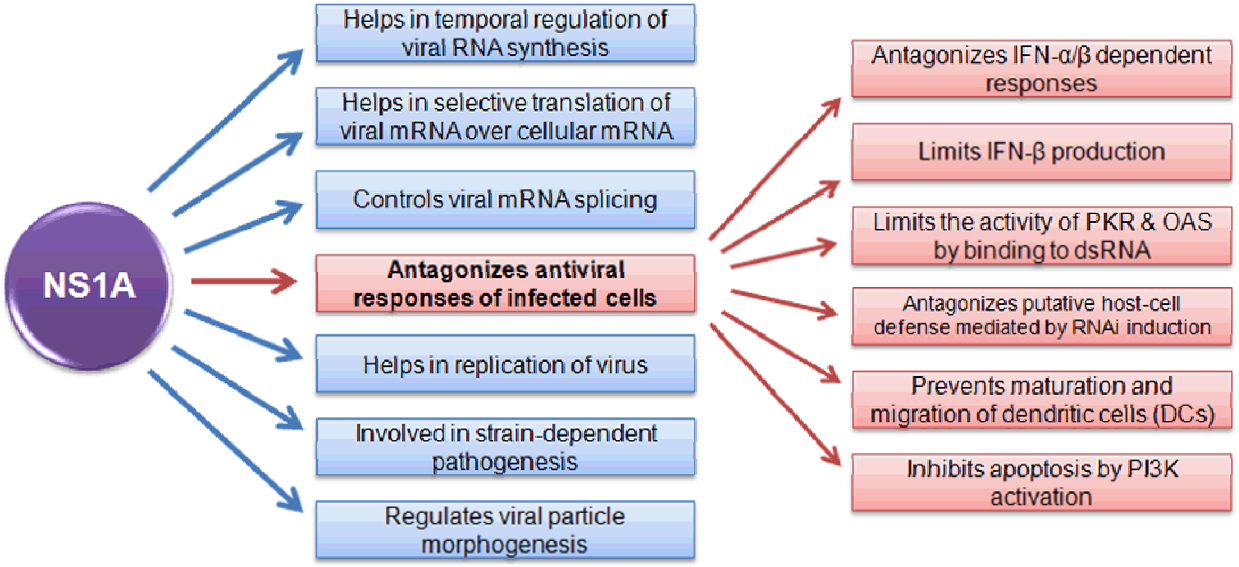

Figure 2. Multiple functions mediated by NS1A protein in influenza infected cells.

Though in influenza virus infected cells NS1A predominantly localizes in the nucleus, later during infection a significant proportion of the protein has also been observed in the cytoplasm. Depending on the strain of influenza virus, NS1A may contain one or two nuclear localization sequence(s) (NLS), and one nuclear export sequence (NES). The first NLS (NLS1) of NS1A is a monopartite NLS that is highly conserved, involving residues R35, R38 and K41. In contrast, the second NLS (NLS2) is a bipartite NLS, which is absent in some viral strains and involves residues K219, R220, R231 and R232 [11]. The cytoplasmic distribution of NS1A may be directed due to the presence of a latent NES sequence located within residues 138-147 [17].

NS1A is known to be post-translationally modified by phosphorylation [18, 19, 20] but the precise biological role of this modification is yet to be elucidated. In recent

studies, this protein also has been reported as the target for ISG15 [21, 22]. ISG15 modification of NS1A at a single residue (K41) is associated with loss of function of NS1A thus affecting efficient viral replication, serving the purpose of antiviral action mediated by the cells [21]. Finally, we recently reported that NS1A is also targeted by cellular SUMOylation [23], as presented in this thesis.

1.3 The cellular SUMOylation system:

SUMOylation is a post-translational modification involving the covalent conjugation of SUMO (Small Ubiquitin-like MOdifier) protein to lysine residue(s) in the target protein. SUMO is synthesized as an immature precursor protein that is proteolytically cleaved by SUMO-specific peptidases (SENP) to expose a C-terminal di-glycine motif required for conjugation. Mature SUMO is activated by a heterodimeric E1 activating enzyme (SAE2/1) in an energy-consuming process. Subsequently, SUMO is transferred to the E2 conjugating enzyme (Ubc9), which conjugates it to the target protein. This step is facilitated by E3 ligases, which increase the specificity and speed of the reaction but are not absolutely required for SUMOylation. SUMO is conjugated to the target protein via an isopeptide bond formed between the carboxyl group of its C-terminal glycine residue and the epsilon amino group of a lysine residue in the target protein. The modified lysine residues are frequently (but not always) located within the consensus motif ΨKXE, where Ψ is a bulky hydrophobic residue, K is the target lysine to be modified, X is any amino acid, and E is glutamic acid. In the final step, Sentrin(SUMO)-specific proteases (SENPs) efficiently cleave the isopeptide bond between SUMO and its target, rendering both available for subsequent rounds of

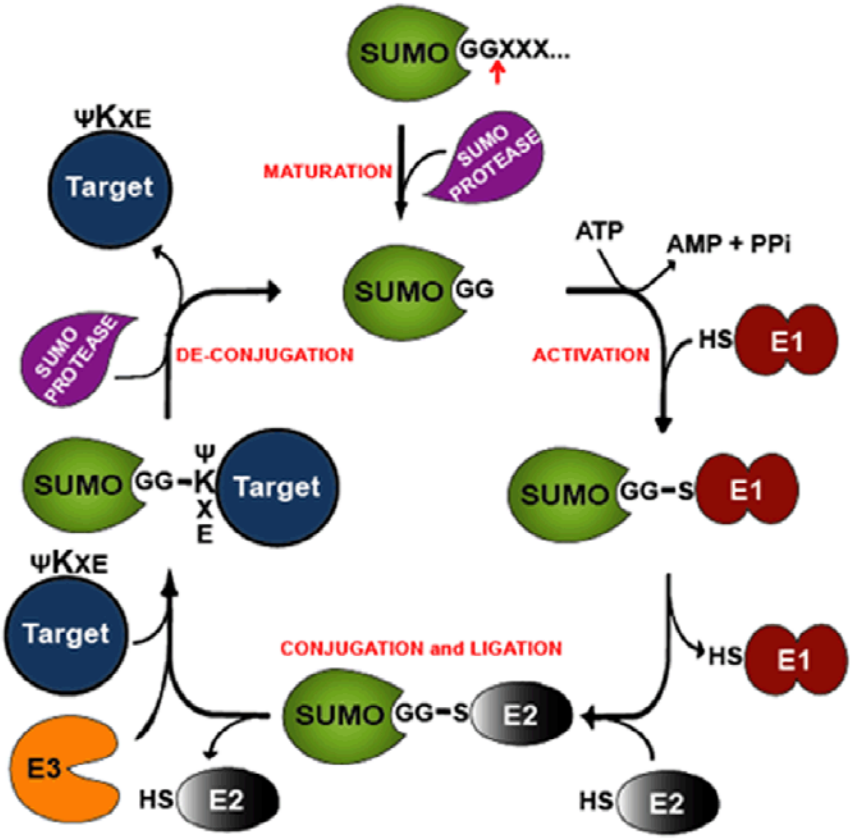

Figure 3. The SUMOylation pathway.
SUMO is synthesized as an immature precursor form whose maturation requires proteolytic cleavage of its C-terminal end. Following that SUMO is activated by a heterodimeric E1 activating protein in an ATP dependent manner and transferred to an E2 conjugating enzyme. The last step of SUMO conjugation includes transferring SUMO from the E2 conjugating enzyme to a lysine residue in the target protein forming an isopeptide bond in a process that is sometimes but not always facilitated by an E3 ligase. Following conjugation,SUMO specific proteases cleave the isopeptide bond between SUMO and its target protein, making the process reversible and dynamic.

modification, and making the pathway a reversible and dynamic process, as shown in Figure 3 [24, 25, 26, 27].

SUMO proteins belong to the Ubiquitin-like Modifier (UBL) family although they share less than 20% similarity with ubiquitin [24]. These proteins are approximately 10 kDa in size and as per our current knowledge, four mammalian SUMO isoforms have been isolated so far, SUMO 1-4, [24]. Among the SUMO isoforms, mature forms of

SUMO2 and SUMO3 are 97% identical with each other, so they are commonly referred as SUMO2/3 [24, 26], but they share only approximately 50% similarity with SUMO1 [24, 28]. Under normal physiological conditions SUMO1 conjugation seems most predominant in the cell while SUMO2/3 remains mostly in the free non-conjugated form [24, 29]; however, SUMO2/3 become heavily conjugated to target proteins under stress conditions such as heat shock or hypoxia [26, 29]. The targets for SUMO1 and SUMO2/3 appear to be distinct from each other, therefore suggesting different functional roles for SUMO1 than SUMO2/3 [24, 27, 29, 30]. In general, SUMO conjugation results from the addition of single SUMO moiety to the target proteins, though SUMO2/3 is known to form poly-SUMO chains owing to the presence of an internal SUMOylation site in SUMO2/3 [24, 27]. Most of all the SUMOylation that occurs in the cell appears to take place in the nucleus, but increasing evidence supports the idea that SUMOylation occurs ubiquitously throughout the normal cell [24], and it exhibits effects on diverse biological functions ranging from signal transduction, sub-cellular localization, DNA damage repair, chromosome segregation, transcriptional regulation, stress response and cell death [26, 31]. An emerging role for SUMOylation is its potential relevance during infections with different viruses [31, 32, 33].

1.4 Development of an 'Artificial SUMO Ligase':

Though SUMOylation was first discovered more than a decade ago, the identification and characterization of new SUMO targets remains challenging due to the very low steady-state levels observed for the SUMOylated form of any given protein in the cell. Except for a few exceptional SUMO targets, at any given time the SUMOylated

form of a protein is only a very small fraction of the total amount of the protein present in the cells (usually less than 1-5%) [34, 35]. Several studies have been performed with the purpose of developing methods that will increase the level of SUMOylation of a particular protein and therefore facilitate the analysis of the functional consequences of SUMOylation. Among other methods, the development of SUMO-Ubc9 fusion [34] and Ubc9 fusion directed SUMOylation (UFDS) [35] have proven quite advantageous in analyzing the function of SUMOylation, though both of these methods have their own caveats. As its name indicates, SUMO-Ubc9 fusions involve the over-expression by transfection of a SUMO protein fused to Ubc9. This method has proven beneficial for the identification of SUMOylated proteins, specifically of those containing a SUMO interacting motif (SIM) [36], but it has very limited use for the analysis of the functional consequence of SUMOylation for a particular target as it increases the global SUMOylation of all proteins in the cell and decreases the specificity of the analysis. In contrast, UFDS involves the fusion of Ubc9 to the specific protein under study, and therefore it dramatically increases the SUMOylation of a particular protein, thus helping its functional analysis [35, 36]. UFDS has proven useful even for the identification of very weakly or transiently SUMOylated proteins [35] but it requires the formulation of an Ubc9 fusion to the target protein and therefore restricts SUMOylation only to the fused target protein and may alter its ability to establish specific protein interactions due to the presence of Ubc9 as a structural component of the fusion protein.

In this study we report the development of the “artificial SUMO ligase” approach that is based on the fusion of Ubc9, not to the SUMO target protein but to the interaction domain from a known interacting partner, which can be a domain responsible for

dimerization in the case of proteins known to form dimers. The fusion here thereof produced works as an artificial SUMO ligase by bringing Ubc9 in close proximity to the SUMOylation site in the target protein and stabilizing its interaction with the target protein, therefore specifically increasing the SUMOylation of the target protein. The most relevant advantage over the other methods provided by the “artificial SUMO ligase approach” is that it increases endogenous SUMOylation of a given protein to a remarkable extent, therefore helping the functional analysis of the effect of SUMOylation in a protein specific manner without increasing the global SUMOylation of the cell.

1.5 Viruses and SUMOylation:

SUMOylation is an important modulator of protein function, and provides a new paradigm of virus-host interactions. Viruses normally interact with the SUMOylation system either by modulating the system to enhance viral propagation or by utilizing the system to secure the proper function of specific viral proteins [32, 33]. Alternatively, the host also may utilize the SUMOylation system as a way to control viral infection. Even though there are four known isofoms of SUMO identified in mammals, viruses appear to interact mostly and preferably with SUMO1 [32]. Currently, there is very little evidence demonstrating interactions of viral proteins with SUMO2/3 other than for Papilloma virus E2 proteins (both human and bovine) [37] and human cytomegalovirus immediate-early IE2 regulatory protein [38].

On the contrary, SUMO1 conjugation has shown the capability to modify a broad range of viral proteins from different viruses such as the Human Cytomegalovirus Immediate-early protein 1 (HCMV-IE1), for which SUMO1 conjugation of the IE1 protein

appears required for the full activity of this protein [39], thus contributing to efficient viral replication. The 86 kDa Immediate-early protein 2 of Human Cytomegalovirus (HCMV-IE2) was also shown to be SUMO1 modified, and SUMO1 conjugation appears to be important for IE2 mediated transactivation of two early viral promoters [40]. The Bovine Papilloma virus E1 Protein (BPV-E1) also serves as a substrate for SUMO1 and for this protein SUMO1 modification appears important for its helicase function and intranuclear distribution, and nuclear export [41, 42]. For the Human T-cell leukemia virus Tax oncoprotein, SUMO1 modification is required for its retention in the nuclear compartment [43]. SUMOylation of the Adenovirus Type 5 early region 1B oncoprotein (Ad5-E1B) is required for its transformation activity [44], and for the Hepatitis delta antigen of the Hepatitis delta virus, SUMOylation modulates viral RNA synthesis [45].

The SUMOylation system also appears to exert some antiviral activities as suggested by the fact that many viruses evolved different mechanisms to inactivate or control the host's SUMOylation system, as exemplified by the Gam1 protein, a viral protein produced by an avian adenovirus. This protein promotes the ubiquitinylation of the SUMO activating enzyme E1, thus leading to its proteasomal degradation and a subsequent block in the system [46]. Similarly, Ebola Zaire virus hijacks the host SUMOylation machinery to suppress innate immunity by SUMOylating IRF-7 [47]. Epstein-Barr virus and Human Cytomegalovirus block the SUMO modification of the PML protein, thus leading to the disruption of the PML nuclear bodies, a step required to induce lytic infections [48, 49]. Finally, the covalent attachment of SUMO1 to Human immunodeficiency virus type 1 (HIV-1) Gag polyprotein (p6 domain) exemplifies

utilization of SUMO modification by the host system against viral infection as SUMOylation of p6 seems detrimental to HIV-1 replication [50].

Recently one genome-wide short interfering RNA (siRNA) screening in human lung epithelial cells (A549) identified SUMOylation as one of the host machineries required by influenza virus for effective viral multiplication [51]. Furthermore, a combinational approach consisting of yeast two hybrid screenings along with genome-wide expression profiling identified Ubc9 (UBE21), the SUMO conjugating enzyme, as a direct interactor sor several influenza viral proteins [52]. Given that influenza virus is one of the few RNA viruses that replicate inside the nucleus of the host cells and that most of the influenza virus proteins bear conserved consensus SUMOylation motif(s) as predicted by SUMOsp prediction software and summarized in Table 2, [53], it seems possible that SUMOylation may play important roles during influenza virus infection.

1.6 Overall goal of this study:

This study pursued two major goals: 1) Determine whether any influenza viral protein is SUMOylated during infection; and 2) Characterize the SUMOylation of one of the viral SUMO targets identified. Throughout its execution, this study has demonstrated a correlation between influenza virus infection and host cell SUMOylation system by identifying several influenza viral proteins targeted by SUMOylation. Additionally, this study has characterized the multifunctional NS1A protein of influenza virus as a *bona fide* target for the SUMOylation system and identified the main SUMOylation sites in this protein. Though the biological implication of SUMOylation for NS1A is yet to be fully elucidated, an artificial SUMO ligase developed during this study to increase the

SUMOylation of NS1A in a specific manner will greatly facilitate its functional analysis in the future. Altogether this line of research provides new insights about the interaction of influenza virus with the cellular SUMOylation system.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cells and viruses used: HEK293A cells (Invitrogen Corp., Carlsbad, CA), A549 cells (ATCC, Manassas, VA), and MDCK cells (ATCC) were maintained in 1x Dulbecco's Modified Essential Medium (DMEM) supplemented with high glucose, L-glutamine, sodium pyruvate, and 10% fetal bovine serum. The cells were maintained in a 37^{o}C incubator at 5% CO_2. Influenza A/PR/8/34 (H1N1) and A/Vic/77 (H3N2) were a gift from Dr. John M. Quarles (Dept. of Microbial and Molecular Pathogenesis, College of Medicine, Texas A&M Health Science Center) and were propagated in MDCK cells at MOI 0.001 using 1x DMEM supplemented with 0.2% bovine serum albumin and 2 µg/mL TPCK-treated trypsin (Worthington Biochemical Corp., Lakewook, NJ).

2.2 Plasmids: The individual expression plasmids coding viral genes were constructed following the method previously demonstrated by Hoffmann et al. [54]. All eight viral genes from Influenza A/PR/8/34 (PB2, PB1, PA, HA, NP, NA, M & NS) were cloned by reverse transcription-PCR (RT-PCR) using first a UNI-12 primer targeting the 12 conserved nucleotides at 3' end of extracted viral RNA from infected supernatants using MagMAX™ viral RNA isolation kit (Applied Biosystems, Foster City, CA), and subsequently with PCR reactions using segment specific forward and reverse primers to amplify cDNA copies of each gene segment of influenza virus and inserted into pcDNA3.1 mammalian expression vector (Invitrogen Corp., Carlsbad, CA) following In-Fusion™ Dry-Down PCR Cloning method (Clontech, Mountain View, CA).

2.3 In-vitro SUMOylation assays: *In vitro* sumoylation assays were performed as reported in earlier studies [55]. All different viral proteins were synthesized from maxipreped DNA and labeled with ^{35}S-Methionine, using a coupled transcription/translation rabbit reticulocyte system under the control of the T7 promoter (Promega Corp., Madison, WI). Subsequently, the ^{35}S-labeled protein products were incubated at 30°C for 90 minutes in the presence of either, 1 µg of the purified catalytic domain of the yeast de-SUMOylating enzyme Ulp1, or a mix of 1.5 µg purified SUMO1, 280 ng E1 activating (SAE2/1) and 1 µg E2 conjugating (Ubc9) enzymes of SUMOylation. To confirm the identity of any extra higher molecular weight band observed in the latter reaction as a SUMOylated form of the target protein, an additional sample was incubated with a mix of purified SUMO1 and the E1 and E2 SUMOylation enzymes in each case, but 30 minutes after the beginning of the reaction, 1 µg of Ulp1 was added to the sample. All reactions were performed in the presence of a previously described SUMOylation reaction buffer containing 50 mM Tris pH 8.0, 5 mM MgCl2, 5 mM ATP, and 0.5 mM DTT [55]. The reactions were stopped by the addition of 4X sample buffers (50mM Tris pH 6.8, 10% glycerol, 4% SDS, 0.01% bromophenol blue, 2% β-mercaptoethanol). The samples were then subjected to be resolved in 10% SDS-PAGE gels and developed by autoradiography. The recombinant construct for the expression and purification of Ulp1403-621 was kindly provided by Dr. Christopher D. Lima (Structural Biology Program, Sloan-Kettering Institute, 1275 York Avenue, New York, NY).

2.4 Transient transfections and transductions with Recombinant Adenoviruses: HEK293A cells were seeded at the density of 10^5 cells/well in 24 well plate, next day transfected with desired combinations of plasmids using TransIT®-LT1 (Mirus Bio LLC, Madison, WI) according to the manufacturer's recommendations and cell extracts were collected either following 24 hours post-transfection when further analyzed by immublotting or 48 hours post-transfection when analyzed for affinity purifications. Transductions with recombinant adenoviruses as described in our previous report [23] were performed in A549 cells at a multiplicity of infection (MOI) of 100, using fully confluent cell cultures. The transduced cells were used for the desired experimental procedure 48 h post-transduction to allow appropriate expression of the adenovirus-encoded transgenes.

2.5 Affinity Purification of SUMOylated proteins: For the affinity purification of all SUMOylated proteins, cells grown and treated as necessary in 6 well plates were collected in 300 µL of denaturing buffer (8 M Urea, 100 mM NaH_2PO_4, 10 mM Tris, 0.2% Triton X-100, 20 mM NEM, pH 8.0), and the resulting cell extract was sonicated to breakdown the chromosomal DNA and decrease the viscosity of the sample. A 50 µL aliquot of the resulting extract was mixed with an equal volume of 4x sample buffer for direct immunoblot analysis of Total Cell Extract (TCE). The remaining 250 µL were diluted with 3,750 µL of dilution buffer (100 mM NaH_2PO_4, 10 mM Tris, 0.2% Triton X-100, 20 mM NEM, pH 8.0), and incubated with 100 µL (wet volume) of S-Protein agarose beads (Novagen, EMD Biosciences Inc.) for 16 h at 4^oC. The diluted cell extract-bead mixture was poured over an empty chromatography column and the packed beads were washed with 30 mL of ice-cold 1x PBS, and the bound proteins

were eluted by the addition of 100 µL of 4x sample buffer, to be analyzed by immunoblotting in 10% SDS-PAGE gels.

2.6 Immunoblot analysis: Monolayers of cells previously treated by transfection, transduction and/or infection as needed were lysed in 2X sample buffer and following breakage of chromosomal DNA using using a 29½ gauge needle were heated for 3 min at 100°C after addition of 10% β-mercaptoethanol. The samples were resolved by 10% SDS-PAGE gels, using either pre-made PAGEgel™ gels (PAGEgel Inc., San Diego, CA) or SDS-PAGE gels made in-house. The resolved proteins were transferred onto a PVDF Immobilon™-P membranes (Millipore Corp, Bedford, MA) and blocked with 3% non-fat milk in 1X PBS with 0.5% Tween for 30 min. at room temperature. After blocking the membrane, in each case the membrane was incubated with primary antibody overnight at 4°C and subsequently incubated for 1 hr. at room temperature with anti-rabbit or anti-mouse secondary antibody. The membranes were developed using the Immobilon™ Western HRP Substrate system (Millipore). In most experiments the membranes were re-used a minimum of three times. Before re-use, the Immobilon™ membranes were stripped by incubation in boiling stripping buffer (1% SDS and 0.2% β-mercaptoethanol) for 10 min, and washed five times with 1x PBS supplemented with 0.05% Tween 20.

The following primary and secondary antibodies were used in this study for western blotting: anti-T7 tag® mouse MAb (Novagen, EMD Biosciences Inc., San Diego, CA) at 1:5,000 dilution; anti-NP mouse MAb clone AA5H (Meridian Life Science, Inc., Memphis, TN) at 1:2,500 dilution; anti-M1 mouse MAb clone GA2B (Meridian Life

Science, Inc.) at 1:5,000 dilution; anti-SUMO1 rabbit MAb Y299 (Epitomics Inc., Burlingame, CA) at 1:5,000 dilution; anti-PB1 goat polyclonal antibody vK-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:2,500 dilution; anti-PB2 amino terminus mouse MAb clone 170-3C12 (obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH) at 1:1,000 dilution; anti-NS1A mouse MAb, clone NS1A-1A7 (BEI Resources) at 1:1,000 dilution; anti-Ubc9 rabbit MAb EP2938Y (Epitomics Inc.) at 1:5,000 dilution; anti-GAPDH mouse MAb 2D4A7 (Santa Cruz Biotechnology, Inc.) at 1:5,000 dilution; anti-SUMO2 rabbit PAb (Invitrogen) at 1:5,000 dilution: anti-mouse IgG goat polyclonal antibody HRP-conjugated (Santa Cruz Biotechnology, Inc.) for detection of all mouse MAbs; anti-rabbit IgG goat polyclonal antibody HRP-conjugated (Santa Cruz Biotechnology, Inc.) for detection of all rabbit MAbs; and anti-goat IgG donkey polyclonal antibody HRP-conjugated (Santa Cruz Biotechnology, Inc.) for detection of all goat polyclonal antibodies.

2.7 SUMOylation site prediction: The SUMOylation site predictions for all different protein components of Influenza virus were done using SUMOsp 2.0 software [53], where the cut-off value for predictions was set to medium except otherwise indicated. Two prediction softwares, SUMOsp2.0 [53] and SUMOplot™ [56], were used for identifying potential SUMOylation site(s) in NS1A.

2.8 Site-directed mutagenesis: A T7 tag (MASMTGGQQMG) was introduced at the N-terminus of NS1A protein for easier detection using Phusion® Site-Directed Mutagenesis Kit (Finnzymes, Woburn, MA) following manufacturer's protocol. For mutagenesis study to map the SUMOylation site in NS1A, same kit (Finnzymes) was

used for generating the SUMOylation deficient mutant clones and so for generation of T7 tagged NS2 construct.

2.9 Immunofluorescence Analysis: For immunofluorescence analyses, cells were fixed with 4% paraformaldehyde and sequentially permeablized with 100% methanol and 0.5% Triton X-100 in 1x PBS for 10 min each. Upon permeabilization, the cells were incubated in blocking solution (1x PBS supplemented with 3% goat serum) followed by incubation with primary antibodies. Following primary antibodies were used for immunofluorescence: anti-T7 tag® mouse MAb (Novagen) at 1:2,000 dilution, anti-Ubc9 rabbit MAb EP2938Y (Epitomics Inc.) at 1:1,000 dilution and anti-NS1A mouse MAb (BEI Resources) at 1:1,000 dilution. Alexa Fluor® 488 goat anti-mouse (highly cross-absorbed) and Alexa Fluor® 594 goat anti-rabbit (highly cross-absorbed) (both from Invitrogen Corp.) were used as the secondary antibodies. All procedures after the addition of the secondary antibody were executed in the dark. Images were captured using a Nikon TS-100 fluorescence microscope and a Nikon DS-2M Color Digital Camera.

2.10 Cycloheximide treatment: For cycloheximide treatment, HEK293A cells were seeded at the density of 10^5 cells/well in 24 well plate, transfected with WT T7NS1A or T7NS1AK70A/K219A, and 24 hours post-transfection treated with 100 µg/ml cycloheximide (Sigma-Aldrich Corp.,St. Louis, MO) for the indicated time periods, after which the cell lysates were collected in 2X sample buffer and prepared for immunoblotting as indicated above.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 All Influenza viral proteins contain conserved SUMOylation site(s):

In silico sequence analysis of all protein components of influenza virus by prediction software SUMOsp 2.0 interestingly revealed the presence of one or more consensus motif(s) in the sequences of all influenza viral proteins tested as listed below in Table 2. Even though there are plenty of proteins reported to be SUMOylated at lysine residues lacking the SUMO consensus motif, and proteins containing the SUMO consensus motif are not necessarily SUMO targets [29], this analysis suggested that there may be some connection between the cellular SUMOylation system and influenza virus.

Table 2: Predicted SUMOylation site(s) in different influenza viral proteins (predicted by SUMOsp 2.0):

Protein	Position	Peptide sequence	Type
PB2	189	TKE**K**KEE	Type: Non-consensus
	190	KEK**K**EEL	Type: Non-consensus
	339	SSV**K**REE	Type: Ψ-K-X-E
	699	ILG**K**EDK	Type: Non-consensus
	718	NLA**K**GEK	Type: Ψ-K-X-E
PB1	176	SMN**K**EEM	Type: Non-consensus
	612	VCL**K**WEL	Type: Ψ-K-X-E
	736	GRI**K**KEE	Type: Ψ-K-X-E
	737	RIK**K**EEF	Type: Non-consensus
	757	RRQ**K*****	Type: Non-consensus
PA	29	EDL**K**IET	Type: Ψ-K-X-E
	139	NKI**K**SEK	Type: Ψ-K-X-E

	158	MAT**K**ADY	Type: Non-consensus
	361	KNM**K**KTS	Type: Non-consensus
HA	521	DGV**K**LES	Type: Ψ-K-X-E
NP‡	7	QGT**K**RSY	Type: Non-consensus
	113	LYD**K**EEI	Type: Non-consensus
NA	242	KIF**K**IEK	Type: Ψ-K-X-E
M1	21	GPL**K**AEI	Type: Ψ-K-X-E
	104	RKL**K**REI	Type: Ψ-K-X-E
	187	TTA**K**AME	Type: Non-consensus
	252	QRF**K*****	Type: Non-consensus
NS1A	70	RIL**K**EES	Type: Ψ-K-X-E
	219	PKQ**K**REM	Type: Non-consensus
NS2(NEP)‡	18	RMS**K**MQL	Type: Non-consensus

‡ The cut-off value for SUMOylation site prediction for these proteins was set to LOW, for all others it was MEDIUM.

3.2 Influenza infection increases overall cellular SUMOylation levels:

To evaluate whether influenza virus infection affected overall cellular SUMOylation following influenza infection in a cell culture system, MDCK cells were infected at different infectious viral particles to cell number ratios (a proportion referred to as Multiplicity of Infection, or MOI), using two influenza viral strains, A/PR/8/34 H1N1 and A/Vic/77 H3N2. Unfractionated (total) cell extracts of the infected cells were collected 15 hrs. post-infection (p.i.) and evaluated by immunoblotting using anti-SUMO1 antibody. An increase in overall SUMOylation (SUMO1 conjugation) following influenza virus infection was observed, as demonstrated by the appearance of new intermediate molecular weight bands and an increase in the intensity of the high molecular weight SUMO bands upon viral infection [Figure 4A]. However, no significant differences were observed in the steady-state levels of the SUMO E2 conjugating enzyme Ubc9 upon viral infection [Figure 4B]. Anti- NS1A immunoblot analysis

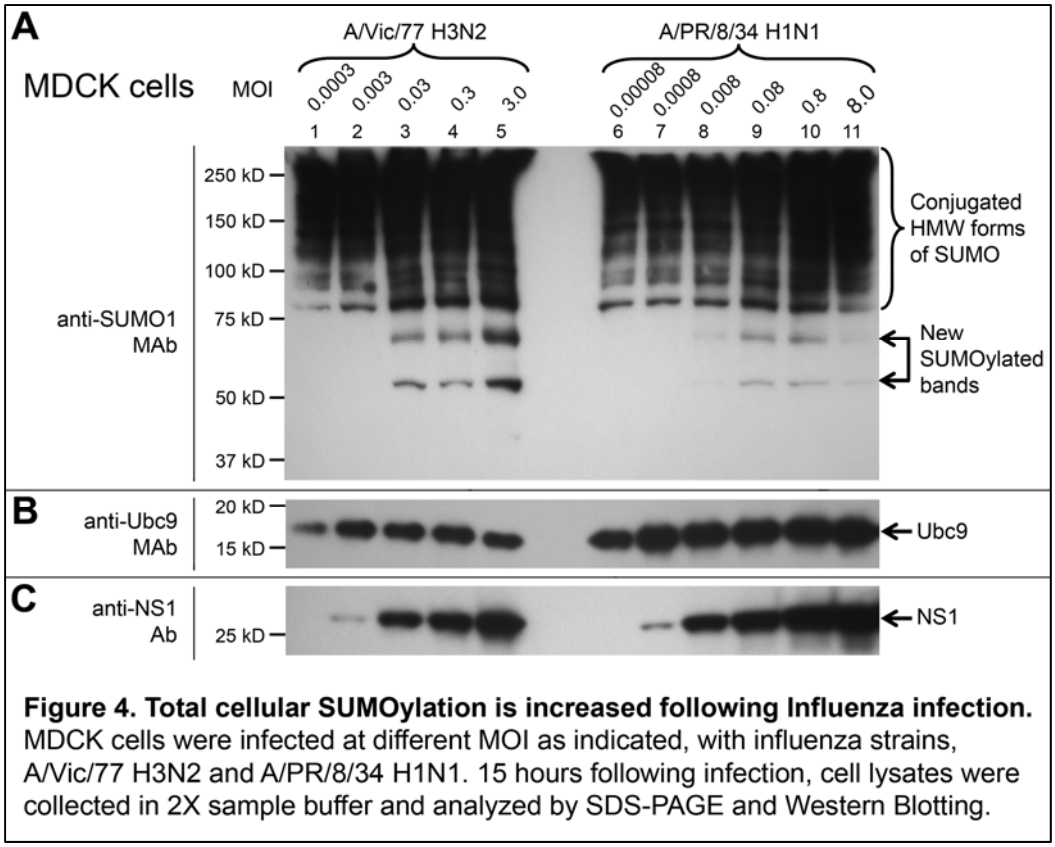

Figure 4. Total cellular SUMOylation is increased following Influenza infection. MDCK cells were infected at different MOI as indicated, with influenza strains, A/Vic/77 H3N2 and A/PR/8/34 H1N1. 15 hours following infection, cell lysates were collected in 2X sample buffer and analyzed by SDS-PAGE and Western Blotting.

confirmed the expected increase in the expression of viral proteins upon increased MOI [Figure 4C]. Altogether, these observations strongly support an interaction between the cellular SUMOylation system and Influenza A virus.

3.3 Most influenza viral proteins are SUMOylated in vitro:

The *in vitro* SUMOylation assay is a quick and convenient screening method to determine if any given protein is modified by SUMOylation. Following the procedure described by Rosas-Acosta et al. [55], and using a set of cDNA expression constructs spanning the full array of influenza virus genes derived from our laboratory strain of Influenza A/PR/8/34 H1N1 (cloned by the author), we tested the SUMOylation potential of most of the virus-encoded proteins. In these assays we found that most of the influenza viral proteins, including the three sub-units of the viral RNA polymerase (PB2,

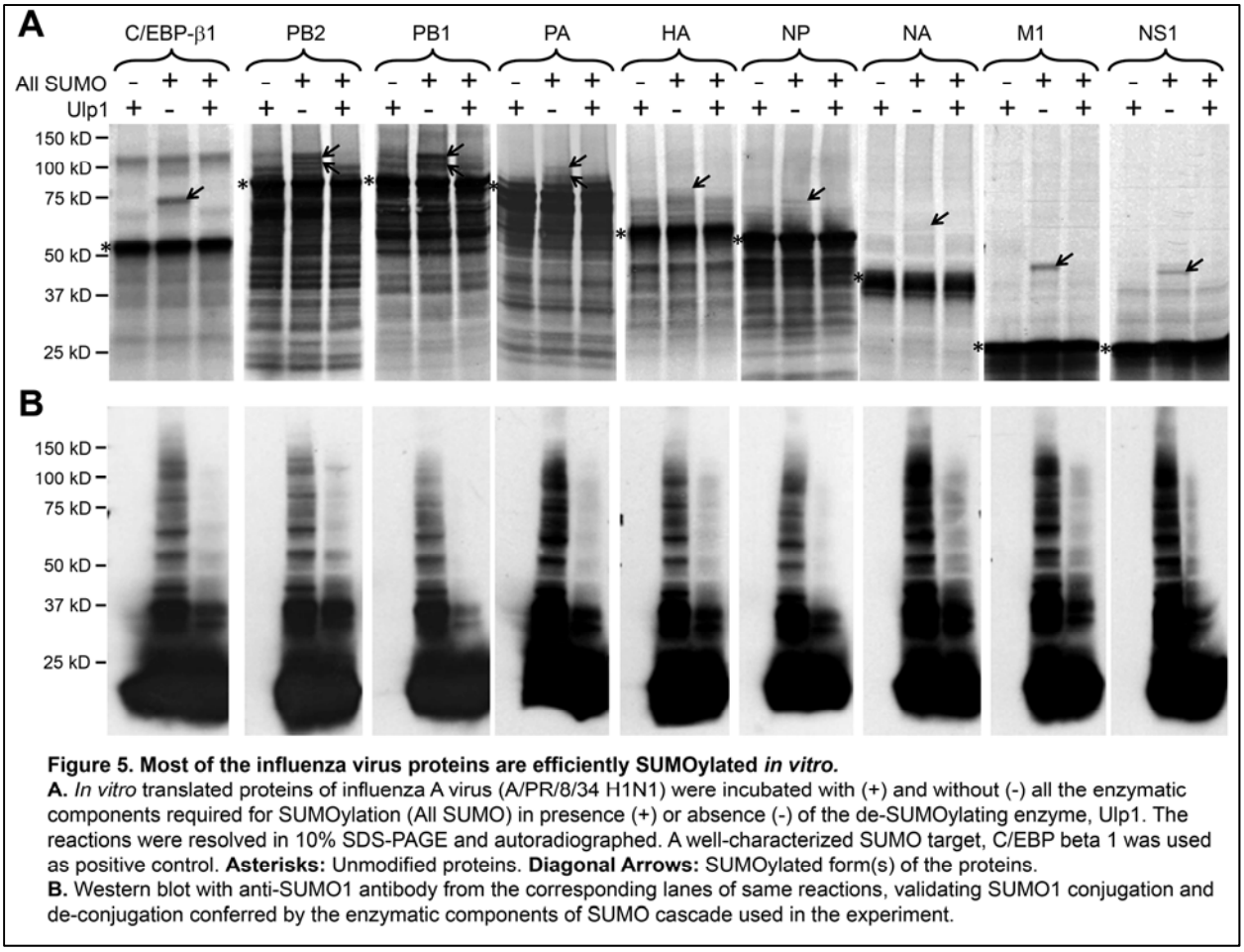

Figure 5. Most of the influenza virus proteins are efficiently SUMOylated *in vitro*.
A. *In vitro* translated proteins of influenza A virus (A/PR/8/34 H1N1) were incubated with (+) and without (-) all the enzymatic components required for SUMOylation (All SUMO) in presence (+) or absence (-) of the de-SUMOylating enzyme, Ulp1. The reactions were resolved in 10% SDS-PAGE and autoradiographed. A well-characterized SUMO target, C/EBP beta 1 was used as positive control. **Asterisks:** Unmodified proteins. **Diagonal Arrows:** SUMOylated form(s) of the proteins.
B. Western blot with anti-SUMO1 antibody from the corresponding lanes of same reactions, validating SUMO1 conjugation and de-conjugation conferred by the enzymatic components of SUMO cascade used in the experiment.

PB1, PA), and the viral proteins HA, NP, M1 and NS1A are all very efficiently SUMOylated *in vitro*. As shown in Figure 5A, the appearance of one or more higher molecular weight form(s) in the middle lane for most of the in vitro translated and ^{35}S-labeled proteins in presence of all SUMO components (indicated by arrows), and its disappearance in presence of all SUMO components following incubation with the de-conjugating enzyme Ulp1, conclusively demonstrated that the higher molecular weight form(s) observed correspond to SUMOylated forms of the proteins being tested, and that most of the viral proteins, at least seven out of the eight protein tested, served as targets for cellular SUMOylation system. Western blot analysis with anti-SUMO1 antibody [Figure 5B] validated the findings, as conjugated higher molecular weight

forms of SUMO1 were observed in the middle lanes of each of the groups in the absence of Ulp1, and the deconjugated free form of SUMO1 was the main product observed following Ulp1 incubation. Altogether, the data indicates that most of the influenza virus proteins of Influenza A/PR/8/34 H1N1 are subjected to modification by SUMO1. In this experiment, C/EBP-β1, a well characterized SUMO target was used as positive control [57].

3.4 Few viral proteins are SUMOylated when over-expressed by transfection:

In a recent report, we demonstrated that dicistronic constructs containing the SUMO1 or SUMO3 ORF followed by the internal ribosomal entry site sequence of the Endomyocarditis virus (EMCV) and the Ubc9 ORF, both under the control of the cytomegalovirus promoter, were able to induce robust SUMOylation, when transfected in mammalian cells and therefore provide an efficient method to identify SUMO targets in a cell culture system [23]. Following co-transfection in HEK293A cells with (+) and without (-) the combinations of indicated plasmids including the dicistronic plasmid for SUMO1 (referred as Dual S1/I/U), and the purification of SUMOylated proteins by affinity chromatography on S-protein agarose beads, owing to the presence of an S-tag at the N-terminus of the SUMO1 encoded in the dicistronic SUMO1 construct, we have found that at least three influenza virus proteins are targeted by the cellular SUMOylation system in a mammalian cell culture model [Figure 6]. Analysis of total cell extracts (TCE) [Figure 6A & B] by western blot revealed the presence of a high molecular weight form for both T7 tagged NS1A and M1 [Figure 6A, diagonal arrows, lanes 5 and 17, respectively], when the indicated protein was over-expressed along with

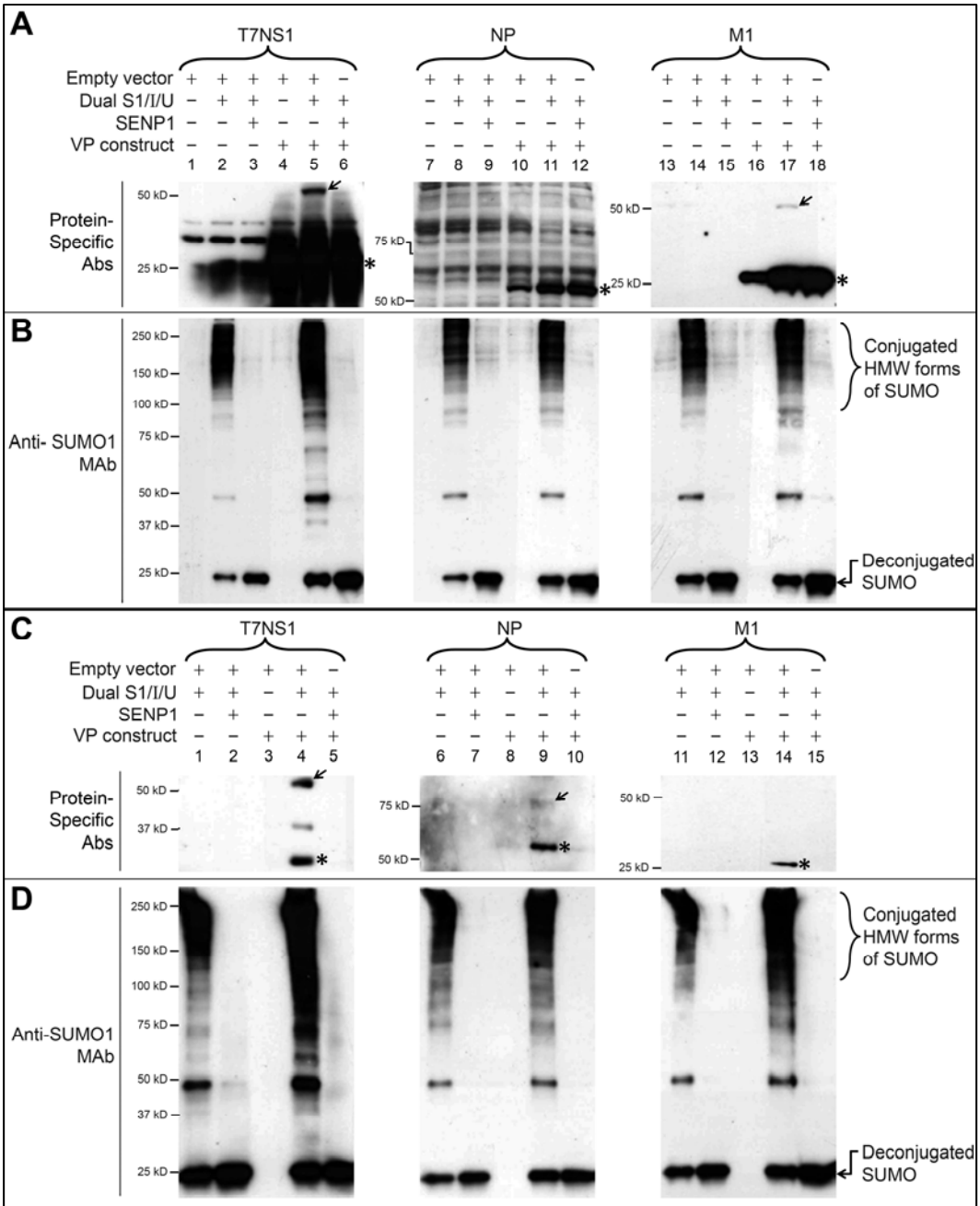

Figure 6. Several Influenza virus proteins (NS1, NP & M1) are SUMOylated in transiently transfected mammalian cells.

Panel A, B. Total cell extracts (TCE). HEK293A cells were transfected with (+) and without (-) the indicated plasmids carrying expression cassettes for abovementioned proteins. 48 hours post transfection, cells were lysed in a denaturing buffer containing 8 M urea, and total cell extracts were analyzed by SDS-PAGE and Western Blotting. Panel A refers to western blots with protein specific antibodies, Panel B refers to western blots with anti-SUMO1 antobody.

Panel C, D. S-beads Purified Proteins. TCE collected in 8 M urea buffer were subjected to further dilution of urea to 500 mM. Protein from resulting lysates were purified on S-beads by affinity chromatography and analyzed by SDS-PAGE and Western Blotting. Panel C refers to western blots with protein specific antibodies, Panel D refers to western blots with anti-SUMO1 antibody.

VP constructs: Contructs encoding viral protein as indicated on top of the profiles. **Asterisks:** Unmodified proteins. **Diagonal Arrows:** SUMOylated form of the proteins.

exogenous SUMO1 and Ubc9 (co-transfected with Dual S1/I/U). The disappearance of

the higher molecular weight form of the protein when co-expressed with the combinationof SUMO1 and mammalian de-SUMOylating enzyme SENP1, conclusively demonstrated that the extra higher molecular weight forms observed in the indicated lanes truly correspond to SUMOylated forms of T7NS1A and M1. Total Cell Extracts (TCE) analysis did not reveal presence of any similar higher molecular weight form of NP but longer exposure of the membrane was hindered by presence of other cross-reactive bands. The global effects on cellular SUMOylation produced by co-expression of SUMO1 and Unc9 in the presence and absence of the de-SUMOylating enzyme SEMP1 are clearly evident on the SUMO-immunoblot presented in Figure 6B.

The protein profile obtained upon purification on the S-protein beads demonstrated that T7NS1A and NP are authentic SUMO targets *in vivo*. In the presence of Dual S1/I/U, a significant proportion of unmodified T7NS1A was purified on the S-beads [Figure 6C, asterisk, lane 4], along with a higher molecular weight form corresponding to the molecular weight of SUMOylated T7NS1A [Figure 6C, diagonal arrow, lane 4]. The absence of the higher molecular weight form of the protein in the presence of excess SENP1, associated with increased de-SUMOylating activity [lane 5], further supported the conclusion that T7NS1A is efficiently SUMOylated in a cell culture system. The presence of unmodified T7NS1A even after affinity purification may be a result of the dimerization of NS1A, thus allowing the purification of unmodified forms along with the modified protein. Alternatively it could be due to the co-purification of de-SUMOylating enzymes on the beads which become activated during the last washes or during the last stages of sample processing in preparation for SDS-PAGE. Interestingly, an extra band between the SUMO-modified and unmodified forms of T7NS1A were

observed, specifically at around ~37 kD. This band might correspond to SUMOylated NS2 (NEP), which is the splice variant of NS1A, as the molecular weight of the band fits nicely with the predicted size of the SUMOylated form of NS2. Subsequence experiments have provided conclusive support to this conjecture.

Similar results were observed for NP, for which a higher molecular weight form, visible in presence of Dual S1/I/U [diagonal arrow, lane 9], disappeared in presence of SENP1, thus indicating that NP is also targeted by the cellular SUMOylation system. Surprisingly, no higher molecular weight form of M1 was visible following S-beads purification [Figure 6C, lane 14], although it was obvious in the immunoblot analysis of the TCE. However, the presence of unmodified form of M1 in presence of Dual S1/I/U [asterisk, lane 14], but not in M1 alone [lane 13] or in presence of excess SENP1 [lane 15], indicated that M1 is also likely to be SUMOylated in cell culture, although the intensity of the SUMO modified and S-beads purified form of M1 may not be as intense as compared to others. Figure 6D showed the global profile of the SUMO1 conjugation and de-conjugation profile and fits nicely the expected profile.

3.5 Several viral proteins are also SUMOylated following infection:

To determine whether any of the influenza virus proteins is SUMOylated during infection, A549 cells were transduced with recombinant adenoviruses carrying either the dicistronic SUMO1 construct (AdV-Dual S1/I/U) or a mutated version of the construct containing a deletion of the two C-terminal glycine residues in SUMO1(AdV-Dual S1ΔGG/I/U). The terminal di-glycine motif is essential for SUMO conjugation, so deletion of the di-glycine motif in AdV-Dual S1ΔGG/I/U renders the over-expressed

SUMO1 non-conjugatable but still capable of binding to Ubc9, therefore producing a block in the SUMOylation cascade. The latter construct (AdV-Dual S1ΔGG/I/U) served as ideal negative control for our study. 48 hours post-transduction, the cells were infected with either of two strains of Influenza A virus, A/PR/8/34 H1N1 or A/Vic/77 H3N2 and samples were collected 15 hours post-infection in a denaturing buffer containing 8M urea as described previously. TCE were subjected to SDS-PAGE and Western Blotting analysis and the SUMO-modified proteins were purified on S-protein agarose beads and analyzed separately by SDS-PAGE and Western Blotting.

As shown in Figure 7, two of the viral proteins (M1 and NS1A) are SUMOylated during viral infection, two (NP and PB1) are likely to be modified by SUMO following infection, and one (PB2) seems not to be modified by SUMO. Importantly, all viral proteins were easily detected in the TCE, but higher molecular weight forms suggestive of SUMO-modification were not observed for any of the viral proteins in samples infected with A/PR/8/34 H1N1 or A/Vic/77 H3N2. In sharp contrast, the S-beads purified samples showed clear evidence of SUMOylation for M1 and NS1A. From the S-beads profile in Figure 7D, it is evident that the M1 protein from both strains of Influenza A virus tested are equally targeted by the cellular SUMOylation system, as implied by the profile observed of SUMO modified M1 bands [Figure 7D, lanes 8 & 11, diagonal arrows]. The absence of the high molecular weight bands in lanes with over-expressed but non-conjugatable SUMO1 (AdV-Dual S1ΔGG/I/U) [Figure 7D, lanes 9 & 12] demonstrated that the bands observed are SUMOylated forms of M1. Along with the SUMOylated form of M1, a significant proportion of unmodified M1 protein was also co-purified from S-beads purification, but the absence of any unmodified form of M1 in

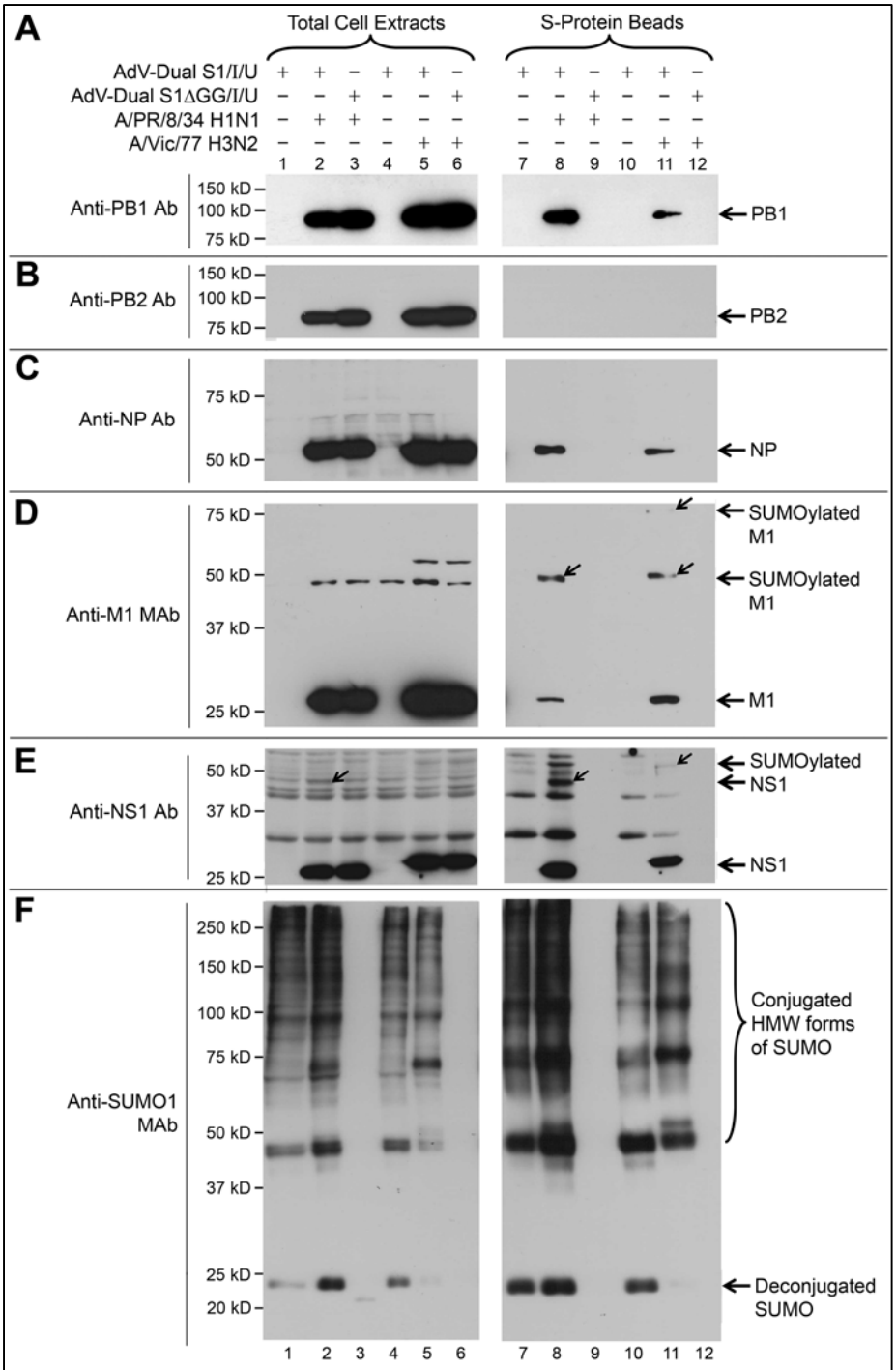

Figure 7. Several viral proteins are SUMOylated in influenza infected cells following over-expression of SUMO1 by adenoviral transduction.
A549 cells were transduced with recombinant adenoviruses carrying Dual S1/I/U and non-conjugatable Dual S1deltaGG/I/U at MOI 100. 48 hours post transduction, the cells were infected with Influenza strains A/PR/8/34 H1N1 and A/Vic/77 H3N2 at MOI 3. Samples were collected 15 hous post-infection in a denaturing buffer containing 8 M urea and Total Cell Extracts (TCE) were analyzed by SDS-PAGE and Western Blotting. Same samples were subjected to further dilution of urea to 500 mM and proteins from resulting lysates were purified on S-protein agarose beads by affinity chromatography and analyzed by SDS-PAGE and Western Blotting (WB).
Panel A-E: WB with Influenza protein specific antibodies. **Panel F:** WB with anti-SUMO1 antibody.
Diagonal Arrows: SUMOylated form of the proteins.

lanes transduced with AdV-Dual S1ΔGG/I/U [Figure 7D, lanes 9 & 12], indicated that

the purification of the unmodified form of the protein in S-beads was likely due to the co-purification of de-SUMOylating enzymes in the S-protein beads. Similar results were observed with NS1A also, where SUMO modified forms of NS1A were observed from samples infected with both strains of Influenza virus [Figure 7E, lanes 8 & 11, diagonal arrows], though the modified higher molecular weight form of NS1A was much more prominent in the sample infected with A/PR/8/34 H1N1, than in the sample infected with the A/Vic/77 H3N2. Along with the modified form, significant amount of unmodified NS1A was also detected when transduced with AdV-Dual S1/I/U but not with AdV-Dual S1ΔGG/I/U, indicating that the potential co-purification of cellular de-SUMOylating enzymes.

Other than the influenza viral proteins discussed above, which are clearly SUMOylated during infection, PB1 and NP are the other proteins that are likely to be targeted also by the cellular SUMOylation system. Although both the TCE and S-protein beads purified protein profiles for either protein did not reveal any high molecular weight forms suggestive of SUMOylation, the co-purification of the unmodified form of the protein in cells transduced with AdV-Dual S1/I/U but not in cells transduced with AdV-Dual S1ΔGG/I/U [Figure 7A and 7C, S-protein beads profile], indicated that SUMO conjugation was responsible for the co-purification of the unmodified form of the proteins. Given that at any time point the proportion of the SUMO modified form of any protein is significantly less than the unmodified form, it was very likely that the SUMO modified form was below the detection level for the antibody. Altogether the data indicated that PB1 and NP are also likely to be authentic SUMO targets.

Interestingly, although PB2 was modified by SUMO1 in the *in vitro* SUMOylation assay, we observed no indication that it was modified during infection, as neither the modified nor the unmodified forms of the protein were detected on the S-protein beads [Figure 7B]. The TCE profile confirmed that the protein was present in the samples infected with both strains, thereby suggesting that PB2 is not targeted by SUMO1 modification during viral infection. The western blot profile obtained with the anti-SUMO1 antibody [Figure 7F] followed the expected profile, showing a substantial increase in high molecular weight SUMO1 conjugates in samples transduced with the AdV-Dual S1/I/U but not with the AdV-Dual S1ΔGG/I/U.

3.6 NS1A is modified even with endogenous SUMOylation:

Among all the viral proteins tested, NS1A seemed the best SUMO target. To determine whether NS1A is modified by endogenous SUMOylation also, another set of transient transfection experiment was performed with over-expressed T7NS1A in absence (-) and presence (+) of exogenous SUMO1, with (+) or without (-) SENP1. As shown in Figure 8, even in absence of extra copies of SUMO1, an extra higher molecular weight band of T7NS1A was observed [Figure 8A, lane 2, diagonal arrow], which disappeared when SENP1 was over-expressed [Figure 8A, lane 3], indicating that the band observed is T7NS1A modified by endogenous SUMO. In the presence of exogenous SUMO1, an extra higher molecular weight band was evident [Figure 8A, lane 4, diagonal arrow], which was shifted slightly higher than the band observed in lane 2. The disappearance of the latter band in the presence of SENP1 conclusively indicates that T7NS1A is SUMO modified, this time with the exogenous SUMO1. The

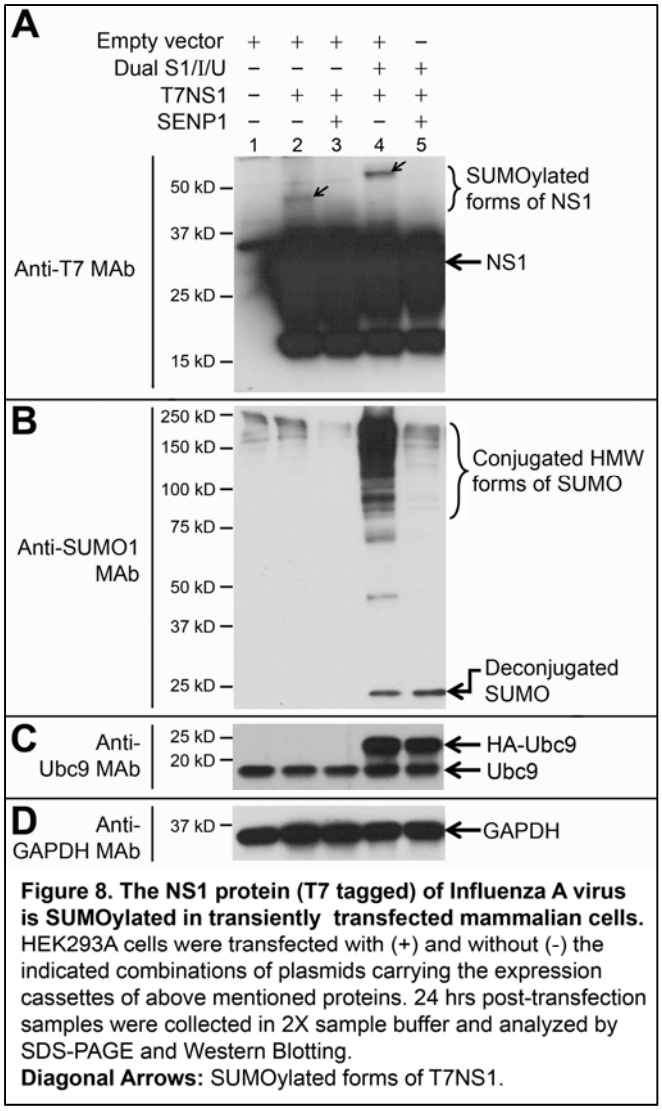

Figure 8. The NS1 protein (T7 tagged) of Influenza A virus is SUMOylated in transiently transfected mammalian cells. HEK293A cells were transfected with (+) and without (-) the indicated combinations of plasmids carrying the expression cassettes of above mentioned proteins. 24 hrs post-transfection samples were collected in 2X sample buffer and analyzed by SDS-PAGE and Western Blotting.
Diagonal Arrows: SUMOylated forms of T7NS1.

difference in molecular weight of both SUMO1 modified bands of T7NS1A is due to the presence of a dual tag (His and S tag) at the N-terminus of the protein encoded by the Dual SUMO1 expression construct (Dual S1/I/U) [23], which was used to over-express SUMO1. As shown in Figure 8B, there was a significant difference in global SUMO1 conjugation between endogenous and over-expressed SUMO1 [Figure B, lane 2 and lane 4]. Remarkably, in spite of the substantially limiting levels of global SUMOylation observed in the absence of over-expressed SUMO, T7NS1A was still efficiently SUMOylated, emphasizing again a possible role of SUMOylation during influenza virus

infection. Figure 8C showed the cellular levels of the endogenous and exogenous Ubc9; the slight shift in molecular weight observed in over-expressed Ubc9 resulted from the presence of an HA tag at the N-terminus of the protein. Figure 8D referred to the loading control of the experiment, the house keeping gene GAPDH. Altogether, the data conclusively demonstrated that NS1A is a *bona fide* target of the cellular SUMOylation system, being modified with endogenous SUMO in a mammalian cell culture system.

3.7 NS1A is modified not only by SUMO1, but also with SUMO2/3:

Some known SUMO targets are preferentially SUMOylated with either SUMO1 or SUMO2/3, whereas others seem to be equally SUMOylated with both main SUMO types [26, 58]. To determine whether NS1A is preferentially conjugated to SUMO1 or SUMO2/3, similar transfection experiments were performed including a dicistronic construct coding for SUMO3 and Ubc9 (Dual S3/I/U) in HEK293A cells. The data obtained [Figure 9] demonstrated that in the presence of exogenous SUMO1, an extra higher molecular weight band was evident [Figure 9A, lane 4], corresponding to SUMO1 modified T7NS1A, as described earlier. Surprisingly, such extra higher molecular weight band was also observed in presence of exogenous SUMO3 [Figure 9A, lane 9] and in this case the band also disappeared in the presence of excess deconjugating enzyme, SENP1 [Figure 9A, lane 10], confirming its identity as a SUMOylated form of T7NS1A. The overall profile of SUMO2/3 conjugation and de-conjugation followed the expected profile as detected with SUMO2 antibody [59] [Figure 9B, lanes 5-10]. The anti-SUMO1 immunoblot presented in Figure 9C validated the overall conjugation and de-conjugation pattern of SUMO1, and in Figure 9D GAPDH level confirmed equal loading in all the

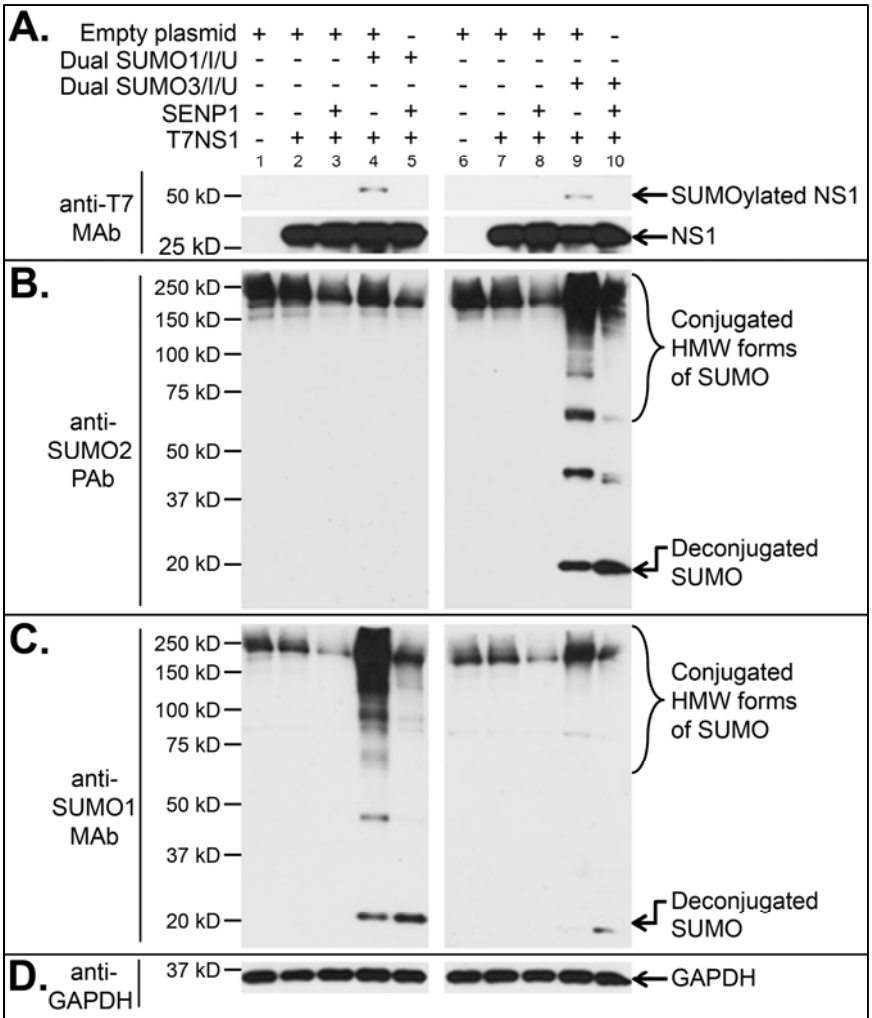

Figure 9. The NS1A protein (T7 tagged) is conjugated to both SUMO1 and SUMO2/3.
HEK293A cells were transfected with (+) and without (-) the indicated plasmid combinations, including expression casettes of SUMO1 (Dual S1/I/U) and SUMO3 (Dual S3/I/U). 24 hrs post-transfection samples were collected in 2X sample buffer and analyzed by SDS-PAGE and Western Blotting.

samples. Altogether, this finding suggested that NS1A has the potential to be equally conjugated by both SUMO1 and SUMO2/3.

3.8 Prediction of potential SUMOylation site(s) in NS1A:

To generate a non-SUMOylatable form of NS1A, it was important to first identify the lysine residue(s) on which NS1A is SUMOylated and then mutate the predicted lysine residues to alanine using a site-directed mutagenesis approach. The probable

SUMOylation site(s) in NS1A were predicted using several SUMOylation prediction softwares. As shown in Table 3, both SUMO prediction softwares used (SUMOplot™ and SUMOsp 2.0) [53, 56], identified lysine residues K70 and K219 as potential SUMOylation sites in NS1A. Out of the two predicted lysine residues, K70 followed the consensus motif for SUMOylation (Ψ-K-X-E) whereas K219 did not.

Table 3: Predicted SUMOylation sites in NS1A (using SUMOplot™ and SUMOsp 2.0):

Prediction software	Position of lysine residue	Peptide sequence
SUMOplot™	K70	IVERI L**K**EE SDEAL
	K219	PLTPK Q**K**RE MAGTI
SUMOsp 2.0	K70	RIL**K**EES
	K219	PKQ**K**REM

However, current prediction softwares were designed based on limited available data and there are several proteins that contain a perfect SUMO consensus motif, but still are not SUMOylated. Similarly, there are several SUMO target proteins that are SUMOylated at sites located outside of a consensus motif [29]. So, the computationally predicted site still needed to be confirmed experimentally.

3.9 Mapping of SUMOylation site(s) in NS1A:

To confirm the site of SUMOylation in NS1A, point mutants bearing K to A substitutions of the residues previously predicted as potential SUMOylation sites were generated. To this end, three mutants, namely T7NS1A-K70A, T7NS1A-K219A and T7NS1A-K70AK219A were generated. As indicated their names, the first two contained single K to A amino acid substitutions, whereas the third contained two K to A substitutions. To analyze the effect of the point mutations on NS1A SUMOylation,

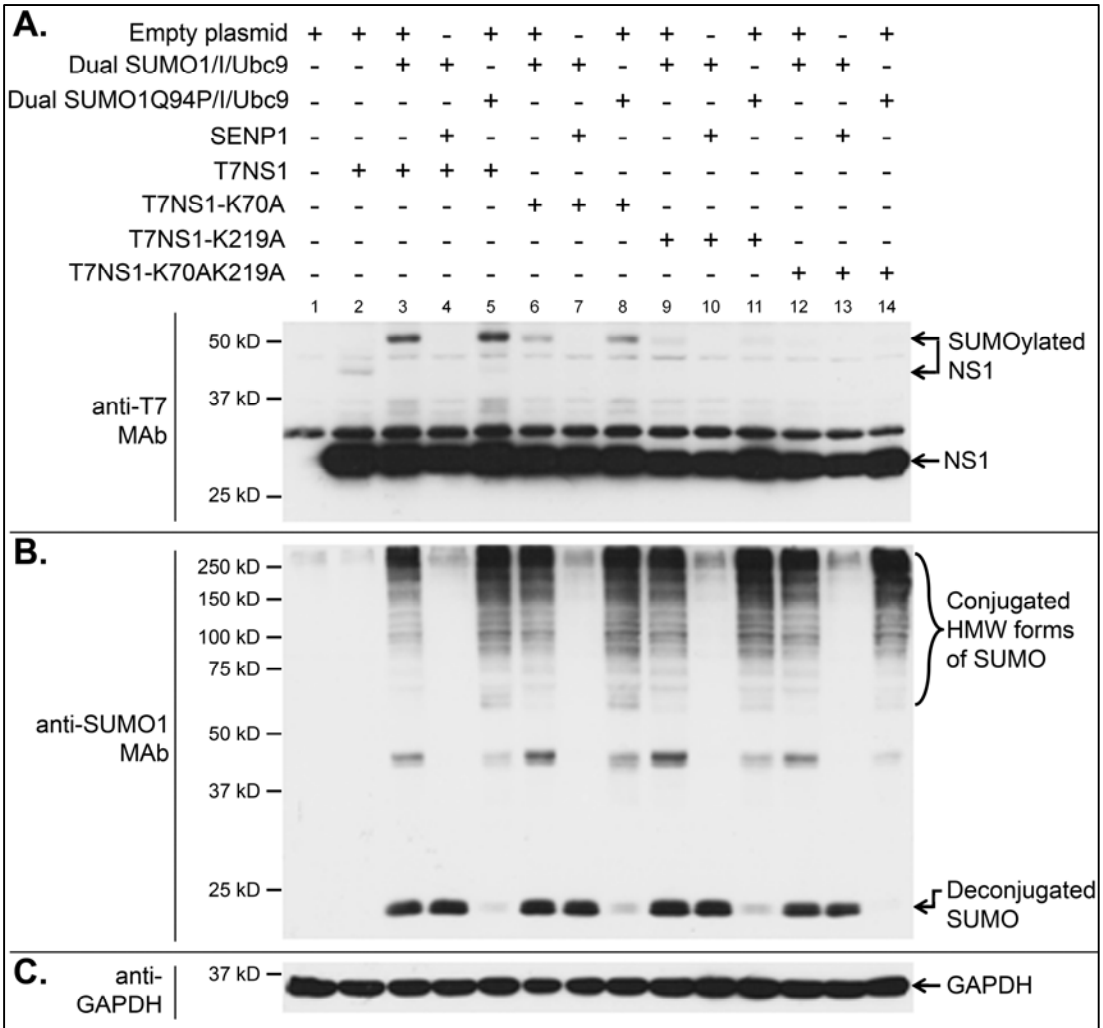

Figure 10. Mapping of the SUMO1-SUMOylation site of NS1A.
HEK293A cells were co-tracsfected with single or double mutants of NS1A (K70A, K219A, K70AK219A) along with combinations of deconjugatable SUMO1/I/U or non-deconjugatable SUMO1Q94P/I/U and SENP1. 24 hours post-transfection samples were collected in 2X sample buffer and were analyzed by SDS-PAGE and Western Blotting.

HEK293A cells were transiently transfected with the T7NS1A mutants along with Dual S1/I/U, in presence (+) or absence (-) of SENP1, or an expression plasmid for a non-deSUMOylatable form of SUMO1 and Ubc9 (Dual SUMO1Q94P/I/U). At 24 hours post-transfection, the cell lysates were collected in 2X sample buffer and the SUMOylatability of the mutants was evaluated by SDS-PAGE and Western Blotting and compared to the parental T7NS1A (hereafter referred as WT T7NS1A).

As shown in Figure 10, when co-transfected with Dual S1/I/U, the T7NS1A-K70A mutant showed a small decrease in its ability to be SUMOylated [Figure 10A, Lane 6], whereas the T7NS1A-K219A mutant showed a significant decrease in SUMOylation [Figure 10A, Lane 9], compared to the level of SUMOylation observed in WT T7NS1A [Figure 10A, Lane 3]. A double mutant containing K to A mutations in both sites (T7NS1A-K70AK219A) was almost not SUMOylated at all [Figure 10A, Lane 12]. Simultaneous analysis performed with non-deSUMOylatable construct Dual SUMO1Q94P validated our observations [Figure 10A, Compare Lanes 8, 11 and 14 from Lane 5]. The immunoblot profile obtained with the anti-SUMO1 antibody [Figure 10B], showed the expected conjugation pattern including the de-conjugation profile conferred by the over-expression of SENP1. In the same experiment, GAPDH was used as the loading control [Figure 10C] and it indicated that similar levels of protein were loaded for all the different samples. Therefore, the data presented here identified K219 as the primary SUMO1-SUMOylation site in NS1A, while K70 appeared to act as a secondary SUMOylation site.

3.10 SUMO2/3 conjugation of NS1A occurs through the same residue as SUMO1:

In order to verify if SUMO2/3 conjugation happens through the same residues as SUMO1, or it follows any other SUMOylation site(s), another set of transient transfection experiments were performed in HEK293A cells with the deconjugatable dicistronic construct expressing SUMO3 and Ubc9 (Dual S3/I/9), in the absence (-) or presence (+) of the deconjugating enzyme SENP1, or a non-deconjugatable dicistronic construct for SUMO3 (Dual S3Q89P/I/U). It was observed that the pattern of SUMOylation of

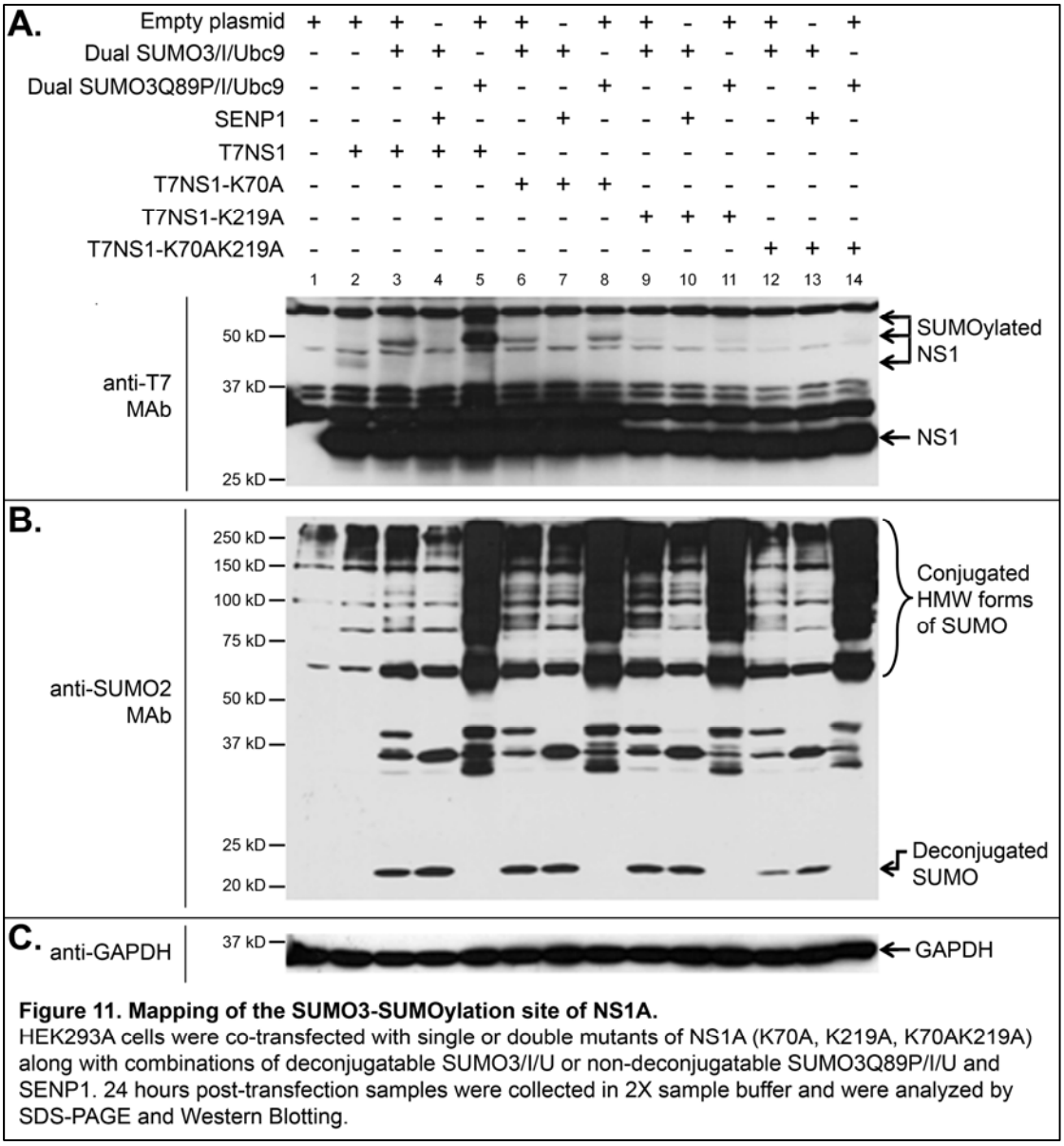

Figure 11. Mapping of the SUMO3-SUMOylation site of NS1A.
HEK293A cells were co-transfected with single or double mutants of NS1A (K70A, K219A, K70AK219A) along with combinations of deconjugatable SUMO3/I/U or non-deconjugatable SUMO3Q89P/I/U and SENP1. 24 hours post-transfection samples were collected in 2X sample buffer and were analyzed by SDS-PAGE and Western Blotting.

T7NS1A with SUMO3 was very similar to the SUMOylation pattern observed with SUMO1, as revealed in Figure 11. Again, the T7NS1A-K70A mutant showed a small decrease in its ability to be SUMOylated by SUMO3 [Figure 11A, Lane 6], while the T7NS1A-K219A mutant showed a significant decrease in SUMOylation [Figure 11A, Lane 9], compared to the level of SUMO3 SUMOylation observed in WT T7NS1A [Figure 11A, Lane 3]. Also, the double mutant (T7NS1A-K70AK219A) was almost lacking in its ability to be SUMOylated. Analysis performed with the non-deconjugatable

mutant, Dual S3Q89P/I/U, further confirmed these observations. Figure 11B represented the overall conjugation and de-conjugation pattern observed with SUMO2 antibody, while the GAPDH level in Figure 11C confirmed equal loading in all samples. Altogether, these findings suggested that the same lysine residues were targeted by SUMO1 and SUMO2/3 conjugation.

3.11 SUMOylation does not seem to affect sub-cellular distribution of NS1A following transfection:

SUMOylation is known to alter the sub-cellular localization of some of its targets [60]. In order to assess if SUMOylation plays any role in the sub-cellular distribution of NS1A, the effect of SUMOylation on the localization of NS1A was evaluated using different SUMOylation-deficient mutants of T7NS1A. To monitor any alteration in localization, HEK293A cells were seeded and 24 hours later co-transfected with Dual S1/I/U and WT T7NS1A or the different SUMOylation-deficient mutants of T7NS1A (K70A, K219A, K70AK219A). At 24 hours post-transfection, the cells were fixed and analyzed by immunofluorescence using a mouse monoclonal antibody against T7 and rabbit monoclonal antibody against Ubc9 as primary antibodies, and Alexa 488 Goat anti-mouse and Alexa 594 Goat anti-rabbit both highly cross-absorbed as secondary antibodies.

As shown in Figure 12, it was apparent that there was no significant change in the localization of the SUMOylation deficient mutants of T7NS1A as compared to WT T7NS1A. As evidenced in the merged images, the localization of SUMOylation deficient

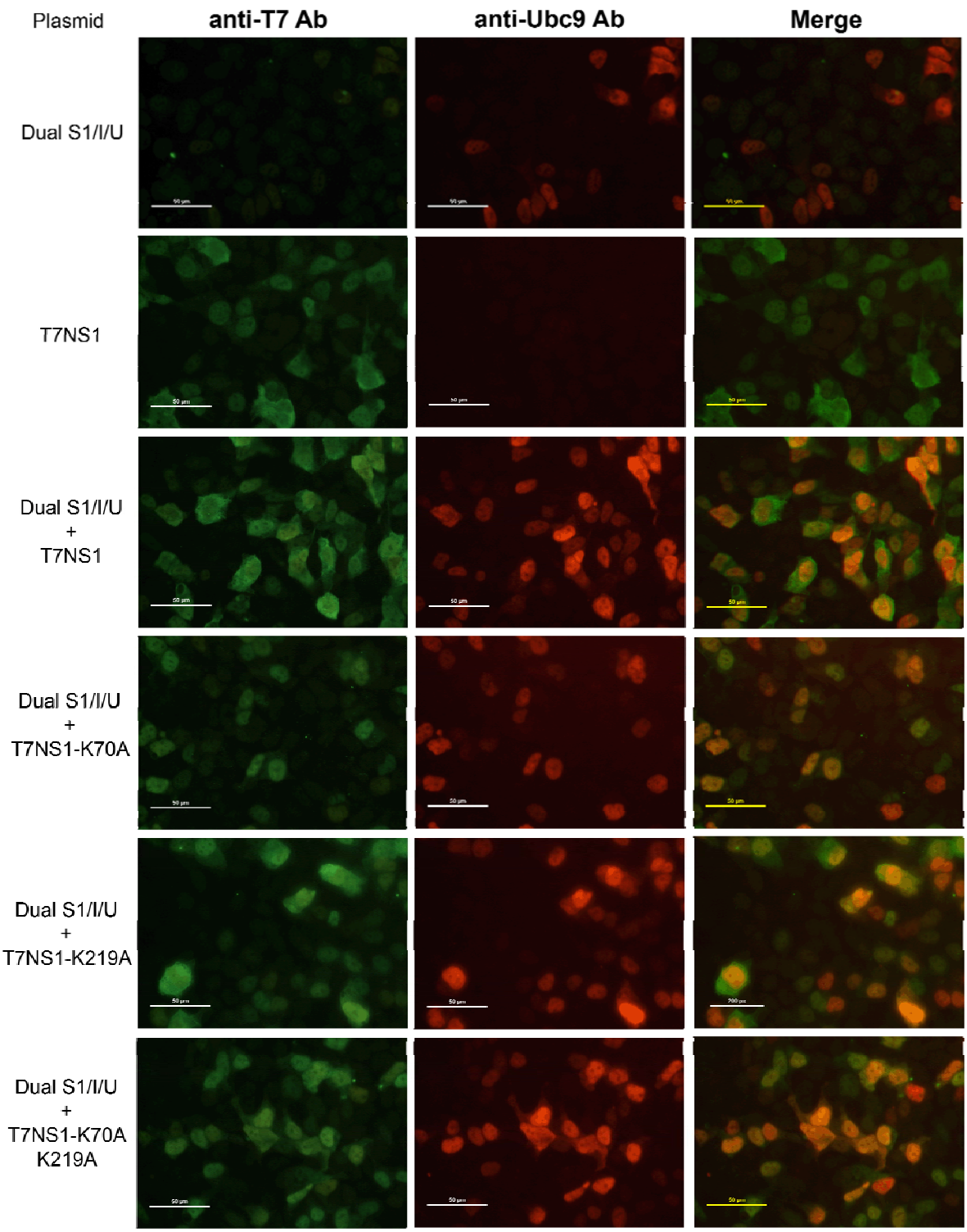

Figure 12. SUMOylation does not seem to affect the localization of T7NS1 following transient transfection.

HEK293A cells were transfected with Dual S1/I/U or T7NS1 or co-transfected with T7NS1 and its mutants (K70A, K219A, K70AK219A) along with Dual S1/I/U. 24 hours post transfection, the cells were fixed and prepared for immunofluorescence. The merged images are shown at the far right. Bar, 50 micrometer.

mutants (K70A, K219A, K70AK219A) was not altered compared to the localization of WT T7NS1A when co-expressed with SUMO1. For WT and all SUMOylation-deficient mutants, there was substantial co-localization with Ubc9 in the nucleus, therefore suggesting that SUMOylation does not play a substantial role in affecting the sub-cellular localization of T7NS1A.

3.12 Up-regulation or down-regulation of SUMOylation does not bear any effect on localization of NS1A following infection:

Next, in order to ascertain if alteration in cellular SUMOylation level affects NS1A's sub-cellular distribution following infection, recombinant adenoviruses engineered to produce well characterized effects on the cellular SUMOylation system were used for transduction in A549 cells. To up-regulate cellular SUMOylation, a recombinant adenovirus carrying the dicistronic SUMO1 construct (AdV-Dual S1/I/U) was used. Similarly, to down-regulate SUMOylation, either the AdV-Dual S1ΔGG/I/U, carrying the non-conjugatable diglycine deletion mutant known to produce a block in the SUMOylation cascade, or the AdV-Dual Ubc9(C93S)/I/Ulp1, carrying an expression cassette for both, a Ubc9 mutant lacking the SUMO conjugating activity, and the yeast de-SUMOylating enzyme Ulp1, were used. 48 hrs post-transduction, cells were infected with Influenza strain A/PR/8/34 H1N1 at a MOI of 0.5 and 10 hrs post-infection the cells were fixed with 4% paraformaldehyde and prepared for immunofluorescence using an anti-NS1A mouse monoclonal antibody as primary antibody and Alexa 488 Goat anti-mouse antibody as secondary antibody.

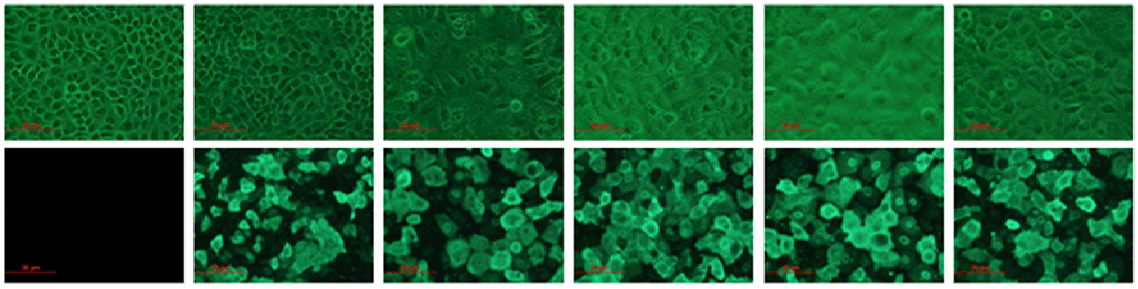

Figure 13. Sub-cellular distribution of NS1A expressed during infection remains unaltered following up-regulation or down-regulation of SUMOylation system.
A549 cells were transduced with recombinant adenoviruses designed for up-regulation (AdV-DS1/I/U) or down-regulation of total SUMOylation level in cells (AdV-DS1deltaGG/I/U or AdV-DU/I/Ulp1), and 48 hrs post-transduction were infected with Influenza A/PR/8/34 H1N1 at MOI 0.5. Cells were fixed at 10 hrs post-infection and prepared for immunofluoresce. Bar, 50 micrometer

As of Figure 13**,** no major difference was observed in the localization of NS1A in cells up-regulated or down-regulated in SUMOylation, in comparison to the control infected samples where there was no adenoviral transduction or transduction with Adv-LacZ, where there were no changes in SUMO level. In all the samples observed, NS1A exhibited nuclear and cytoplasmic distribution, indicating that the alterations in the SUMOylation system produced by transduction with different recombinant adenoviruses did not affect the sub-cellular localization of NS1A expressed during infection.

3.13 SUMOylation does not seem to affect NS1A's stability:

Among other numerous biological functions, SUMOylation is also known to alter protein stability by two different mechanisms: 1) by working as a ubiquitin antagonist competing with the ubiquitin system for modification of specific lysine residues; and, 2) by enhancing the recognition by ubiquitin ligases able to recognize poly-SUMO2/3 chains. The first mechanism increases protein stability, whereas the second one decreases it [26, 27, 61]. To investigate, whether SUMOylation affected the stability of

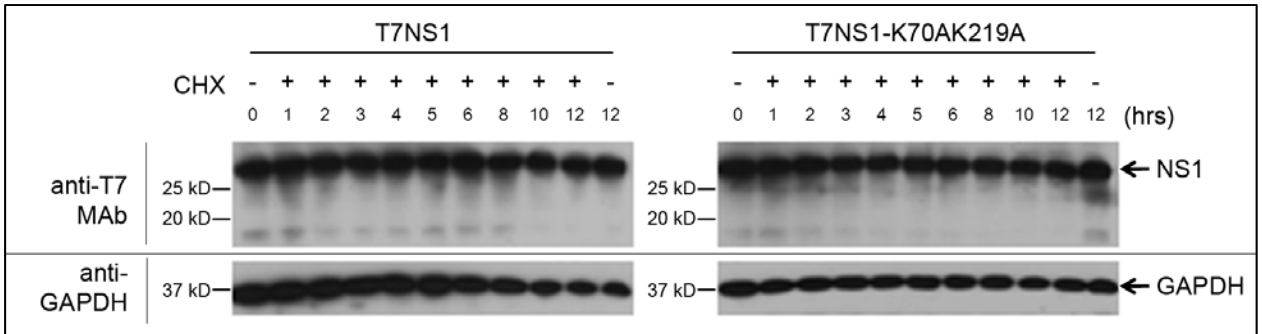

Figure 14. SUMOylation does not seem to affect NS1A's protein stability.
HEK293A cells were transfected with WT T7NS1 and SUMOylation deficient double mutant, T7NS1-K70AK219A. 24 hrs post-transfection the cells were treated with cycloheximide (CHX) and cell lysates were collected with 2X sample buffer at indicated time points (hrs) post-CHX treatment and subsequently analyzed by SDS-PAGE and Western Blotting.

NS1A, WT T7NS1A and its SUMOylation deficient double mutant T7NS1A-K70A/K219A were over-expressed by transfection in HEK293A cells, and the cells were subsequently treated with cycloheximide to stop overall protein synthesis. Samples collected at different time points post-cycloheximide treatment were then analyzed by SDS-PAGE and Western blotting.

Our data (shown in Figure 14) indicated that NS1A is a very stable protein and that SUMOylation does not affect its stability in any obvious manner. Following cycloheximide (CHX) treatment, both WT and mutant T7NS1A showed a similar time-dependent decrease in protein levels, and in both cases the decrease was minimal even at 12 hrs post CHX treatment, exhibiting cellular levels similar to those observed in the untreated samples used as control. Altogether these observations indicated that SUMOylation does not seem to affect NS1A's protein stability.

3.14 The alternative spliced form of NS gene, NS2 (NEP) is also SUMO1-modified:

The 8th and last segment of vRNA segment of Influenza A virus encodes two non-structural proteins, one unspliced form and one alternatively spliced form, [62]. The alternative splicing of NS gene results into formation of a protein of 121 amino acid

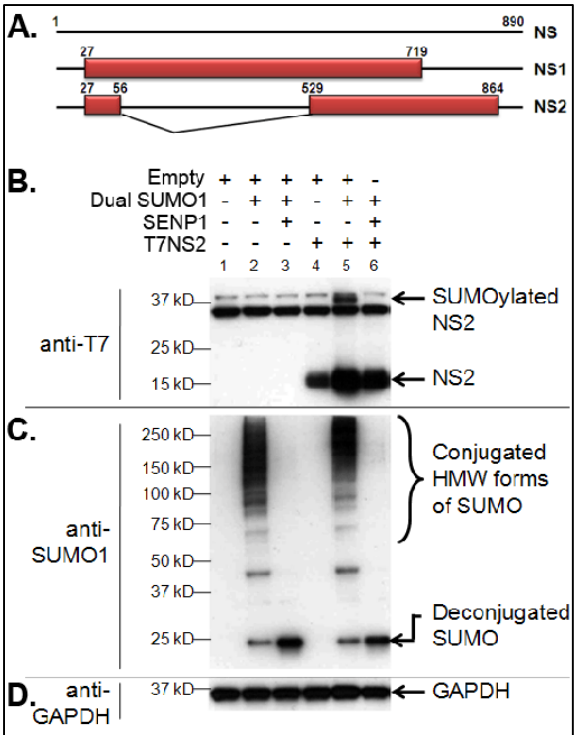

Figure 15. Schematic diagram showing alternative spliced product of NS gene (NS2/NEP) and evidence of SUMOylation of NS2.
A. Schematic diagram illustrating the coding region of NS1 and NS2. **B-D**. HEK293A cells were transfected with indicated plasmids and 24 hrs post-transfection samples were collected and analyzed by SDS-PAGE and Western blotting.

residues and approximately 14 kDa in size, known as NS2 [Figure 15A]. To determine if NS2 is also targeted by SUMOylation a T7 tagged NS2 clone was constructed using site-directed mutagenesis approach. Then, HEK293A cells were co-transfected with the expression plasmid for T7-tagged NS2 and different combinations of other plasmids, including Dual SUMO1/I/U, in the absence (-) and presence (+) of the SUMO de-conjugating enzyme SENP1. Samples were collected and analyzed as before by immunoblotting.

As shown in Figure 15, co-transfection with Dual SUMO1 led to the appearance of a higher molecular weight form of T7NS2 [Figure 15B, lane 5], which disappeared in the presence of SENP1 [Figure 15B, lane 6], demonstrating that NS2 is also modified

by SUMO1 in mammalian cells. Overall SUMOylation level was revealed by SUMO1 antibody profile [Figure 15C], while GAPDH in Figure 15D confirmed equal loading in all the samples. The data thus obtained unequivocally confirmed the SUMO modification of NS2.

3.15 The artificial ligase, $NS1A_{1-87}$_Ubc9 fusion, dramatically increases the endogenous SUMOylation of NS1A:

As mentioned earlier, at any given point the SUMOylated form of a given protein is only a very small fraction of its unmodified form [36, 37], thus studying the functional consequences of SUMOylation of a given protein is fairly challenging. In order to increase the endogenous SUMOylation of T7NS1A, an 'artificial SUMO ligase' was developed by crafting a fusion of the RNA-binding domain of NS1A including the linker region ($NS1A_{1-87}$), with Ubc9. To this end, the coding region of Ubc9 was cloned downstream of the $NS1A_{1-87}$ ORF, generating the $NS1A_{1-87}$_Ubc9 fusion. The 'artificial ligase' idea was based on the fact that NS1A is well known to form dimers. If $NS1A_{1-87}$_Ubc9 works appropriately as a ligase, it should increase the level of endogenous SUMOylation of T7NS1A when co-transfected in mammalian cells. Furthermore, according to the findings of this study, the major SUMOylation site in NS1A is K219, thus the artificial ligase ($NS1A_{1-87}$_Ubc9) should not be able to SUMOylate itself. To test our hypothesis, HEK293A cells were co-transfected with $NS1A_{1-87}$_Ubc9 fusion and T7NS1A, in the absence (-) or presence (+) of the de-conjugating enzyme SENP1. As positive control and to provide a model for comparison, co-transfection with Dual S1/I/U

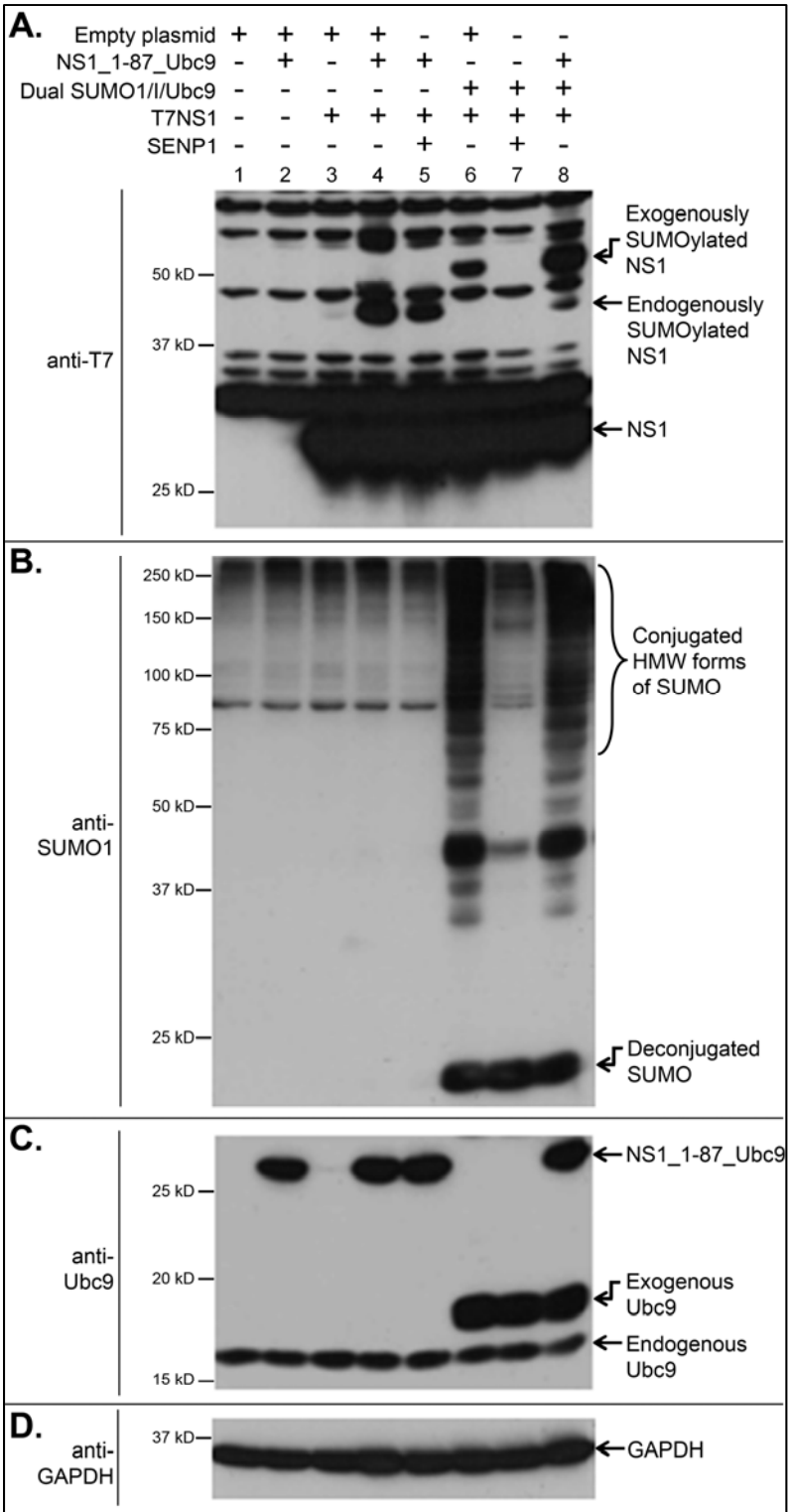

Figure 16. The 'articial SUMO ligase', NS1_1-87_Ubc9 dramatically increases endogenous SUMOylation of T7NS1.
HEK293A cells were transfected with indicated plasmid combinations and 24 hrs post-transfection cell lysates were collected in 2X sample buffer and analyzed by SDS-PAGE and Western blotting.

and T7NS1A was also performed. 24 hrs post-transfection, cell lysates were collected in 2X sample buffer and analyzed by SDS-PAGE and Western Blotting.

As shown in Figure 16, $NS1A_{1-87}$_Ubc9 worked perfectly in its capacity as a ligase, as it dramatically enhanced the level of endogenous SUMOylation of T7NS1A [Figure 16A, Compare Lane 4 with Lane 3]. The level of SUMOylation achieved with $NS1A_{1-87}$_Ubc9 was even more than the level observed upon up-regulating cellular SUMOylatiopn with the Dual S1/I/U construct. In the presence of SENP1, the amount of SUMOylated form of T7NS1A was decreased but not abolished [Figure 16A, Lane 5], contrary to the effect of SENP1 observed with Dual S1/I/U [Figure 16A, Lane 7], indicating that $NS1A_{1-87}$_Ubc9 fusion dramatically shifts the balance of the SUMOylation-deSUMOylation events affecting NS1A toward its rapid SUMO conjugation, surpassing its de-conjugation, which is otherwise normally the predominant event. Interestingly, over-expression of $NS1A_{1-87}$_Ubc9 did not lead to the global increase of cellular SUMOylation observed with Dual S1/I/U [Figure 16B], which suggest that the effect observed is highly specific. Figure 16C demonstrated the level of endogenous Ubc9 as well as the relative position of Dual S1/I/U and Ubc9 fused to $NS1A_{1-87}$, while Figure 16D confirmed equal loading in all different samples. Altogether the data demonstrated that the $NS1A_{1-87}$_Ubc9 is functioning adequately as a new developed tool to specifically increase the endogenous level of SUMOylation without altering the global SUMOylation pattern.

3.16 Determining the specificity of NS1A$_{1-87}$_Ubc9 fusion on SUMOylating a substrate:

In order to further assess the specificity of the 'artificial SUMO ligase' described above, we evaluated the SUMOylation of the SUMO-deficient mutants of T7NS1A. As

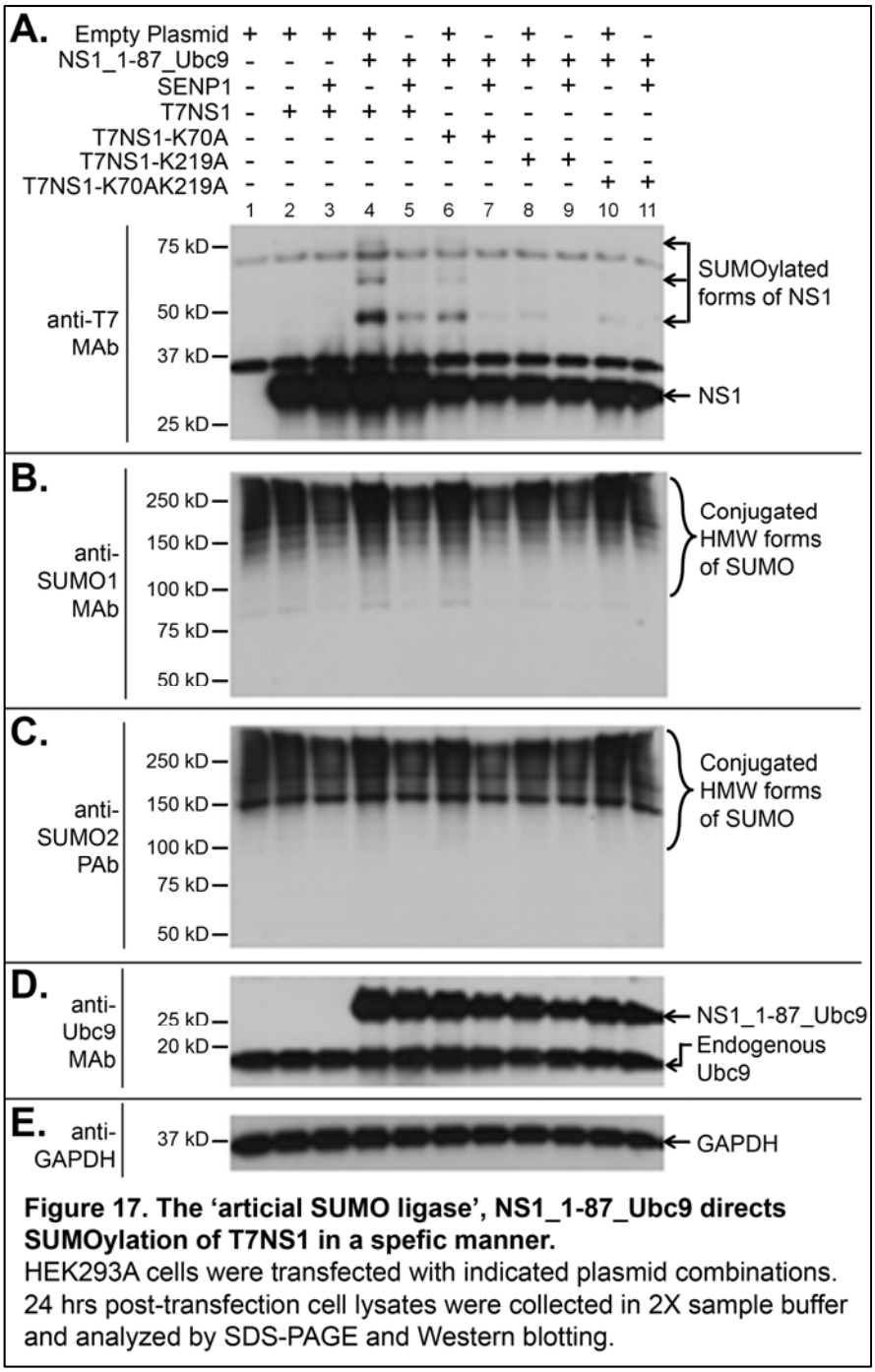

Figure 17. The 'articial SUMO ligase', NS1_1-87_Ubc9 directs SUMOylation of T7NS1 in a spefic manner.
HEK293A cells were transfected with indicated plasmid combinations. 24 hrs post-transfection cell lysates were collected in 2X sample buffer and analyzed by SDS-PAGE and Western blotting.

the artificial ligase is increasing T7NS1A's ability to be SUMOylated significantly, it was needed to test if SUMOylation occurred at the previously mapped SUMOylation sites or in other residues. To this end, HEK293A cells were transfected with the $NS1A_{1-87}$_Ubc9 fusion together with WT and SUMOylation deficient mutants of T7NS1A, in the absence (-) or presence (+) of SENP1. Cell lysates were collected 24 hrs post-transfection with 2X sample buffer and analyzed by SDS-PAFE followed by western blotting.

As shown in Figure 17, when the SUMOylation deficient mutants were used for co-transfection, the level of SUMOylation mediated by the $NS1A_{1-87}$_Ubc9 fusion was dramatically reduced compared to the SUMOylation level of WT T7NS1A [Figure 17A, Compare Lanes 6, 8 and 10 with Lane 4], indicating that the artificial ligase targeted its substrate at the same lysine residues previously mapped as the main SUMOylation sites in NS1A. Blotting with SUMO1 [Figure 17B] and SUMO2 [Figure 17C] again confirmed that there was no global increase in overall SUMO conjugation in cells. Immunoblotting directed against Ubc9 revealed the level of endogenous Ubc9 and relative level of $NS1A_{1-87}$_Ubc9, and the level of GAPDH established the loading control of the experiment. Altogether, this experiment confirmed the specificity conferred by $NS1A_{1-87}$_Ubc9 fusion in SUMOylating a substrate at specific sites and provided a powerful tool to enhance endogenous SUMOylation level of T7 tagged NS1A specifically to help analyzing its biological role(s).

CHAPTER 4: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary and Conclusions:

Current anti-influenza weapons include annual vaccinations and antiviral drugs. Such measures normally target structural components of the virus, thus are prone to encounter viral resistance over relatively short time-span. Vaccination is a validated and widely used option against influenza, but for immunocompromised patients its effectiveness is minimal. In addition, the strain specific nature of the annual vaccine limits its ability to hinder any new strain variant generated as a result of antigenic drift or antigenic shift. Thus, the ever-changing nature of influenza virus constitutes the major limiting factor for the effective prevention of influenza by vaccination. The major antiviral drugs against influenza belong to two main groups, neuraminidase inhibitors (Oseltamivir and Zanamivir) and M2 ion channel pump inhibitors (Adamantanes i. e. Amantadine and Rimantadine) [1, 2]. Both types of drugs target structural components of virus, and therefore are prone to favor the rapid selection of resistant viral strains produced by the high mutation rate associated to the error-prone viral RdRp. In a recent study, it was shown that a single spontaneously arising mutation in Neuraminidase (H274Y) is responsible for the virtually 100% prevalence of Oseltamivir (TAMIFLU)-resistant viruses among all H1N1 viral isolates characterized in the USA during the 2008-2009 influenza season [63]. So, the leading antiviral against influenza has lost its effectiveness against seasonal influenza. Surprisingly, in a previous epidemiological study it was observed that a single amino acid substitution affecting any one of five amino acids in the transmembrane domain of M2 protein is capable of generating Amantadine resistant strains of influenza A virus. Furthermore, the fitness of the

resistant virus generated is equivalent with the wild-type (WT) virus [64]. While it was previously demonstrated that during 2005-2006 influenza season, 90% of H3N2 viruses and 15% of H1N1 viruses analyzed were resistant to Adamantanes [2], according to CDC, the circulating H3N2 viruses analyzed during last influenza season of 2008-09 showed practically 100% resistance against Adamantanes [65].

From this perspective considering the potential drawbacks of available measures, there is an urgent need for a more generalized approach to combat influenza. One possible alternative is to target cellular systems either required for viral growth or able to neutralize viral growth, which will be less prone to be affected by the emergence of resistant viruses. Additionally to our advantage, targeting cellular systems will be able to work irrespective of the type, strain and antigenic properties of the virus, which will be quite advantageous considering the broad array of subtypes of influenza virus. As the virus is dependent on the host cells for its multiplication, targeting the host cellular factors essential for influenza infection may provide an attractive alternative for antiviral therapy. The concept of inhibiting specific cellular functions, which are indispensable for the virus for its efficient infection, but not required by the host cells for short time period, has already been proposed as a way to limit the generation of resistant viruses [66]. Though in recent years a wide variety of different antiviral approaches have been explored, targeting cellular factors seems to have the most promising outcome [67].

In recent studies, ISG15 conjugation to NS1A protein is reported as one of the antiviral mechanisms mediated by host cells, therefore restricting viral replication by some loss-of essential functions performed by this protein [21, 22]. On the contrary, it

was shown previously that influenza virus also developed some mechanisms to overcome host anti-interferon response as exemplified by NS1A targeting ubiquitin ligase TRIM25, therefore inhibiting TRIM25 mediated ubiquitination of CARD domain of RIG-I that is a viral RNA sensor and facilitator of type-I interferon production [68]. Altogether both examples establish an entire new arena of complex virus-host interactions where post-translational modifications of the host cells play some important roles in context to influenza infection, sometimes mediating an anti-viral response, or being exploited by the virus to override the host defense.

Several lines of evidence already have established the fact that some DNA viruses utilize the host SUMOylation machinery for producing optimal environment for infection. SUMOylation also appears to facilitate infection by modulating activity or localization of some of the viral proteins [41-44].

As mentioned by the recent genome-wide RNAi screening, SUMOylation appears to be an important cellular factor required by influenza virus for its efficient multiplication [51]. Our current study reveals that upon influenza infection the cellular SUMOylation level is increased. From unpublished observations of our lab, it seems SUMOylation promotes influenza infection as down-regulation of SUMOylation precluded production of some viral proteins those are important for its efficient infectivity or release of new virions. Along with those observations, as mentioned in this thesis, there are more than one SUMO-modified proteins of influenza virus including NS1A that serves as an important virulence factor and one of the main weapons of influenza virus to counteract cellular defense. Though the underlying mechanism how SUMOylation is able to advance viral infection remains elusive, altogether it is indicative that this post-

translational modification may serve as an attractive target to impede influenza viral infection in future.

Additionally, as mentioned before, NS1A was identified in this study as an authentic target for SUMOylation system, even being modified with endogenous level of SUMOylation present in cells. The mutagenesis study presented herein identified K219 of NS1A protein as the main SUMOylation site for both SUMO1 and SUMO2/3 while K70A was recognized as the secondary site. Interestingly, a multiple sequence alignment [Figure 18] form an array of different Influenza A virus subtypes identified K219 as a totally conserved residue among all human subtypes, which is unanticipated from such a variable virus. Some of strain-specific NS1A were truncated but ending on

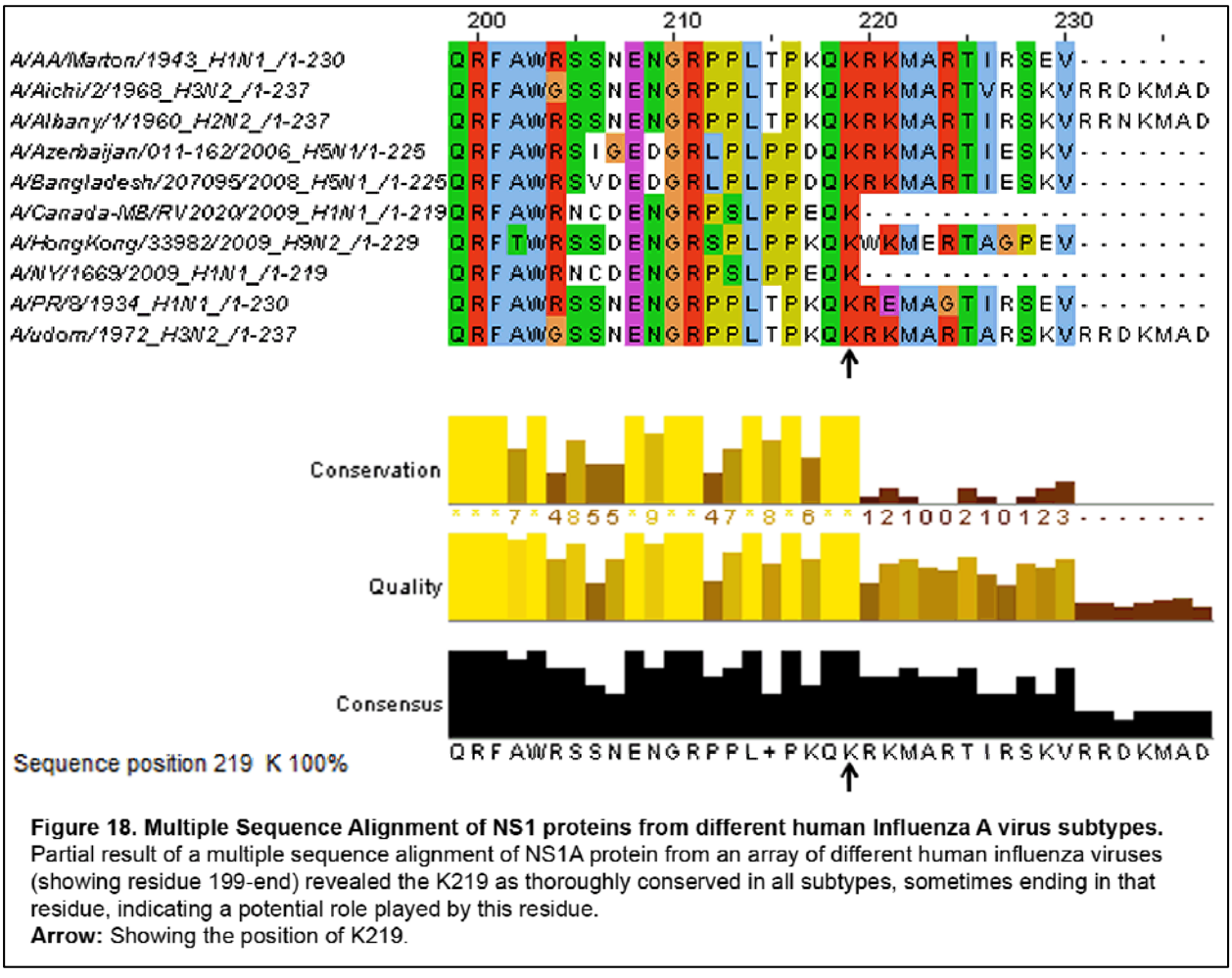

Figure 18. Multiple Sequence Alignment of NS1 proteins from different human Influenza A virus subtypes. Partial result of a multiple sequence alignment of NS1A protein from an array of different human influenza viruses (showing residue 199-end) revealed the K219 as thoroughly conserved in all subtypes, sometimes ending in that residue, indicating a potential role played by this residue.
Arrow: Showing the position of K219.

residue K219, signifying this residue plays some important roles for the virus. Importantly, a recent study by Zhao et al. showed that recombinant viruses with a substitution of K219R in NS1A could not be generated [21], and in the same study K219 was also shown as a secondary site for ISG15 modification, implying K219 as a very crucial residue for the virus survivability as well.

Although the biological implications of SUMOylation for NS1A still remain elusive, with the new “artificial SUMO ligase” approach generated in this study, it is possible to enhance the level of endogenous SUMOylation of NS1A to a dramatic extent, which essentially will help in analyzing the functional consequences of NS1A SUMOylation in a protein-specific manner.

Collectively, this study extended the proposed interplay of influenza virus with the cellular SUMOylation system, reporting several influenza viral proteins as SUMO targets for the first time and casting insights on a new paradigm of virus-host interactions.

4.2 Future Directions:

To this point, influenza still remains as one of the oldest diseases with the potential to cause major life threatening pandemics. The increasing resistance of the viruses against the available antivirals necessitates development of novel generic therapeutic approaches without the side effects of conferring resistance. The idea of targeting cellular factors necessary for viral survival is gradually gaining popularity to impede the progression of this disease. Along with other cellular factors, SUMOylation appeared as a crucial factor necessary for efficient viral infection [51, 52]. Analyzing how influenza virus interacts with the SUMOylation system and the consequences of

this interplay may provide us with new tools to develop a broad spectrum antiviral therapy. In this study, NS1A has also been identified as an authentic SUMO target being modified primarily in a thoroughly conserved residue in all human influenza A virus sub-types. It will be intriguing to analyze the possible roles played by SUMOylation for the multifunctional protein NS1A in order to further understand the virus-host interactions and find new approaches to hinder some essential functions performed by this protein to help the virus evade the cellular defense mechanisms. Though the precise mechanism of functional consequences of SUMOylation for NS1A is still undefined, the accumulating evidence suggests that exploring this new arena will shed lights in the process of developing and designing novel prevention strategy against influenza virus.

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CURRICULUM VITAE

Sangita Pal was born in Berhampore, West Bengal, India. The youngest daughter of Dr. Ranjan Pal and Puspa Pal, she graduated from Barasat High School in Barasat, West Bengal in Spring of 2002. Upon graduation, she entered the West Bengal University of Animal & Fishery Sciences in Kolkata, India to pursue her undergraduate study. She was awarded the University Merit Scholarship throughout her undergraduate study. In Spring 2007, she served as an intern in different state veterinary hospitals in West Bengal. Upon her graduation in Spring 2007, she was accepted into the Master's program in Biology Department at The University of Texas at El Paso to start in Spring 2008. She was selected for poster presentation at the 28th American Society for Virology (ASV) organized at University of British Columbia, Vancouver, Canada and at the 5th International Conference SUMO Ubiquitin, UBL Proteins: Implications for Human Diseases organized at University of Texas MD Anderson Cancer Center, Houston, Texas. She received a travel award from ASV committee for attending ASV meeting. Moreover, she was awarded Graduate School Professional Funding Award from UTEP to excel in research. She published a co-first author paper in Journal of Virological Methods in 2010 with her results. In 2010, she was accepted into the PhD program at University of Texas MD Anderson Cancer Center at Houston to continue her education.

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