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Influenza A Virus Multiplication and the Cellular SUMOylation System

Andrés Santos, Jason Chacón and Germán Rosas-Acosta

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"Medical care was making little difference anyway. Mary Tullidge daughter of Dr. George Tullidge, died twenty-four hours after her first symptoms. Alice Wolowitz, a student nurse at Mount Sinai Hospital, began her shift in the morning, felt sick, and was dead twelve hours later."

"In ten days - *ten days!* - the epidemic had exploded from a few hundred civilian cases and one or two deaths a day to hundreds of thousands ill and hundreds of deaths each day."

Excerpts from Chapter Nineteen, "The Great Influenza" by John. M. Barry.

It is impossible to predict with absolute certainty whether we will ever face another pandemic like that of the 1918 Spanish flu. But nevertheless, we must be prepared to fight it in the event that it might happen. Increasing our knowledge of the molecular biology of the virus is by far the best way to get ready for it. In fact, it might be the only way.

GRA.

1. Introduction

1.1. A coated agent with a fragmented genome: The infectious viral particle of Influenza A Virus

Influenza A virus (IAV), a member of the Orthomyxoviridae [1], is an enveloped, negative-sense, RNA virus that contains a segmented genome composed of eight different viral RNA strands (vRNAs), each coding for one or two different viral proteins [2]. The viral particle is composed of a phospholipid bilayer membrane derived from the plasma membrane of the host, decorated by two single transmembrane domain viral proteins, the hemagglutinin

(HA) and neuraminidase (NA) proteins, and one pore-forming protein, the matrix 2 (M2) ion channel protein [3]. The viral envelope surrounds a relatively irregular viral nucleocapsid formed by the viral matrix (M1) protein, which in turn surrounds eight viral ribonucleoproteins (vRNPs) that appear to be placed in a well organized cylindrical arrangement [4]. Each vRNP is made of a single stranded viral genomic RNA segment covered by numerous copies of the viral nucleoprotein (NP), and a single copy of the viral RNA-dependent RNA-polymerase (RdRp), which is associated to a double stranded RNA structure formed by 12 and 13 complementary nucleotides at the 5' and 3' end regions in each gene segment, respectively. The viral RdRp is a trimeric complex made of single copies of the basic one, basic two, and acid RNA polymerase proteins (referred to as PB1, PB2, and PA, respectively). Also associated to the infectious viral particle is the nuclear export protein (NEP), formerly known as non-structural protein 2 (NS2), a protein involved in the export of vRNPs from the nucleus of infected cells (Reviewed by Nayak et al [5], and Rossman and Lamb [3]) (Figure 1). Finally, recent analyses have demonstrated the incorporation of several host-cell encoded proteins into the mature viral particle [6].

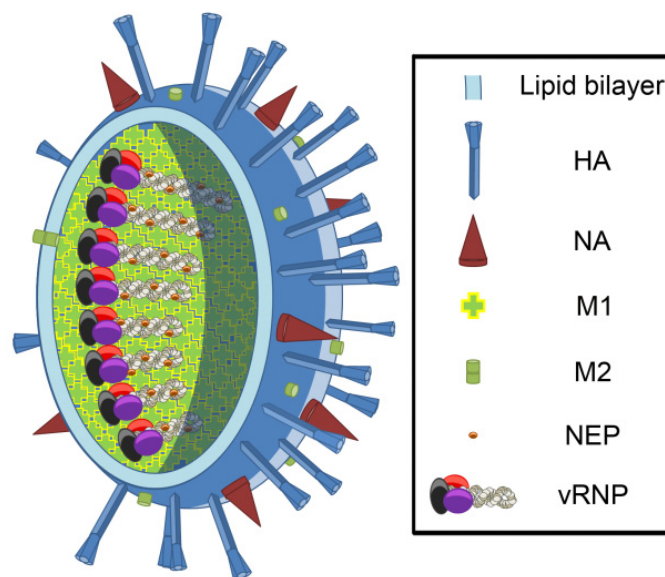


Figure 1.

1.2. A nuclear RNA virus: The life cycle of influenza A Virus

The infectious cycle of the virus starts with the binding of the infectious viral particle to the plasma membrane of the host cell. This initial binding is mediated by the viral HA protein, which exhibits high binding affinity for sialic acid molecules coating the extracellular domains of integral membrane proteins located at the plasma membrane. Upon binding, the virion enters the cell by receptor mediated endocytosis. Acidification of the resulting endocytic vesicle triggers the release of the vRNPs from the viral nucleocapsid and the fusion of the viral envelope with the endocytic membrane, thus allowing the discharge of the vRNPs into the cytoplasm of the infected cell [1]. Then, in a process mediated by cellular karyopherins, the vRNPs move rapidly into the nucleus (reviewed by Boulo et al. [7]). This

nuclear translocation is a very peculiar and intriguing property of influenza virus, which makes it quite unique among RNA viruses, as most RNA viruses remain in the cytoplasm, where they carry out most of their functions, including viral replication. However, influenza virus requires access to the cell nucleus for successful transcription and replication of the viral genome. Once in the nucleus, the incoming RdRp associated to the vRNPs initiates synthesis of viral mRNA and produces the first round of viral protein synthesis, which leads to the expression of the early viral proteins PB1, PB2, PA, NP, and non-structural protein 1 (NS1) [1]. NS1, plays a critical role during infection by neutralizing cellular antiviral responses and increasing viral protein synthesis, while decreasing the synthesis of cellular proteins (reviewed by Hale et al. [8], Lin et al. [9], and Krug et al. [10]). The role of neutralizing antiviral defenses has been recently shown to be shared by another viral protein produced by a limited number of viral strains: The so-called PB1-F2 protein, a 90 amino acid protein produced by an alternative ORF contained within the PB1 gene segment [11-12].

Newly synthesized RdRps drive the synthesis of mRNA coding for late viral proteins, including HA, NA, M1, NS2, and M2, and later on undergo a shift in activity, from transcription to viral replication. Upon synthesis of new vRNA, the newly synthesized vRNA gene segments are incorporated into vRNPs that are exported out of the nucleus thanks to complex interactions established with the viral proteins NEP and M1, and the cellular karyopherin CRM1 [13]. The vRNPs are then incorporated into new viral particles by a budding process that takes place at lipid rafts located at the apical surface of the plasma membrane, in an area referred to as the budozone (reviewed by Vait and Thaa [14]). These lipid rafts contain the viral integral membrane proteins HA, NA, and M2, and the interactions established between the cytosolic tails of these proteins, particularly HA and NA, the viral structural protein M1, and vRNPs, appear to play a critical role as drivers of the budding process (reviewed by Nayak et al. [5], and Rossman and Lamb [3]). The budding process is completed by membrane scission, a process that involves the activity of M2 [15], and the infectious viral particles are released into the extracellular environment thanks to the activity of NA, which cleaves sialic acid molecules off the cell surface, therefore freeing the virus from interacting with its cellular receptor [3].

1.3. How influenza affects humans: Clinical effects of influenza in the human host

Seasonal epidemic influenza usually causes an acute but self-limited infection of the respiratory tract, characterized by acute febrile symptoms, sudden onset of sore throat, nonproductive cough, rhinorrhea, myalgia, headache, and general malaise. Uncomplicated influenza usually resolves within 3 to 7 days of the first appearance of symptoms, although the cough and the general malaise may persist for up to a couple of weeks [16]. Complicated influenza is usually associated to the presence of other pre-existing medical conditions in the infected person, including pulmonary or cardiac disease, diabetes, obesity, and hypertension. Complicated influenza may result in primary influenza viral pneumonia, lead to secondary bacterial pneumonia, or exacerbate other underlying medical conditions, most frequently of pulmonary or cardiac nature. Most of the complicated cases of influenza occur

among people 65 years old and over. In the United States alone, seasonal epidemic influenza is estimated to be responsible for approximately 200,000 hospitalizations and 34,000 deaths every year [17-18]. Influenza has been shown to have strong pro-thrombotic and pro-inflammatory effects in the host. Therefore, a significant number of deaths associated with seasonal influenza infection are likely to be a consequence of cardiovascular events facilitated by pre-existing cardiovascular disease but triggered by influenza infection [19].

In contrast with the relative mildness of seasonal epidemic influenza, pandemic influenza usually has a more aggressive presentation. Pandemic influenza is associated with the introduction into the human host of a virus exhibiting an avian-type HA gene segment showing limited similarity to pre-existing human influenza viruses [20]. This determines that most humans will have few or no pre-existing cross-reactive antibodies against the avian-type HA protein that coats the viral particle, thus allowing unobstructed viral multiplication in the human host during the initial hours after infection. The fast viral growth that ensues under such conditions leads to a more aggressive disease presentation, accompanied sometimes by an unregulated cytokine production, a situation referred to as a "cytokine storm", that leads to an exaggerated pro-inflammatory response in the lungs [21]. Lung inflammation may in turn result in rapid progression to viral pneumonia, and in some cases to acute respiratory distress syndrome (ARDS), a condition that requires mechanical ventilation and may lead to death [20].

1.4. Our anti-influenza arsenal: Current therapeutic and prophylactic options against influenza

Currently, our most effective weapon against influenza virus is vaccination. However, effective vaccination requires a close antigenic match between the prevailing viral type being transmitted throughout the population and the viral strain used for vaccine development. The high mutational rate associated to the error prone viral RdRp, introduces frequent changes in the antigenic makeup of the virus. This imposes the need for an annual vaccination aimed at stimulating the immune system with a vaccine virus that closely resembles the changes occurring in the prevalent viral population. Selection of the viral strain to be used and vaccine production itself are both time consuming processes, and vaccine production against a novel pandemic virus may easily take 3 to 6 months, therefore keeping vaccination from playing an important role in the prevention of the first wave of viral infections during a pandemic, as recently evidenced during the 2009 H1N1 "swine" flu pandemic [22]. Further complicating the rapid production of anti-pandemic influenza vaccines is the potential undesired selection of viral variants that grow well in embryonated eggs, the current preferred media for amplification of the viral particles used for vaccine production. This may lead to the unintended selection of viral strains that are mismatched with the circulating viral strain [23].

A truly promising development related to influenza vaccines is the possibility of developing "universal" vaccines capable of triggering the production of broad-spectrum neutralizing antibodies effective against most (if not all) influenza strains. In addition to M2 and NP, the

targets classically pursued for the development of universal influenza vaccines [24], it has been recently demonstrated the existence of two regions within the viral HA molecule that are capable of triggering broadly neutralizing antibodies: The stem and the receptor-binding pocket. Animal models have shown substantial progress in the ability to enhance the production of antibodies against the stem region, which is normally ignored by our immune system due to its inaccessibility [25]. However, it is still uncertain whether the success observed so far in animal models will be achievable in humans.

Antiviral drugs constitute our second most valuable weapon against influenza virus. Currently, there are two main types of FDA-approved anti-influenza drugs: Those targeting the viral M2 proton ion channel, which are chemically derived from Adamantane and therefore referred to as Adamantanes; and those targeting the viral neuraminidase protein, referred to as neuraminidase inhibitors. There are two M2 ion channel inhibitors: Amantadine (Symmetrel®) and Rimantadine (Flumadine®). These drugs act by blocking the ion channel formed by the M2 transmembrane protein, therefore preventing the acidification of the viral particle and precluding the release into the host cell cytoplasm of the vRNPs away from the viral matrix upon membrane fusion. Similarly, there are two neuraminidase inhibitors that are widely used in the clinic: Oseltamivir (Tamiflu®) and Zanamivir (Relenza®). These drugs act by inhibiting the enzymatic activity of the viral neuraminidase (NA) protein, therefore blocking viral release at the plasma membrane and preventing the spread of infection throughout the respiratory tract [26].

Although the two types of drugs indicated above have proven helpful at preventing and treating complicated cases of influenza, and as prophylactic tools to prevent influenza transmission among members of the same household, their clinical use has been limited by the viral ability to develop resistance against them. Resistance against the Adamantanes in tissue culture settings was rapidly noted after their initial development, but it was not recognized as a real issue until 2003 when widespread resistance to Adamantanes was first observed among clinical viral isolates in the USA. By 2005, resistance to Adamantanes was so predominant that their use was discontinued [27]. This situation did not change as a consequence of the 2009 pandemic, as the M2 gene segment in the 2009 H1N1 pandemic virus originated from an Adamantane resistant strain. Therefore, today virtually all circulating influenza strains in the USA are considered to be resistant to Adamantanes and their clinical use is no longer suggested by the CDC [28]. Resistance to Oseltamivir was initially considered to be much more difficult to develop and disseminate among circulating strains, mainly because the mutations involved in providing resistance to it were thought to reduce transmissibility and overall viral fitness [29]. However, over 99% of all clinical isolates of H1N1 influenza virus during the 2008-2009 influenza season were found to be resistant to Oseltamivir during the 2008-2009 influenza season [30-31]. Luckily, the NA gene incorporated into the H1N1 2009 pandemic virus was derived from an Oseltamivir-susceptible strain, and Oseltamivir resistance became substantially less predominant as the pandemic strain displaced and completely substituted the previous seasonal H1N1 strain. Nevertheless, resistance has already been reported among several clinical isolates of the currently circulating 2009 H1N1-derived influenza strain; therefore, it is likely that Oseltamivir resistance will become widespread among circulating viruses once again [32].

The vision that emerges from the brief review of our current anti-influenza arsenal presented above is one that emphasizes the need for alternative weapons. Vaccination will likely continue to play an essential role in minimizing the damaging effects of seasonal influenza, but unless we succeed in developing a truly universal anti-influenza vaccine, vaccination is unlikely to play a major role in the control of a highly pathogenic pandemic. Similarly, current antivirals may not be that useful either against a new pandemic due to the virus' ability to develop resistance against drugs targeting viral components. One promising alternative is to target cellular factors required for viral growth and multiplication for the development of new host-targeted antiviral agents [33-34]. One obvious drawback of this strategy is that drugs targeting host factors may exert toxic or substantial secondary effects in the host. However, host-targeted antiviral agents are likely to be effective against a broad range of viruses, (as most viral strains rely on the same cellular factors for their growth), and offer very limited chances for the development of viral resistance. In our opinion, these potential advantages associated to the implementation of host-targeted antivirals far outweigh their drawback and provide further justification for continuous investments aimed at a better understanding of influenza biology.

1.5. A hot affair that is about to happen emphasizes the need for further basic research: A highly pathogenic bird virus is likely to generate the next influenza pandemic

While many viruses are capable of producing severe disease in humans, few have the ability to generate pandemics with the potential to devastate human society. Influenza virus is one of them, and its ability to bring havoc into our society has been clearly demonstrated at least once in our recent historic record. The 1918 H1N1 "Spanish flu" influenza pandemic killed more than 50 million humans and probably infected over 30% of the human population at the time [35]. To date, the 1918 H1N1 pandemic is still considered the most damaging pandemic ever faced by humanity.

The isolation and characterization of the full array of viral gene segments of the 1918 pandemic virus, obtained from frozen and formalin-fixed tissue samples collected from victims of the 1918 pandemic, allowed the reconstruction of the virus [36]. This outstanding accomplishment has provided important insights into the molecular nature of the "Spanish flu" pandemic virus. Among others, those studies have revealed the relevance of specific gene segments, and of the full gene constellation as a whole, for the high pathogenicity exhibited by the virus (reviewed by Taubenberger and Kash [37]). However, numerous questions remain unsolved, including the origin of the virus. It has been postulated that the 1918 virus likely resulted from the direct adaptation of a bird virus to humans, based on molecular signatures of its genome, such as the high GC content [38]. However, others consider it more likely that the virus had been introduced initially into swine, therefore allowing it to gradually become adapted to mammals [39]. In the absence of a viral repository that could provide detailed knowledge of the predominant viral strains in the years before the 1918 pandemic, it is very likely that the actual origin of the 1918 pandemic will remain unknown.

The lack of evidence demonstrating that the 1918 pandemic virus jumped directly from birds into humans, the apparent adaptation of all subsequent pandemic viruses (including the recent 2009 H1N1 "swine flu" pandemic) in swine before their introduction in humans, and the notion that only three HA subtypes (H1, H2, and H3) are normally observed in humans, out of the sixteen different HA subtypes present in nature, helped maintain for decades the belief that avian influenza viruses could not be transmitted directly from birds to humans. This belief came to an end in May 1997 when a 3 year old boy in Hong Kong was infected and killed by what was subsequently determined to be an H5N1 avian virus [40-41]. Subsequent events of direct transmission of H5N1 avian viruses into humans have accounted for up to 597 human cases reported to the World Health Organization (WHO) as of 19 March 2012, 351 of which have been lethal, for a mortality rate of about 59% [42]. Furthermore, direct transmission from birds to humans has also been reported for H9N2 and H7N7 viral types[20].

It has been calculated that the mortality rate for the 1918 pandemic was approximately 2.5% [35]. Therefore, the apparent pathogenicity of H5N1 viruses for humans, suggested from the number of lethal cases, appears to surpass by a wide margin that of the 1918 "Spanish flu". However, the real mortality rate associated to H5N1 infections may be orders of magnitude lower, as suggested by numerous studies performed on the prevalence of antibodies against H5 (a measurement indicative of exposure to H5N1 viruses) among individuals in regions where clinical cases of H5N1 influenza have been reported [43]. Nevertheless, the high pathogenicity of the H5N1 virus for humans is unquestionable.

The factor that has precluded H5N1 from triggering a highly pathogenic human pandemic is its inability to be efficiently transmitted among humans. It has been almost 15 years since the first demonstrated case of direct transmission of H5N1 to humans, and throughout this time there has been arguably only one case of apparent direct H5N1 transmission among humans [44]. This suggests that it might be extremely difficult for the virus to become fully adapted for human transmission. Unfortunately, this may not be the case: Two recent reports, one published in *Science* by the group lead by Dr. Fouchier [45], and one published in *Nature* by the group lead by Dr. Kawaoka [46], and whose publication triggered substantial controversy due to the potential dual use of the data reported [47], provide proof that a few mutations affecting a limited number of viral gene segments are sufficient to allow highly pathogenic H5N1 viruses to become capable of direct transmission among ferrets, the animal model that best recapitulates the major features of influenza virus infections in humans. As the mutations identified in the *Nature* and *Science* reports could evolve spontaneously among viruses that propagate in the wild, these reports stress the relative high likelihood that humanity may face a highly pathogenic H5N1 pandemic sometime in the future. Importantly, other studies have also indicated that the novel 2009 H1N1 "swine flu" exhibits high genetic compatibility with highly pathogenic H5N1 viruses [48], thus also increasing the odds of H5N1 producing a human adapted high pathogenicity strain via reassortment with 2009 H1N1. Considering the weaknesses of our current anti-influenza defenses, these observations emphasize the utmost relevance of intensifying basic research in influenza biology with the goal of identifying and developing new prophylactic and therapeutic options against this virus.

2. Transcription and replication of the influenza virus genome

In the 1970's, Stephen C. Inglis, from the University of Cambridge at England, was the first to discover that the Influenza viral polymerase was a heterotrimeric protein complex composed of PB1, PB2, and PA. Later, further studies determined that NP, which encapsidates vRNA or complementary RNA (cRNA), forms large RNA-protein complexes when it associates with a viral polymerase and a cRNA or vRNA segment. In the past 40 years, the extensive characterization of each subunit of the viral polymerase, including NP, and the conserved sequences of the vRNA gene segments have provided substantial information contributing to our knowledge of the mechanisms employed by Influenza A virus for transcription and replication. However, despite the collective efforts of the influenza scientific community, some ambiguity still exists about how some steps take place during viral transcription and replication. In order to achieve a complete and detailed molecular knowledge of these processes, it is important to keep in perspective the basic knowledge previously established by the founders of the field and the most recent findings related to the regulation of the viral polymerase, all of which are briefly presented below.

2.1. The engine behind a pathogenic machinery: Nuclear accumulation and 3D structure of the viral RdRp

The release of the vRNPs from the infectious viral particle is perhaps the most critical step in the initiation of viral replication. This event requires the acidification of the viral particle core for the disruption of the intermolecular interactions shared between the vRNPs and the M1 viral protein [49]. The viral complex responsible for the acidification of the virion's core is the one formed by the tetramer of the M2 viral protein, which constitutes the smallest ion channel discovered to date [50]. The acidic environment then triggers the HA-mediated fusion of the viral and endosomal lipid bilayers [51-53], which allows the free vRNPs to migrate from the virion's core into the cytoplasm of the infected cell. Since influenza transcription and replication take place in the cell nucleus, the incoming vRNAs must then be imported into the nucleus, a process mediated by two nuclear localization signals (NLS) present in NP, (spanning residues 1-13 [NLS1] and residues 198-216 [NLS2]) [54]. Recent studies, performed using digitonin-permeabilized cells in the presence of exogenous cytosol and energy-regenerating systems, have suggested that the directionality of nuclear traffic of the vRNPs during infection is determined by the exposure or masking of NLS1 in NP [55]. However, even in the presence of an antibody against NLS1 in NP, vRNPs still migrate to the nucleoplasm, indicating that NLS2 in NP also contributes to the nuclear accumulation of vRNPs [54]. Additional studies are needed in order to assess the contribution of NLS2 in NP during the nuclear accumulation of the vRNPs.

The nuclear transport of the *de novo* synthesized viral polymerase proteins takes place in a highly organized manner and is critical for viral transcription and replication. The nuclear transport of PB2 occurs in the absence of any additional viral protein, while dimerization of PB1 and PA in the cytoplasm is required for their efficient nuclear trafficking [56-58]. Recent fluorescence cross-correlation spectroscopy (FCCS) studies demonstrated that the trimeric

structure of the RdRp is only present inside the cell nucleus [57]. This finding led to the discovery of a new function for the N-terminal domain of PA; preventing the formation of these trimeric structures in the cytoplasm [59]. Unfortunately this inhibitory mechanism has not been fully characterized, but it is speculated that the flexible linker region separating the N- and C-terminal domains of PA allows for a conformational change that prevents binding of PB2 while residing in the cytoplasm. Should this be true, it would then be important to characterize the molecular mechanism triggering the conformational change in PA that takes place upon its entry in the nucleus.

The three subunits comprising the viral RNA-dependent RNA-polymerase (RdRp) are encoded by the three largest vRNA gene segments of Influenza A virus. PB2 is the largest subunit of the polymerase and the carrier of the 7-methylguanosine 5'-cap binding site [60]. The second largest subunit in the viral polymerase is PB1, and this is the only subunit that has the four conserved amino acids distributed among four different motifs present in all RNA-dependent RNA-polymerases and RNA-dependent DNA-polymerases [61]. Lastly, the smallest subunit, PA, has a characteristic PD-(D/E)-XK motif present in type II endonucleases, and is responsible for the cleavage of the 5'-cap of cellular mRNA [62-63]. These three viral factors form a very compact protein complex that still manages to maintain a high degree of flexibility.

Several different 3D conformations have been observed using cryo- and negative staining electron microscopy for the purified recombinant influenza viral polymerases in the presence or absence of vRNA, NP, and both [64-67]. Those studies demonstrated that the polymerase has a globular hollow conformation that becomes compacted upon association with vRNA alone or vRNA in the presence of NP oligomers [64-67]. Additionally, PB1, PB2, and NP have been identified as the main viral proteins mediating the RNA-dependent interaction of the viral polymerase and the RNA-protein complex formed between NP and vRNA [65]. The diverse array of quaternary structures portrayed by the molecular structures predicted for the viral polymerase in the studies mentioned above, provide strong evidence of the existence of multiple conformational stages for the polymerase, likely associated to its various functional properties during transcription and replication. However, multiple other structures such as those associated to the cRNA-bound stage, the 5'-cap interacting stage, and the oligomeric stage formed by the polymerase inside the nucleus, still remain unresolved.

2.2. Transcribing the blueprint to assemble the engine: Viral mRNA transcription

The transcription of the viral genome begins as early as one hour post-infection and takes place inside the cell nucleus [68] (Figure 2A). Early during infection, transcription of viral mRNAs is coupled to their translation and seems to plateau at 2.5 hours post-infection, whereas viral protein translation continues increasing throughout infection [68]. This provides evidence that a not fully characterized post-transcriptional regulatory mechanism ensures continued translation of the mRNA produced early during infection. Given that Influenza depends on the endogenous 5' cap-dependent cellular ribosomal machinery to

translate its gene products, influenza has developed a mechanism called “cap-snatching” to prime the synthesis of all viral mRNAs [69]. According to *in vitro* reconstitution assays the cap-snatching event seems to be dependent upon binding of the viral polymerase complex on the viral promoter at the 3′-end of vRNA molecules [63, 69-70]. This interaction between the viral RdRp and the 3′-end of the vRNA triggers a conformational change within the RdRp, and allows PB2 to bind the 7-methylguanosine 5′-cap from cellular mRNAs [60, 69]. Additionally, the interaction between the viral RdRp and the cellular mRNA brings into close proximity the cellular 5′-cap with the PD-(D/E)-XK motif in PA, which then enzymatically cleaves 9-17 nucleotides downstream of the 5′-cap at guanine or adenine residues [63, 71] (Figure 2B). Through the use of site directed mutagenesis, vRNP reconstitution assays, and primer extension analysis it has been further shown that the coordination of a divalent ion (preferably manganese or magnesium) by the N-terminal domain of PA is required for the cap-snatching event [62-63]. The divalent cation required for the endonucleolytic activity of PA is stabilized by a cluster of four residues (H41, E80, D108, E119) residing in the N-terminal domain of PA [62-63]. Moreover, the crystal structure of these four residues seems to be unusually distant from the catalytically active Lysine residue at position 134, when compared to other crystal structures of type II endonucleases [62-63]. As mentioned above, the 5′-cap from cellular mRNAs is used by the viral polymerase to prime the transcription of viral mRNA during infection [69, 72](Figure 2C). This phenomenon has been demonstrated by *in vitro* transcription assays in which the chemical removal of the 5′-cap from β -globin mRNA abolished the synthesis of viral mRNA [72]. Consistently, recapping of the β -globin mRNA through the use of a Vaccinia virus guanylyl or methyl transferase rescued the production of IAV mRNA [72].

While the 5′-cap from cellular mRNAs primes the transcription of viral mRNAs, the addition of ~150 adenines at the 3′ end, independent of the cellular poly-adenylation machinery, finalizes viral mRNA elongation. Polyadenylation of viral mRNAs is mediated by “stuttering” of the viral polymerase upon reaching a uridine-rich sequence usually made of 5-7 uridines, located 17 to 22 nucleotides away from the 5′ end of the vRNA template [73] (Figure 2D). At first, it was hypothesized that the formation of a panhandle structure between the complementary regions located at the 3′ and 5′ ends of the vRNA created sufficient steric hindrance to prevent the viral polymerase from transcribing the complete vRNA molecule, allowing the polyadenylation of viral mRNAs [73-74]. Later, *in vitro* transcription assays, performed using vRNA segments with various point mutations at the 5′-end as templates for transcription, determined that the formation of a duplex between the 3′- and 5′-ends of the vRNA, along with the binding of the viral polymerase to the 5′-end of the vRNA are both required for transcription [75]. This finding led to a refined hypothesis in which the viral polymerase binds to the panhandle structure formed between the complementary regions of the vRNA, recognizes the viral promoter, and subsequently elongates viral mRNA transcripts while remaining bound to the 5′-end of the template [75]. Because the viral polymerase is bound to the 5′-end of the template throughout the course of transcription, the viral polymerase is not capable of transcribing the 5′-end of the vRNA [75]. In other words, upon reaching the uridine-rich region at the 5′-end of the vRNA, the restraints inflicted by the intermolecular interactions shared between 5′-end of the vRNA

and the viral polymerase will produce polymerase stuttering, and this in turn will allow polyadenylation to occur [75]. Subsequent studies proved that transcription of viral mRNA was mediated primarily by the incoming vRNP-bound polymerase and not by polymerases added in *trans* [76]. This unexpected but important data was obtained during vRNP transfection experiments in which the transcription of viral RNA introduced into the cell as a component of purified vRNPs formed in the presence of a mutated (transcriptionally inactive) PB2 subunit could not be rescued by the addition of recombinant functional polymerases [76]. Moreover, vRNP co-transfection experiments have shown that the transcription of viral gene segments could not take place by polymerases present on adjacent vRNPs [76]. Altogether, the results discussed above strongly suggest that transcription of viral mRNA is driven by the polymerases that accompany the vRNA into the nucleus in the form of vRNPs early during infection.

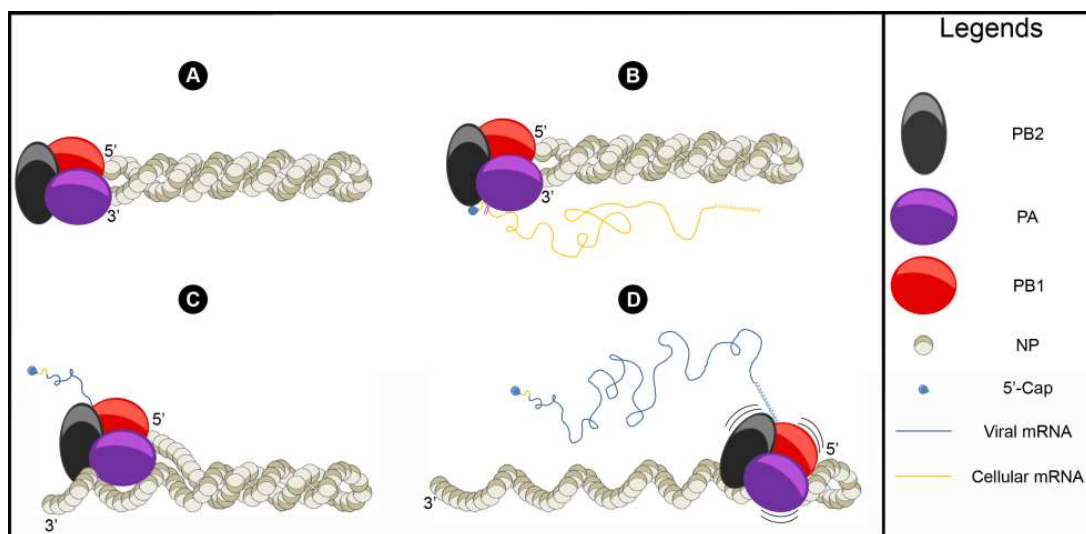


Figure 2.

2.3. The time and place to make the mistakes that might help future viral generations: Viral genome replication

The replication of the influenza virus genome requires the vRNA to first be transcribed into an additional type of viral RNA known as complementary RNA (cRNA). cRNA has a positive polarity and, as its name implies, it possesses a sequence that is complementary to that of the vRNA molecule. It is imperative for genome replication that the cRNA molecule contains the complete sequence of the vRNA, since it will be used as the template that will drive the synthesis of new vRNA late during infection. Early studies demonstrated that, in sharp contrast with the transcription of mRNA, the transcription of cRNA can be initiated in a primer-independent manner, while also using the same 13 and 12 nucleotides at the 5'- and 3'-end of the vRNA respectively as a promoter for transcription [77-78]. However, despite cRNA molecules being used for the replication of the viral genome, the accumulation of cRNA is very limited at early time points during infection [77]. It was also shown that the transcription of complete vRNA segments from cRNA templates could not be achieved in the absence of NP during *in vitro* transcription assays [79]. Subsequently, it

was demonstrated that the absence of cRNA at early time points post-infection could be explained by the lack of free NP protein early during infection, because the artificially driven expression of NP within the cell before infection allowed the accumulation of cRNA at early time points post-infection [80]. The model developed based on these observations proposed the formation of a “stabilization complex”, in which the binding and coating of the cRNA molecule with NP monomers prevents its degradation by cellular RNAses [80]. Surprisingly, even after 30 years of research, not very much information has been gathered on the molecular mechanisms involved in the elongation of cRNAs or how the steric hindrances faced by the viral polymerase during transcription are evaded to permit the complete transcription of the cRNA segment.

2.4. Knowing when to stop and accelerate leads to a more pathogenic performance: Mechanisms regulating transcription and replication

The meticulous orchestration of the multiple molecular complexes formed between the viral polymerase, vRNA, cRNA, other viral proteins, and cellular factors throughout the various steps during viral transcription and replication, provides additional molecular targets and mechanisms to regulate the activity of the RdRp. Those interactions are constantly triggering conformational changes on the flexible structure of the viral polymerase, which in turn dictate the specific functionality that it should exert. In order to further exemplify how different molecular interactions can exert a regulatory function on the viral polymerase, the structure of the viral promoter and the role of other viral proteins interacting with the RdRp will be discussed briefly below.

i) The structure of the viral promoters: Through the use of Nuclear Magnetic Resonance (NMR) and calculations for determining the 3D structure of the vRNA promoter, it has been shown that the vRNA promoter has a terminal stem, followed by an internal loop, and a proximal stem [81-82]. The terminal stem displays an inherent bend that allows the viral promoter to be easily melted, therefore possibly playing a regulatory role during the initiation of RNA synthesis [82]. This intrinsic bending of the terminal stem might be of great relevance for transcription initiation, since the viral RdRp does not appear to have a helicase activity associated to it [82]. Additionally, the nucleotides involved in the formation of the internal loop within the vRNA promoter correspond to the same nucleotides previously identified as the binding sites for the viral RdRp [82-83]. Most of the residues constituting the internal loop have a highly dynamic structure and their binding to the viral polymerase creates an energetically favorable reaction by reducing the high entropy displayed by this structure [82]. Therefore, it is suggested that this internal loop in the vRNA promoter positions the catalytic core of the viral polymerase at the transcription initiation site [82].

The structure of the cRNA promoter used for genome replication has also been determined by NMR and exhibits a somewhat comparable but very distinct structure to that of the vRNA promoter [84]. Importantly, the structural differences between the two viral promoters might allow the viral polymerase to distinguish between the transcription of mRNA/cRNA and vRNA [84]. This idea is further supported by the fact that the viral

polymerase binds to the cRNA promoter through the central region of PB1, while the vRNA promoter interacts with the C-terminal region of PB1 [85]. Interestingly, in both cases the internal loop regions of the vRNA and cRNA are involved in the binding of the RdRp, suggesting that the structure of the internal loop might be more relevant than the actual sequence itself for positioning the viral polymerase to initiate transcription [84-85]. Nonetheless, even though the internal loop regions share general structural similarities, their individual conformations are quite distinct. The comparison of both structures showed that the internal loop of the cRNA promoter is very unstable when compared to the internal loop present in the vRNA promoter [84]. The lack of stability of the internal loop region in the cRNA is due to the improper stacking of the bases belonging to the nucleotides located in that region, which contrasts sharply with the proper stacking observed for the nucleotides located in the equivalent regions of the vRNA promoter [84]. However, the relevance of such great difference in stability for both the vRNA and cRNA loop regions still remains unknown.

ii) Viral proteins involved in the regulation of the viral polymerase: As mentioned above, viral proteins different from those constituting the vRNP complexes can also play an important regulatory role in the function of the viral polymerase. To date, the molecular mechanisms underlying the viral polymerase switch from transcription to replication still remain unresolved. However, the involvement of several viral factors has been characterized to some degree. So far, only one viral protein has been identified as a regulator of the transcriptase activity of the viral polymerase. Primer extension analyses, have shown that the mRNA/vRNA ratio generated during vRNP reconstitution assays performed in the presence of NP and the viral RdRp (but no other viral protein) is substantially larger than the mRNA/vRNA ratio normally generated during viral infection [86]. Testing the effect of overexpressing individual Influenza A viral proteins on the transcription of multiple vRNA gene segments during vRNP reconstitution assays allowed the identification of a transcriptional regulatory function associated with NEP [86]. Subsequently, it was shown that the presence of NEP leads to the generation of Influenza A virus-derived small viral RNAs (svRNA) [87]. These svRNAs correspond to the 5'-end of each vRNA genomic segment and have different lengths, ranging from 22 to 27 nucleotides, depending on the gene segment they are derived from, and seem to interact directly with the viral polymerase [87]. More importantly, their accumulation in the cell is coupled to a shift from viral transcription to replication, while their depletion results in loss of vRNA synthesis for their parental gene segment [87]. These results suggest that these svRNAs prime the initiation of transcription for genomic vRNA, as previously observed in cellular DNA-dependent RNA-polymerases. Altogether, both NEP and svRNAs seem to be playing a critical role in the switch from transcription to replication. Nevertheless, no direct interaction between the polymerase and NEP has been observed through the use of immunoprecipitation assays, therefore leaving the exact molecular mechanism involved in this regulatory process unresolved. An important lesson derived from these studies is that, in the absence of supplementary proteins (e.g. NEP) the limited number of viral factors (PB1, PB2, PA, and NP), commonly employed during vRNP reconstitution assays, might prevent us from realizing the authentic effects imposed by chemical inhibitors or point mutations on the

transcriptase activity of the RdRp, since under such conditions these assays do not accurately recapitulate the events involved in viral transcription and replication during influenza infections.

3. Cellular factors important for viral multiplication

The first identification of cellular factors involved in influenza viral infection took place as early as 1959 [88]. However, up to fairly recent times, most studies related to influenza replication and multiplication focused exclusively on the mechanistic assessment of processes mediated by viral proteins and put little emphasis on the cellular factors or pathways involved in influenza replication. During the last few years, the use of multiple approaches such as protein pull-downs, chemical inhibition studies, yeast-two hybrid screening, affinity purification, and RNA interference (RNAi), have led to the identification of multiple cellular factors essential for influenza viral replication [89-97]. In the sections below, we will review the most recent findings related to the relevance of specific host cellular proteins and systems for influenza transcription and replication. Despite the substantial progress achieved during the last few years, the overall status quo of the field is that the functions of the characterized viral-cellular protein interactions remain mostly unclear. Table 1 summarizes the cellular factors required for influenza replication discussed in this section.

3.1. Downloading a virus: Viral egress from endocytic vesicles

In order for the vRNPs to reach the nucleus, which constitutes their final destination within the host cell, they have to be released from the incoming viral particle. This process requires the acidification of the viral particle and fusion of the endosomal and viral membranes. The decrease in pH within the endosomal compartment requires the cellular vacuolar proton ATPases (v-(H⁺)ATPase) to hydrolyze ATP and transport protons inside the vesicle [98]. Three large screenings for cellular factors relevant for influenza infection, performed by Hao et al. [91], Konig et al. [92], and Karlas et al. [95], identified members of the v-(H⁺)ATPase family as critical host factors required for the progression of influenza infection. Before the screenings, it had been established that treatment of cells with concanamycin A, a well known inhibitor of v-(H⁺)ATPases, was enough to halt the production of viral proteins at an early stage post-infection. However, the addition of concanamycin A one hour after infection had no effect on the production of viral proteins, suggesting that the inhibition of endosome acidification affected only the earliest stages of viral infection [99]. Subsequent studies using diverse inhibitors and RNAi approaches confirmed the involvement of v-(H⁺)ATPases in early stages of infection [100-101] and provided further details of their specific role during influenza infections [102]. Studies by Marjuki et al. [102] revealed that, upon entering the cell, influenza activates the ERK and PI3K pathways. These pathways activate the E subunit of the v-(H⁺)ATPase V1 domain, upregulating its proton pumping activity and leading to a more rapid acidification of the endosome [102], a process needed for the fusion of the viral and endosomal membranes.

The non-clathrin-coated vesicular coat COP-I proteins have also been identified as important cellular factors required for efficient viral replication in three large viral-cellular interaction screenings (Table 1.), thus supporting a relevant role for this protein family in the influenza life cycle. However, it is still uncertain the specific events in the viral life cycle that are affected by this protein family. Because one of the screenings that identified the COP-I proteins as relevant for viral infection used the transcription of early viral genes as output, and the known role of COP-I proteins in retrograde traffic of Golgi vesicles back to the ER [103], it is likely that the COP-I proteins are necessary for some stage of viral entry.

Onward from endocytosis of the viral particles, it has been suggested that a member of the lysosome-associated membrane glycoprotein family, LAMP3, is involved in the release of the vRNPs from the endosomal compartments and in facilitating vRNP nuclear import. However, the hypothetical pivotal role suggested for LAMP3 during infection is somewhat surprising, since LAMP3 is interferon induced and up-regulated during viral infection and therefore expected to exert an anti-viral activity. Nevertheless, siRNA knockdown of LAMP3 significantly reduced levels of NP expression during infection [104], thus supporting the enhancing role postulated for LAMP3.

3.2. A molecular hijacker: Nuclear shuttling of vRNPs

Once inside the cytoplasm, viral RNA transcription begins only upon arrival of the viral ribonucleoprotein (vRNP) complexes into the nucleus. The shuttling across the nuclear pore complex of both, the largest macromolecular complexes formed by the virus during infection, i.e. the vRNPs, as well as of its individual protein components, rely on the cellular nucleocytoplasmic trafficking machinery. The importin α/β pathway is the classical nuclear import pathway, transporting cargo proteins from the cytoplasm into the nucleus upon recognition of a nuclear localization sequence (NLS) by karyopherins, typically importin α . Most cargo proteins directly interact through their NLS with the adapter protein importin α , which then binds importin β for nuclear import. Transport of the importin α/β -cargo protein complex is facilitated by interactions with nucleoporins (Nups), the structural components of the nuclear pore complex [105]. Expectedly, Nups were one the major categories of factors identified in large scale screenings for cellular proteins required for efficient influenza infections, having been identified in 4 different screenings [92-93, 95, 97]. Their fundamental role as regulators of nucleocytoplasmic traffic allows them to dictate the nucleocytoplasmic transport of viral transcripts, proteins, and RNPs.

The PB2 and NP viral proteins have been shown to interact with importin $\alpha 1$ for their nuclear trafficking. As previously described, NP contains two NLS, both of which are of great relevance for proper nuclear import of vRNPs, since mutating either of the two NLS domains results in decreased accumulation of vRNPs within the nucleus [54]. As for PB2, its interaction with importin $\alpha 1$ was initially characterized through targeted mutations in the aspartic acid at position 701 and asparagine at position 319. These two point mutations on PB2 have the ability to further enhance its affinity for importin $\alpha 1$, and consequently, its nuclear accumulation [106]. Furthermore, studies using Fluorescence Cross-Correlation Spectroscopy (FCCS), in which the live transient trafficking of transiently expressed viral

Reference	Mayer et al. (43)	Jorba et al. (48)	Hao et al. (47)	König et al. (46)	Brass et al. (45)	Shapira et al. (44)	Karlas et al. (42)	Bortz et al. (10)	Tafforeau et al. (11)	Literature Based
Cellular Interactors Identified by	RdRp purification (PB1/PB2/PA)	RdRp purification (PB1/PB2/PA)	RNAi Approach	RNAi Approach	RNAi Approach	Yeast Two-Hybrid	RNAi Approach	RNAi Approach	Yeast Two-Hybrid with vRdRp (PB1/PB2/PA)	Host Factors Discussed, Not Present in Screens
Endocytic vRNP Release			ATPVOD1	ATP6AP1, ATP6AP2, ATP6V1A, ATP6V1B2, ATP6VOB, ATP6VOC, ATP6VOD1			ATP1A2, ATP6AP1, ATP6AP2, ATP2C1, ATP6VOC, ATP6VOD1, ATP6V1A, ATPV1B2			LAMP3
Nuclear Import	KPNA1, KPNA2, KPNA3				KPNA1, KPNA3, KPNA6		KPNA1, KPNA3, KPNA6	KPNA1, KPNA4, KPNA3		
Transcription and Replication	POLII(CTD)	SFPQ/PSF		EIF2AK2	POLRH2, EIF2S1		POLRH2, POLR2L, EIF4A3, EIF3A, EIF3C, EIF3G	SFPQ	POLR2A, EIF3S6IP	TFIIH, P-TEFb, PSF
vRNA Splicing	UAP56						CLK1			SF2/ASF
Nuclear export and Membrane Migration			NXF1	PRKC1, MAPEK/MEK, MAPK1/ERK, RAB11, RAB17, RABEP1, NUP214, NUP153, NTRK2	NXF1, NUP62, NUP88, NUP98, NUP107, NUP214, NUP153, NUP1	RABGEF1	NXF1 XPO1(CRM1), RAB6B, NUP98, NUP205,		NUP54	URH49, REF/ALY, RAF/MEK/ERK, PDGFR, PABP1, FPPS, CDK1, CDK2, CDK5
Apoptosis	HSP90			HSP90, PRKC1, PRKCD, PRKACA, PRKAG2			HSP90			
Other Viral-Host Pathways	PARP1			SUMO1, SUMO2, SUMO4, SAE1, ARCN1 (COPD), COPA, COPB, COPG	ARCN1 (COPD), COPA, COPB1, COPG, COPZ1, COPS6	SUMO1P1, UBE21	UBAC2, COPA, COPB1, COPB2, COPG,	PARP1	COPS5	ERK, PI3K

Table 1. Summarized Host Factors Involved in Influenza Replication and Viral Processes.

proteins is recorded, confirmed the interaction between PB2 and importin α during its nuclear import. In contrast with the use of the classical nuclear traffic pathway by PB2, the FCCS studies also noted that nuclear traffic of the remaining viral polymerase subunits (PB1 and PA) occurs through a non-conventional importin α -independent pathway [57], with PB1 forming a heterodimer with PA, which is required by PB1 and PA to gain access to the nucleus (as stated in section 4a) and using it in conjunction with Ran binding protein 5 (RanBP5) as the carrier [107].

3.3. Someone's in the kitchen: Cellular dependent transcription and vRNA synthesis

The activation of RdRp driven vRNA transcription relies heavily on the host cell transcriptional machinery. Four different viral-host interaction screenings [89, 93, 95, 97] identified subunits of the cellular RNA Polymerase II (Pol II), which further reinforced previous work performed on the proposed mechanisms for the role of Pol II on viral transcription. During the normal cycle of cellular RNA transcription, the promoter associated Pol II is phosphorylated by TFIIH to initiate transcription. Transcription, however, can be temporarily paused by negative elongation factors, allowing time for the addition of the 5' cap to the short segment of pre-mRNA transcript already synthesized. Subsequent phosphorylation of the C-terminal domain of the paused Pol II, mediated by P-TEFb, relieves the pause and re-activates transcription [108]. Before the onset of viral transcription, the viral PB2 protein, in association with PB1 and PA, is brought into close proximity to the cellular Pol II by TFIIH, allowing it to bind the 5'-cap structure of a cellular pre-mRNA transcript [109]. As described in the previous section, PA steals the 5'-cap along with a 9-17 nucleotide extension in a mechanism referred to as "cap-snatching." The 5'-cap oligonucleotide then serves as a primer for the polymerase subunit of the RdRp, PB1, to initiate vRNA transcription and elongation [110]. Elongation ends when the RdRp reaches a 5 to 7 uridine base-pair extension towards the 5' end of the vRNA template, which signals for polyadenylation of the transcript [111-112]. Cell splicing factors also appear to be vital for efficient vRdRp-dependent transcription of the viral genome. In a reporter assay, knockdown of the nuclear Splicing Factor Proline-Glutamine Rich (SFPQ/PSF) reduced levels of viral transcription, but had no effect on viral genome replication. Moreover, *in vitro* analysis of viral transcription revealed about a ~5-fold reduction in the fraction of viral polyadenylated (positive sense) transcripts, suggesting that SFPQ/PSF seems to facilitate the polyadenylation of viral transcripts [113]. UAP56, (Bat1/Raf-2p48), a known viral-interactor protein that was also identified in a vRdRp interaction screening by Mayer et al. [89], is a fairly well established RNA helicase involved in spliceosome assembly [114], facilitating the nuclear export of cellular mRNA [115], and is a constituent of the transcription export complex which delivers pre-mRNAs bound to the exon-junction complex to nuclear export factor 1 (NXF1) [116]. Although UAP56 is clearly a factor in cellular RNA splicing, *in vitro* studies indicate that UAP56 forms heterodimers with NP in the absence of vRNA. Upon addition of vRNA the heterodimer dissociates and, through an unknown mechanism, facilitates vRNA synthesis [117] suggesting higher affinity interaction of one of these proteins for vRNA. Altogether, this not only suggests a pro-viral functionality of UAP56 in

enhancing vRNA production, but that efficient viral replication exploits multiple functionalities of cellular host factors in a well orchestrated manner.

3.4. Cutting and mincing the viral transcripts: Host splicing machinery and influenza viral RNA

Splicing of viral mRNA, needed for the production of M2 and NS2, is fully dependent on the host splicing machinery due to the lack of splicing factors encoded within the viral genome. However, the splicing of the M viral segment to produce the M2 transcript is orchestrated by both viral and cellular components. CDC-like kinase 1 (CLK1), a protein responsible for the regulation of pre-mRNA splicing, plays a key role in the production of M2 vRNA spliced transcripts as demonstrated through RNAi knock-down and chemical inhibition studies [95]. CLK1 has been implicated in regulating the splicing of M1 mRNA [95], by phosphorylating the serine/arginine rich splicing factor, SF2/ASF [118]. SF2/ASF is a member of the serine/arginine rich splicing factor family, which are key factors in both alternative and constitutive pre-mRNA splicing, (reviewed in [119]) and is a critical splicing factor involved in the production of M2. Although cellular transcripts most often rely on consistent excision of intronic sequences, influenza virus replication depends on precise proportions of spliced and unspliced transcripts, as observed in the processing of the M1 mRNA transcript. For the duration of infection, splicing factor SF2/ASF is associated to a purine-rich enhancer sequence on the 3' end exon of the M1 transcript. In early stages of infection, the 5' mRNA₃ splicing donor site of the M1 RNA transcript is the highly preferred splicing donor site by the host splicing machinery, thus causing the disfavored M2 5' splicing donor site, ~40 nucleotides downstream, to be ignored. As infection progresses, a newly synthesized viral RdRp binds the 5' end of the unspliced M1 mRNA, blocking access of the cellular splicing machinery to the mRNA₃ 5' end splice donor site. [120]. The host splicing machinery then associates with the M2 5' splice donor site, awaiting activation by SF2/ASF to initiate production of M2 mRNA transcripts [121]. Host splicing factors involved in the processing of the eighth gene segment, NS segment, have yet to be specified. Although it appears that the competition between the splicing machinery and the nucleocytoplasmic transport machinery regulate the production of NEP, it has been established that viral factors associated with viral infection do not regulate splicing of the NS transcript [122-123].

3.5. Unexpected delivery: Nuclear export of Viral mRNA and the vRNP

Since IAV has the unique capability among RNA viruses of replicating in the nucleus, there are additional processes such as viral mRNA and vRNP nuclear export that are essential for viral infection. Due to the nature of the "cap-snatching" event that takes place during infection, nuclear cap-binding complexes have the ability to interact with viral mRNAs and aid in the recruitment of nuclear export regulatory factors such as REF/Aly [124]. This was shown through the use of simple interaction studies in which viral mRNA was able to co-immunoprecipitate with translational machinery factors such as cellular cap binding proteins, RNA and export factor-binding protein REF/Aly, cellular poly(A)-binding protein 1 (PABP1), the 20 kDa subunit of the nuclear cap-binding complex (NCBP2), and the

eukaryotic translation initiation factor 4E (eIF4E) [124]. Additional cellular factors involved in viral mRNA nuclear export were also identified by multiple RNAi screenings. These studies revealed that independent viral mRNA transcripts exploit specific nuclear export factors for their individual export into the cytoplasm. It was not surprising that NXF1, a cellular protein involved in the nuclear export of cellular mRNAs, was found to be of relevance for influenza replication in three independent screenings [91, 93-95]. siRNA knockdowns against NXF1 revealed that mRNAs coding for the early protein NS1, and the late proteins HA, NA, M1, and M2, depend on NXF1 for their nuclear export [91, 93-95]. The mRNAs encoding for the viral polymerase proteins PB1, PB2, PA, and NP also show dependence on NXF1 for their nuclear export, but to a lesser extent [125]. On the other hand, the nuclear export of mRNAs coding for M1, M2, and NS1 show dependence on the previously mentioned RNA helicase, UAP56, for their efficient nuclear export [126]. The dependence of these transcripts on UAP56 was demonstrated by knocking down UAP56, or its high sequence identity paralog URH49. The limited availability of either UAP56 or URH49 triggered an accumulation of influenza dsRNA at the nuclear membrane and an increase in IFN production [126]. However, even when the multiple RNAi screenings have identified cellular factors like NXF1 and UAP56 as essential for viral mRNA nuclear export, additional factors such as those involved in the nuclear export of the mRNA for NEP still remain unknown. Also, we still need to identify the other proteins forming the complexes involved in nuclear export of viral mRNAs, to facilitate the complete characterization of the molecular mechanism driving this fundamental process.

As mentioned above, vRNP nuclear export is also a process of great relevance for the assembly of infectious viral particles. So far, we know that mature vRNP complexes depend on the CRM1 pathway for their nuclear export. CRM1 mediated nuclear export is carried out through the recognition of a nuclear export signal (NES) present in the amino acid sequence of the cargo protein [127]. In the case of the vRNPs, CRM1 binds to the NES located at the N-terminal region of NEP. Interestingly, after mutating the NES present in NEP, its nuclear export was impaired without affecting its interaction with CRM1 [13]. Crystallographic studies of the M1 binding domain present in NEP suggested that the nuclear export of vRNPs required the formation of a “daisy-chain complex” in which, RanGTP-loaded CRM1 associates with the N-terminal NES of NEP, and NEP is associated via its C-terminal domain to an M1 molecule bound C-terminally to the vRNP [128]. Furthermore, several studies have identified a structural component of the nuclear pore complex involved in CRM1-dependent export, Nucleoporin 98 (Nup98), as a critical host factor during influenza A viral replication [91, 93, 95, 129]. A recent study demonstrated the interaction between a GLFG repeat domain located within the Nup98 protein and NEP [129]. This interaction suggests that Nup98 facilitates nuclear export of the vRNPs during influenza infection, via its interaction with NEP [129]. To confirm the relevance of the GLFG domain in Nup98 during viral infection, a mutant form of Nup98 lacking the GLFG domain was overexpressed by transfection and led to a substantial decrease in viral titers [129]. Even though the studies described above provide evidence that Nup98 is an essential cellular factor for viral infection, a more detailed knowledge of the molecular events mediating vRNP nuclear export is still missing.

Lastly, after leaving the nucleus, the vRNPs need to migrate towards the apical surface of the cell to become encapsidated into new virions. Previous studies demonstrated through the use of live imaging microscopy that, upon entering the cytoplasm, vRNP complexes associate with a pericentriolar recycling endosome marker called Rab11, which is involved in endosomal recycling and trafficking [130]. The accumulation of vRNPs at the microtubule-organizing center after nuclear export, allows them to interact with Rab11-positive recycling endosomes and migrate along microtubules to the sites of budding at the apical surface of the plasma membrane [131-133].

4. Influenza virus and the cellular SUMOylation system

4.1. A cellular system with a funny name: Generalities of the SUMOylation system

SUMOylation, the post-translational conjugation of the Small Ubiquitin-like MOdifier (SUMO) to a protein, involves the formation of an isopeptide link between the carboxyl group located at the C-terminal glycine residue in SUMO and the epsilon amino group in a lysine residue located internally in the target protein. There are four different SUMO molecules in humans, SUMO 1-4. SUMO2 and SUMO3 are 95% identical to each other, and in consequence are usually referred to simply as SUMO2/3, whereas SUMO1 shares only approximately 50% identity with them (reviewed by Geiss-Friedlander and Melchior [134], and Dohmen [135]). Besides their sequence differences, SUMO2/3 and SUMO1 appear to be functionally different as well: First, the pool of proteins that are SUMOylated with SUMO2/3 is only partially overlapping with the pool of proteins that are SUMOylated with SUMO1 [136-137]; second, SUMO2/3 is capable of forming poly-SUMO2/3 chains *in vivo*, whereas SUMO1 is not (reviewed by Ulrich [138]). The ability of SUMO4 to be conjugated to proteins *in vivo* is still being debated and therefore its biological role is still uncertain.

The enzymatic pathway involved in SUMOylation resembles that required for the conjugation of its related protein, Ubiquitin, and consists of an E1 activating and an E2 conjugating enzymes, a set of E3 ligases, and a set of SUMO-specific peptidases and isopeptidases (Figure 3). However, the specific enzymes required for SUMOylation are distinct from those involved in Ubiquitylation, and therefore the Ubiquitin and SUMO pathways are independent from each other and subject to different regulatory mechanisms. Interestingly, while in the Ubiquitin pathway the conjugation of Ubiquitin to a substrate has an absolute requirement for the involvement of an E3 Ubiquitin ligase, in the SUMO pathway the E1 and E2 activities (performed by the heterodimeric protein SAE2/SAE1 and Ubc9, respectively) are sufficient for SUMO conjugation, without the absolute need for an E3 SUMO ligase. Nevertheless, numerous E3 SUMO ligases have been identified and are considered to play an important regulatory role for the SUMOylation of specific targets *in vivo* (reviewed by Dohmen [135], Geiss-Friedlander and Melchior [134], and Wilson and Heaton [139]). The SUMO peptidases and isopeptidases make SUMOylation a reversible modification, and their high intracellular concentration and activity is thought to be responsible for the low cellular concentration of the SUMOylated form for any given protein

(for most SUMO targets, the SUMOylated form of the target represents less than 5% of the steady-state cellular level of that protein) [140].

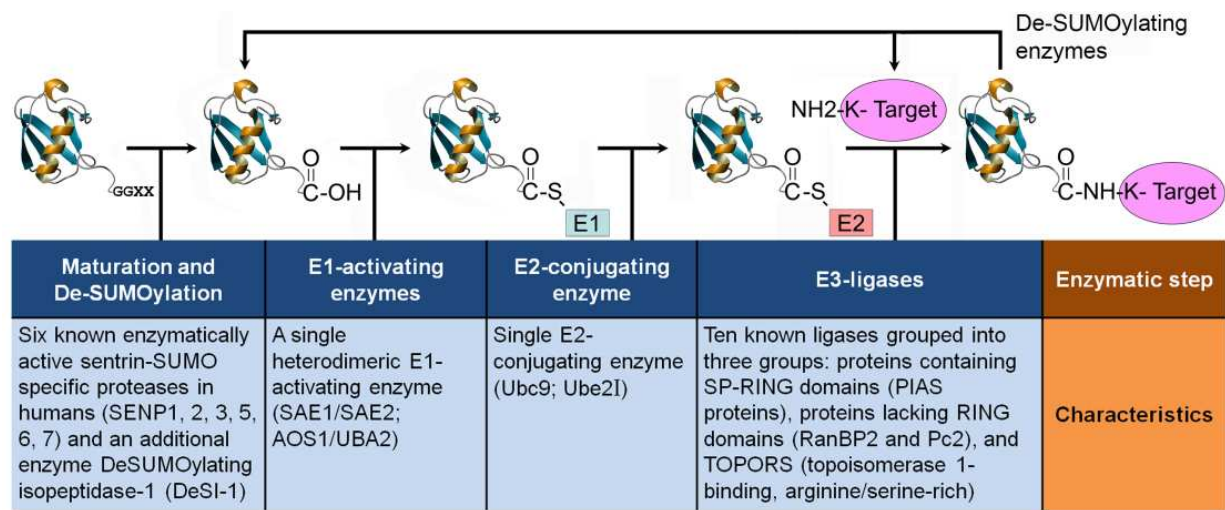


Figure 3.

4.2. A "molecular matchmaker": The effects of SUMOylation on its target proteins

SUMOylation is known to affect an ever increasing number of cellular proteins, some of which were initially identified as SUMO targets during large-scale proteomic analyses of cellular SUMOylation [136-137, 141-142]. The effects produced by this post-translational modification on its target proteins are numerous and appear to be protein specific. Among others, SUMO has been shown to alter its target proteins by affecting their cellular localization, protein stability/half-life, and protein activity (reviewed by Hay [140], Hilgart et al. [143], Gill [144], and Wilkinson and Henley [145]). The unifying theme behind the wide range of activities mediated by SUMOylation appears to be SUMO's ability to regulate the intermolecular interactions established between its targets and other macromolecules, sometimes enhancing them and sometimes blocking them. In either case, it seems that whatever the effect mediated by SUMO might be, its effects last longer than the actual SUMOylated state of the target. In other words, the protein interactions facilitated or prevented by SUMOylation are maintained even after the protein has been de-SUMOylated. A simple explanation for this fact is that SUMO may act as a "molecular matchmaker", introducing proteins to each other and allowing them to establish long-lasting interactions, probably stabilized by the recruitment of other protein partners that can only interact with the interacting pair but not with the individual members of the pair. Those long-lasting interactions remain even after a de-SUMOylating enzyme takes away the SUMO molecule that allowed the initial interaction. As a consequence, mutations affecting the ability of a protein to become SUMOylated will have dramatic effects on protein function, despite the fact that the SUMOylated form of the protein may represent only a small fraction of its total in the cell.

4.3. A post-translational modification known to wrestle with many infectious organisms: The interplay between the cellular SUMOylation system and various infectious agents

While numerous infectious organisms are now known to interact with the cellular SUMOylation system by having some of their proteins being modified by SUMOylation, there are a few specific examples of infectious organisms that produce global changes in the activity of the cellular SUMOylation system. Interestingly, for such organisms capable of affecting the overall activity of the SUMOylation system, the predominant picture observed is their tendency to decrease both, the activity of the SUMOylation system and the overall number of SUMOylated proteins present within the host [146]. This trend suggests that the SUMOylation system may have an intrinsic effect as a cellular defense mechanism against some infectious agents. Two well characterized proteins encoded by two different infectious organisms, a virus and a bacterium, exemplify and support the predominant picture indicated above and, therefore, will be briefly discussed below.

i) Gam1: Gam1 is a protein encoded by the so-called chicken embryo lethal orphan (CELO) avian adenovirus. This protein was initially characterized as an anti-apoptotic viral protein, as well as an inhibitor of the deacetylating enzyme HDAC1 [147]. Subsequently, it was observed that Gam1 was also capable of inducing both, the loss of Pro-Myelocytic Leukemia Nuclear Bodies (PML-NBs), a nuclear structure believed to play antiviral activities and whose formation depends on the SUMOylation of the PML protein, and a global decrease in host cellular SUMOylation [148]. Follow up studies demonstrated that Gam1 inactivates the heterodimeric SUMO activating enzyme SAE2/SAE1, therefore triggering its degradation by a proteasomal-dependent pathway. The degradation of the E1 activating enzyme for the SUMO pathway also leads to the degradation of the E2 conjugating enzyme, Ubc9, therefore producing a complete block in SUMOylation [149]. Importantly, Gam1 has been demonstrated to be essential for CELO viral growth and multiplication, therefore suggesting that the SUMO inhibitory activity mediated by Gam1 is essential for viral fitness [150].

ii) Listeriolysin O (LLO): LLO is a pore forming toxin produced by the intracellular bacterial pathogen *Listeria monocytogenes*, the causative agent of human listeriosis. In a study aimed at evaluating the potential ability of *L. monocytogenes* to alter global cellular SUMOylation, it was observed that HeLa cells infected with this bacterium exhibited a dramatic decrease in global cellular SUMOylation. This was not true for HeLa cells infected with its non-pathogenic cousin *L. innocua*. Subsequent analysis of various bacterial mutants identified LLO as the factor responsible for the de-SUMOylating activity associated to *L. monocytogenes*. Specifically, LLO was shown to trigger the degradation of the E2 conjugating enzyme Ubc9, therefore leading to a global decrease in cellular SUMOylation. The relevance of this decrease for bacterial infection was demonstrated by showing that over-expression of SUMO1 or SUMO2 in HeLa cells before *L. monocytogenes* infection led to a substantial decrease in the number of intracellular bacteria produced by 7 h post-infection, thus supporting a role for decreased cellular SUMOylation in enhancing bacterial growth [151].

In contrast with the examples presented above, there are also reports of proteins encoded by viral pathogens that are endowed with the ability to enhance the SUMOylation of specific

cellular proteins as a way to regulate cellular systems that play an important role during viral infection. Two important examples of this type of interaction between viral agents and the SUMOylation system are presented below.

iii) K-bZIP: The basic leucine zipper protein of Kaposi's sarcoma-associated herpesvirus (KSHV), K-bZIP, is one of the earliest proteins expressed after acute infection or reactivation by KSHV. K-bZIP, a transcriptional regulator of viral gene expression, was initially shown to interact with the SUMOylation system by acting as a SUMO target, being SUMOylated at residue K158. K-bZIP SUMOylation appeared to affect its transcriptional activity [152]. Subsequently, it was shown that K-bZIP contains a SUMO2/3-specific SUMO Interacting Motif (SIM) that allows K-bZIP to enhance its own SUMOylation and that of other cellular proteins known to interact with it, including the tumor suppressor proteins p53 and pRB [153]. In consequence, it has been postulated that K-bZIP acts as a viral encoded SUMO ligase that helps ensure the maintenance of the proper cellular environment needed for viral multiplication by triggering the SUMOylation and subsequent activation of p53, which in turn triggers cell cycle arrest in G1 [153].

iv) VP35: The VP35 protein encoded by Ebola Zaire virus (EBOV) is one of two EBOV viral proteins known to be involved in suppressing the type I interferon (IFN) response in the cell. The mechanism responsible for IFN inhibition was postulated to involve VP35's ability to bind to dsRNA therefore preventing retinoic acid induced gene I (RIG-I) activation [154]. However, a mutant form of VP35 incapable of binding dsRNA was found to retain its IFN blocking activity, suggesting the existence of an alternative mechanism for IFN inhibition by VP35. Type I IFN production is ultimately triggered by the transactivational activity of NF- κ B and two additional transcription factors: Interferon regulatory factors 3 and 7 (IRF3 and IRF7, respectively). VP35 was demonstrated to interact with the SUMO ligase PIAS1, the E2 SUMO conjugating enzyme Ubc9, and IRF7. Furthermore, VP35's ability to interact simultaneously with all of these factors was shown to enhance the SUMOylation of IRF7 and IRF3. In turn, the enhanced SUMOylation of IRF7 and IRF3 was demonstrated to diminish their ability to transactivate type I IFN production, therefore leading to a substantial decrease in type I IFN production during EBOV infection [155]. In consequence, VP35 represents a viral protein that enhances the activity of a SUMO ligase as a way to neutralize the transcriptional activity of specific cellular factors, therefore leading to decreased type I IFN production and diminished cellular antiviral responses during infection, an outcome that favors viral multiplication.

Altogether, the four proteins presented above, encoded by four different pathogens, exemplify some of the most important interactions established between infectious organisms and the cellular SUMOylation system and provide a framework to understand the potential roles played by SUMO during influenza virus infection.

4.4. The beginning of the wrestling match: Initial insights into the relevance of the cellular SUMOylation system for influenza virus infections

Our laboratory was arguably the first in studying the potential interplay between the cellular SUMOylation system and influenza virus. Our initial studies on this topic, dating

back to 2005, were motivated by the well established relevance of SUMO for the biological activities of numerous proteins encoded by DNA viruses [156]. The unifying theme at the time was that SUMO appeared to regulate numerous viral proteins targeted to the nucleus of the cell. Because influenza replication occurs in the nucleus of the infected cell, it seemed plausible that some influenza viral proteins would turn out to be SUMO targets. Our initial test of this hypothesis involved *in vitro* SUMOylation reactions in which ³⁵S-methionine labeled viral proteins produced in a coupled transcription/translation system were incubated with a fully purified reconstituted SUMOylation system. The data produced by these experiments were surprising and unexpected as they showed that most of the 9 viral proteins tested were SUMOylatable *in vitro*. The only viral proteins that were not tested were PB1-F2 and M2, and the only two proteins that did not appear to be SUMOylated were the two membrane viral proteins tested, HA and NA [157].

Although our personal experience has indicated direct correlation between *in vitro* SUMOylation and *in vivo* SUMOylation, it was necessary to demonstrate that the viral proteins identified as SUMO targets *in vitro* could also become SUMOylated when expressed in mammalian cells. This proved more challenging than expected, partially due to the limited pool of free SUMO normally present in the cell, and required the development of a set of dicistronic expression constructs capable of simultaneously inducing large increases in the cellular concentrations of SUMO accompanied by slight increases in the cellular pool of its conjugating enzyme Ubc9. The development and implementation of such dicistronic expression constructs allowed us to demonstrate that the non-structural influenza protein NS1 is a *bona-fide* SUMO target *in vivo*, being SUMOylated both, when over-expressed by transfection, as well as when expressed at normal physiological levels during viral infection. On the day this finding was published online, 14 November 2009, it constituted the first published report supporting the potential relevance of SUMOylation for influenza virus [158], and the dicistronic constructs described therein have subsequently been proven invaluable as key tools in numerous studies related to the SUMOylation of other cellular and viral proteins [157, 159-160].

Just one month after the publication of our report, further evidence supporting an important role for the cellular SUMOylation system during influenza virus infection was provided by two papers reporting the outcome of large scale screenings aimed at identifying cellular proteins required for efficient influenza virus infection. In the first report, published online on 21 December 2009, König et al. reported the identification of SUMO2, SUMO1, and SAE1 among the cellular genes whose down-regulation by RNAi in a human lung epithelial cell line (A549) led to a substantial decrease in viral transcription/translation, as assessed using a Renilla luciferase reporter system placed in substitution for the HA protein in a recombinant A/WSN/1933 H1N1 viral strain [92]. In the second report, published online on 24 December 2009, Shapira et al. identified Ube2I (i.e. Ubc9, the SUMO conjugating enzyme) as one of the cellular proteins capable of establishing direct physical interactions with the viral proteins PB1 and NS1 derived from the A/Puerto Rico/8/1934 H1N1 viral strain, and the NS1 protein derived from the A/Udorn/307/1972 H3N2 viral strain. In that report, the significance of those interactions, initially detected during the implementation of a large yeast two hybrid screening for cellular proteins capable of interacting with viral proteins, was further

validated by using an RNAi approach [94]. Altogether, the two large scale screenings for cellular proteins required for efficient influenza virus infection confirmed the relevance of the SUMOylation system for influenza suggested by our initial studies.

4.5. Wrestling with the interferon response of the host: Relevance of the cellular SUMOylation system for the biological functions of the non-structural viral protein NS1

Almost exactly one year after the initial publication of our report on the SUMOylation of NS1, a collaborative effort involving personnel distributed across ten institutions in two different countries, China and Germany, led to the publication of a report, authored by Xu et al., on the SUMOylation of the NS1 protein of the highly pathogenic avian influenza A/Duck/Hubei/L-1/2004 H5N1 viral strain (published online on 3 November 2010) [161]. According to that report, the authors initially identified Ubc9 as a host-cell protein capable of interacting with a truncated form of NS1 (ending at position 162) in a yeast two hybrid screening executed with the goal of identifying novel host cell protein interactors for NS1. The authors then demonstrated that the H5N1 NS1 is SUMOylated *in vivo* when over-expressed by transfection, as were the NS1 proteins encoded by most viral strains tested, with the sole exception of the NS1 derived from the A/Sichuan/1/2009 H1N1 2009 pandemic strain. The authors also demonstrated that over-expression of the SUMO-deconjugating enzyme SENP1 abolished NS1 SUMOylation, and provided data indicative of NS1 SUMOylation during infection. Subsequent analyses mapped the SUMOylation site in the H5N1 NS1 at residue K221, but indicated that residue K219 could provide an alternative SUMOylation site when K221 was mutated [161].

One of the most important functions associated to NS1 during infection is its ability to neutralize the cellular type-I interferon (IFN) response. In the report by Xu et al., the authors compared the type I IFN-inhibiting activity of the wild-type (wt) NS1 with that of the non-SUMOylatable mutant and found a slight decrease in the non-SUMOylatable mutant's ability to neutralize the IFN response. Subsequent cycloheximide analyses indicated that the non-SUMOylatable NS1 appeared to have a substantial reduction in its stability, therefore accumulating to significantly lower levels in the cell. This property was then considered to be responsible for the non-SUMOylatable mutant's decreased ability to neutralize the type I IFN response. Finally, to study the potential effects of SUMOylation on viral growth, the authors developed a recombinant virus carrying a non-SUMOylatable NS1 in which K219 and K221 were changed to glutamic acid to prevent introducing mutations in NS2, which is encoded by a spliced transcript of the NS gene segment. The resulting mutant virus grew to almost identical titers as the wt virus, but exhibited a 10 fold decrease in viral production at 8 hours post-infection [161].

Recent data obtained by our group further supported the relevance of SUMOylation for NS1, although slight differences with the data presented in the paper by Xu et al. were observed. Such differences provide alternative mechanistic scenarios to explain the molecular effects of SUMOylation on NS1 function. First, our mapping analyses yielded

somewhat different data: According to our findings (Santos et al., manuscript in revision), the primary SUMOylation site in NS1 is not residue K221 but residue K219, which shows partial conservation among different viral strains. Our analyses have also mapped a secondary SUMOylation site in NS1, located in residue K70, a residue that is almost perfectly conserved among all influenza A viral strains. Thus, to abrogate NS1 SUMOylation, it is necessary to simultaneously mutate both, K219 and K70. To this end we developed a double mutant form of NS1, hereafter referred to as NS1K70AK219A, in which both lysines were substituted by alanine. Second, protein stability analyses using cycloheximide also produced slightly different data: Despite numerous repeats, our experiments did not show differences between the stability of the non-SUMOylatable NS1K70AK219A mutant and that of wt NS1 (Santos et al., manuscript in revision). Therefore, our data indicates that SUMOylation does not appear to regulate NS1's stability. Finally, viral replication assays demonstrated that NS1's ability to become SUMOylated exerts more dramatic effects on viral multiplication (as evidenced by the viral titers produced during infection) than those observed by Xu. et al. During the execution of these experiments, we decided to prevent introducing mutations affecting NS2 in the virus carrying the NS1K70AK219A mutant form of NS1 by mutating the splicing acceptor site located in NS1 and moving it, together with a full copy of the second exon for NS2, downstream from the stop codon for NS1. This strategy had been previously developed and successfully implemented by Varble et al. and offers the advantage of allowing the independent manipulation of NS1 and NS2 while still allowing NS2 to be produced as a splicing product of the primary transcript transcribed off the NS gene segment [162]. The NS gene segment generated in this way, hereafter referred to as NS1K70AK219A~NS2wt, produces a non-SUMOylatable NS1 and a wt NS2. To allow proper comparison of growth characteristics in the absence of other potential effects due to the alterations introduced in the NS gene segment, we also developed an equivalent NS gene segment coding for wt (SUMOylatable) NS1, hereafter referred to as NS1wt~NS2wt. The resulting recombinant viruses generated with those NS gene segments, produced by reverse genetics, exhibited striking differences in growth: The virus carrying the NS1wt~NS2wt gene segment produced viral titers that were more than two orders of magnitude (i.e. 100 fold) higher than the virus carrying the NS1K70AK219A~NS2wt gene segment, therefore supporting a very important biological role for the SUMOylation of NS1.

To better understand the effects mediated by SUMOylation on NS1, in addition to producing the non-SUMOylatable form of NS1, we also developed an artificial SUMO ligase (ASL) specific for NS1 (hereafter referred to as NS1-ASL). This innovative tool allows NS1 SUMOylation to be dramatically increased in the absence of any other noticeable change in the SUMOylation of any other protein within the cell (Pal et al., manuscript in preparation). Using this tool we have recently demonstrated that a ~4 fold increase in the fraction of SUMOylated NS1 produces a 25% decrease in NS1's ability to block type I IFN production, whereas blocking NS1 SUMOylation (by introducing the K70A and K219A mutations) produces a 60% decrease in NS1's ability to inhibit type I IFN production. This intriguing observation suggests that there is an intrinsic optimal balance in NS1's SUMOylation levels, and that whenever such balance is disrupted, whether by increasing or decreasing the

proportion of SUMOylated NS1 present in the cell, NS1's IFN blocking activity is diminished. A likely explanation for this model relates to SUMO's ability to modulate the protein interactions established by its targets. NS1 is known to interact with numerous viral and cellular proteins, potentially forming a large number of different protein complexes, each associated to specific roles mediated by NS1. More than one of such complexes may affect NS1's ability to neutralize type I IFN production. For instance, a homo-multimeric NS1 complex might be ideal for coating the viral RNA, therefore preventing RIG-I activation, whereas a complex of NS1 and CPSF might be needed to down-regulate the production of mature cellular mRNAs, including those coding for type I IFNs. Then, it is possible that the levels of SUMOylated NS1 dictate the types of complexes formed by NS1 and their proportion, so that altering the levels of SUMOylated NS1 will affect NS1's function by altering the proportion and nature of the different complexes formed by NS1. Native gels performed with cell extracts derived from cells expressing either NS1wt or NS1K70AK219A, with and without co-expression of the NS1-ASL, have provided experimental support by this hypothetical model by demonstrating SUMOylation-dependent changes in the protein complexes formed by NS1 (Pal et al., manuscript in preparation).

Altogether, our data indicates that the molecular effects mediated by SUMOylation on NS1 are likely to be complex and multifactorial, and that a molecular characterization of the complexes formed by SUMOylated and non-SUMOylated NS1 will be needed to truly understand how SUMOylation affects NS1 function. Furthermore, our data also indicates that it will be necessary to explore in detail the effects of SUMOylation upon NS1 proteins derived from numerous viral strains, as it is likely that the differences observed between the data reported by Xu et al. and our own analyses may reflect strain-specific effects mediated by SUMOylation on NS1 function. Such differences may in turn reflect differences in the distribution of SUMOylation sites in NS1.

4.6. A SUMO-dependent matrix: Relevance of the cellular SUMOylation system for the biological functions of the viral matrix protein M1

An important addition to the history of the interactions established between the SUMOylation system and influenza virus was published online on 20 April 2011 by We et al. [163]. In their manuscript, Wu et al. reported a critical role for SUMO in viral assembly, mediated by the SUMOylation of the M1 protein. The study was initiated by experiments aimed at evaluating whether knocking down the cellular expression of Ubc9 by an RNAi approach affected influenza virus multiplication in Huh7 cells (a hepatoma cell line). Interestingly, cells exhibiting an almost complete knock-down of Ubc9 (achieved by transducing the cells with a lentivirus carrying an shRNA against Ubc9 followed by puromycin selection of the transduced cells) exhibited a decrease of two orders of magnitude in viral production when compared with cells transduced with a lentivirus lacking an Ubc9-specific shRNA. Subsequent analyses demonstrated that the changes in viral production were not mirrored by similar changes in viral protein synthesis. More surprisingly, vRNA accumulation within the Ubc9 knocked-down cells appeared increased, therefore suggesting a defect in viral release and a potential role for SUMOylation during

viral maturation and assembly. The authors then focused their attention on M1, an important player in viral assembly, and found it to be SUMOylated at position K242 [163].

To determine the role of M1 SUMOylation during viral infection, the authors developed a recombinant A/WSN/1933 H1N1 virus carrying a non-SUMOylatable form of M1 containing a lysine to glutamic acid substitution at position K242, hereafter referred to as M1K242E. The lysine to glutamic acid substitution was chosen to prevent introducing any mutations on M2, the other protein encoded by the M gene segment. The mutant virus produced final viral titers two orders of magnitude lower than those produced by the wt virus. Interestingly, the cellular distribution of the NP protein appeared to be substantially altered in cells infected with the mutant virus, displaying a mostly nuclear localization even late during infection, a time when most of the NP signal is usually localized in the cytoplasm and in close proximity to the plasma membrane. However, according to the authors, the cellular localization of M1 was not affected. This suggested that M1 SUMOylation could potentially affect the nuclear export of vRNPs by enhancing their interaction with M1. A subsequent series of immunoprecipitation analyses showed that, in the presence of the non-SUMOylatable form of M1, the interactions between M1 and two proteins normally associated with vRNPs, PB2 and NP, were substantially decreased. This provided empirical support to the role of M1 SUMOylation in enhancing the interaction between M1 and vRNPs. Further support was provided by transmission electron microscopy data showing that, in cells infected with the mutant virus, a high number of empty virus-like particles and viral particles with abnormal morphology were produced, defects normally associated with a weak M1-vRNP interaction [163].

Altogether, the report by Wu et al. presented a very compelling story demonstrating a role for M1 SUMOylation in enhancing the interaction between M1 and the vRNP. We have also observed the SUMOylation of M1 in our own studies, a fact that we reported in our paper published online on 3 March 2011, although we did not succeed in mapping its SUMOylation site. It will be interesting in the future to determine the specific protein interactions that are modulated by M1 SUMOylation, as M1 is known to establish multiple interactions, not only with other viral proteins but also with cellular proteins. A molecular characterization of such interactions may help define new druggable targets in cellular proteins.

4.7. The future of the wrestling match: The potential relevance of the SUMOylation system for the development of innovative antiviral therapies

In addition to the unquestionable role played by SUMOylation for the NS1 and M1 viral proteins, described in the sections above, data generated in our laboratory supports the idea that the interactions between the cellular SUMOylation system and influenza virus are even more complex than already indicated. This statement is supported by two main findings: First, in addition to NS1 and M1, our analyses have demonstrated that other viral proteins, namely PB1, NP, NEP [157], PB2, and PB1-F2 (Santos et al., manuscript in preparation) are also SUMOylated during infection. Second, analyses performed looking at the global profile of cellular SUMOylation at different points post-infection have demonstrated that influenza infection causes a global increase in cellular SUMOylation, characterized by the appearance of two new SUMOylated proteins of ~70 kD and 52 kD [157].

Out of the new viral proteins identified as SUMO targets, our recent studies have already indicated that SUMOylation plays an important regulatory role for the viral polymerase subunit PB1, and ongoing analyses suggest that the same might be true for NEP (unpublished observations). This implies that, besides helping regulate NS1's IFN-neutralizing activity and M1's role in viral morphogenesis (two well established effects of SUMOylation on influenza infections as discussed above), the cellular SUMOylation system may also regulate PB1's role in viral transcription and replication, and NEP's role in the nuclear export of vRNPs. Our studies have demonstrated the existence of an optimal proportion of SUMOylated and non-SUMOylated NS1 that maximizes its IFN-inhibitory activity. It is likely that a similar optimal proportion between the SUMOylated and non-SUMOylated forms may also exist for all the other viral proteins targeted by the cellular SUMOylation system. This possibility emphasizes one important feature of the SUMOylation system that makes it an especially attractive target for the development of new antiviral therapies: Alterations affecting the activity of the SUMOylation system, whether increasing it or decreasing it, will affect the proportions of SUMOylated and non-SUMOylated forms of not just one but several viral proteins. This will likely result in pronounced alterations in the proportions of the various protein complexes made by each viral protein during infection, which will in turn decrease viral multiplication by affecting multiple stages of influenza's life cycle. Therefore, the SUMOylation system constitutes one cellular target whose disruption would likely have multiple damaging effects on viral multiplication without having immediate deadly consequences for the cell. Proof of principle for this idea has already been provided by two studies that have demonstrated substantial decreases in viral multiplication when the SUMO conjugating enzyme is targeted by an RNAi approach [92, 94]. From this perspective, it is conceivable that the ongoing NIH-sponsored efforts by other laboratories to develop drug-like specific inhibitors of the cellular SUMOylation system may, in the long run, yield a new generation of anti-influenza drugs, some of which may prove useful against other viral diseases as well.

Viruses have developed numerous strategies to gain control over the cellular environment of the host as a way to maximize their own fitness. Many of the cellular systems whose activity is increased during viral infections are purposely increased to facilitate viral multiplication. However, other cellular systems are increased as a way to neutralize viral infection, as exemplified by the large number of cellular genes that are turned on by the IFN response. Our analyses have demonstrated that influenza A infections produce a dramatic increase in the activity of the cellular SUMOylation system [157]. This has been demonstrated for a large number of viral strains and cell lines, and therefore is likely to represent a general feature of influenza A infections. We have also proven that the global increase in cellular SUMOylation requires the presence of a transcriptionally active virus, as UV inactivated viruses are unable to trigger it [157]. Additional analyses have also indicated that IFN stimulation is neither sufficient nor required to trigger the increase, as direct addition of recombinant IFN- β to uninfected cells does not result in a global increase in cellular SUMOylation, and cells lacking the ability to produce IFN (such as Vero cells) also exhibit the increase upon viral infection [157]. Although these findings strongly suggest that

the global increase in cellular SUMOylation is triggered by a virus-dependent mechanism, it is uncertain whether the increase itself corresponds to a viral strategy to enhance viral growth, or a cellular attempt at neutralizing viral infection. It appears tempting to choose the first scenario, particularly because of the already demonstrated relevance of SUMOylation for a number of viral proteins [161, 163] and the demonstrated decrease in viral multiplication observed upon targeting Ubc9 by RNAi [92, 94]. However, at this point we consider that it is still possible that some of the SUMOylation events that are induced during infection may have antiviral effects. Our ongoing studies have already identified the minimal set of viral components required to trigger the global increase in cellular SUMOylation described above (Chacon, Santos, et al., manuscript in preparation), and recent data have revealed an unanticipated twist in the story. We are confident that further characterization of the molecular mechanisms involved in the global increase in cellular SUMOylation by influenza virus will lead to a new paradigm for the interactions established between viruses and the SUMO system.

5. Conclusions

IAV remains one of the most damaging viruses for humans. Our knowledge of the molecular mechanisms involved in viral transcription and replication has increased dramatically, and with it, we have also gained new insights into how the host cell is affected by the virus to enhance viral functions, and how cellular functions affect viral replication. However, numerous questions remain, particularly in areas related to the dynamic interplay that takes place between the numerous cellular systems affected by the virus and the viral proteins that trigger those effects. A better understanding of the interplay established between the virus and its host cell is likely to result in the development of new therapeutic agents for the treatment and prevention of complicated influenza infections, which is, in our opinion, one of the most urgent medical needs of our time due to the impending threat of new pandemics. Research started by our group about 7 years ago has begun to unveil some of the multiple roles that the cellular SUMOylation system plays during the life cycle of IAV. The variety and significance of those roles for viral fitness identifies the SUMO system as one of the most promising targets for the development of novel broad-spectrum, host-cell targeted, anti-influenza therapies, which could also be applicable to the treatment of other acute viral diseases.

Author details

Andrés Santos** and Jason Chacón **

Department of Biological Sciences, The University of Texas at El Paso (UTEP), USA

Germán Rosas-Acosta*

Border Biomedical Research Center (BBRC), The University of Texas at El Paso (UTEP), USA

Department of Biological Sciences, The University of Texas at El Paso (UTEP), USA

* Corresponding Author

** These authors contributed equally to this work

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