MOLECULAR BIOLOGY OF HOST - INFLUENZA VIRUS NUCLEOPROTEIN (NP) INTERACTIONS AND THEIR RELEVANCE IN THE PATHOGENESIS OF INFLUENZA VIRUS

By

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Abstract

Influenza viruses are the most successful and omnipresent respiratory pathogens capable of causing pandemics. The interplay between influenza virus and host factors to support the viral life cycle is well documented. Influenza A virus (IAV) proteins interact with an array of cellular proteins and hijack host pathways which are at the helm of cellular responses to facilitate virus invasion. The fifth segment of the 8 segmented negative strand RNA genome codes for the nucleoprotein (NP) that acts as an adaptor protein to mediate plethora of binding interactions. One of the functions of NP is to induce cellular apoptosis in the infected host cells. Host factor RING finger protein 43 (RNF43), a RING-type E3 ubiquitin ligase, is a novel interactor of NP and an essential partner to induce NP-driven p53-mediated apoptosis in IAV-infected cells. IAV leads to attenuation of RNF43 transcripts and hence its respective protein levels in the cellular milieu whereas in RNF43 depleted cells, viral replication escalates several folds. Moreover, RNF43 polyubiquitinates p53 which further leads to its destabilization resulting in a decrease in induction of the p53 apoptotic pathway, a hitherto unknown process targeted by NP for p53 stabilization and accumulation. Collectively, these results conclude that NP targets RNF43 to modulate p53 ubiquitination levels and hence causes p53 stabilization which is conducive to an enhanced apoptosis level in the host cells. Moreover, an imperative role of NP/RNF43 interaction in IAV mediated cell cycle arrest is seen where RNF43 enhances the expression of various S phase promoting genes. In conclusion, our study unravels a novel strategy adopted by IAV for utilizing the much conserved ubiquitin proteasomal pathway and also unravels a promising host target for antiviral intervention.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Student signature: **Date:** 26/02/2016

Main Supervisor signature: **Date:** 26/02/2016

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Abbreviations and Symbols

%	Percent
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
β-ME	Beta-mercaptoethanol
Aº	Angstrom
aa	Amino acid
Amp	Ampicillin
APS	Ammonium persulphate
ATCC	American type culture collection
ATP	Adenosine triphosphate
BD	Binding domain
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHX	Cycloheximide
CSK	Cytoskeleton
cRNA	Complementary RNA

C-terminal	Carboxy-terminal
dATP	Deoxyadenosine triphosphate
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
h (s)	Hour(s)
h.p.i.	hours post infection
HPAI	Highly pathogenic avian influenza
HRP	Horse radish peroxidase
IAV	Influenza A Virus
IFA	Immunofluorescence assay
Ig	Immunoglobulin

Ig G	Immunoglobulin G
IP	Immunoprecipitation
Kan	Kanamycin
Kb	Kilo base pairs
kDa	Kilo dalton
LiAc	Lithium acetate
LB	Luria-Bertani broth
LiCl2	Lithium chloride
LAIV	Live attenuated influenza vaccine
M	Molar
MAPK	Mitogen-activated protein kinase
M1	matrix protein
MCS	Multiple cloning site
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
NA	Neuraminidase
NP	Nucleocapsid/ Nucleoprotein
NP-40	Nonidet P-40
NS1	Nonstructural protein 1

Nt	Nucleotide
NT	Non-targeting siRNA
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PI3K	Phosphatidyl inositol 3' kinase
PBS	Phosphate buffer saline
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBST	Phosphate buffer saline with Tween 20
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
RNF43	RING Finger Protein 43
RNP	Ribonucleoprotein
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction

SDS	Sodium dodecylsulphate
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA
TEMED	N, N, N′,N′-Tetramethylethylenediamine
Tm	Melting temperature
T.P.I	Time post infection
Ub	Ubiquitin
UPP	Ubiquitin proteasomal pathway
vRNA	Viral RNA
WHO	World Health Organization
WT	Wild-type
Y2H	Yeast two hybrid
YPD	Yeast extract peptone dextrose
Zeo	Zeocin antibiotic

CHAPTER 1
Introduction and Objectives

From the disease of one the whole flock perishes. This quote by roman poet Decimus Iunius Iuveniles holds entirely true for viruses. Viruses are the most successful and omnipresent pathogens parasitizing a wide range of hosts, being notorious for their inherent capability to manipulate themselves and their host to the fullest. They represent the best evolutionary models; continuously evolving to become more "biologically powerful" and challenging the monotony of their hosts' defense.

Influenza A viruses (IAV) are the most common and most significant human respiratory pathogens, causing both seasonal, endemic infections and periodic, unpredictable pandemics. Every year, influenza epidemics cause a wave of distress and result in a traumatic loss of lives and economy. Influenza infection in humans is characterized by acute infections rather than latent or chronic ones. Annual influenza virus epidemics follow a seasonal pattern reaching a peak between January and April, in northern hemisphere, when humidity is relatively low thus prolonging the virus survival in aerosols. In the southern hemisphere, influenza A virus outbreaks arise from May to September. A rather persistent virus activity is detected in tropical and subtropical regions.

Seasonal influenza viruses that spread from person-to-person through sneezing, coughing, or touching contaminated surfaces, can cause mild to severe illness and even death, particularly in some high-risk individuals that include pregnant women, the very young and very old, immune-compromised people, and people with chronic underlying medical conditions. Typically, about 10% of the population contracts an influenza infection leading to an average of 20,000 deaths and 114,000 hospitalizations per year.

Influenza virus pandemics are the outrageous manifestation of the virus evolution resulting in the simultaneous infection of 20-40% of the world's population. Highly pathogenic strains of influenza A virus have emerged occasionally in recent history, producing pandemics such as the one in 1918, which resulted in the death of 20–40 million people worldwide.

Influenza A virus is an enveloped negative-strand RNA virus with segmented genomes containing seven to eight gene segments. The 8 segments are encapsulated by the nucleocapsid/nucleoprotein (NP) and encode 13 known proteins.

The segmented RNA genome provides evolutionary advantages leading to the emergence of novel viral strains through intermingling mingling of segments of two different strains as was demonstrated in the 1918, 1957, 1968 and 2009 pandemics. The accumulation of mutations and reassortment events enable emerging viruses to evade host immunity acquired from previous IAV infections or vaccinations and to develop resistance against available antiviral agents that target M2 (amantadine and rimantadine) and NA (oseltamivir and zanamivir). Moreover, resistant strains emerge even faster under antiviral treatment. Thus, targeting the cellular factors involved in IAV replication represents a novel antiviral strategy that could counteract viral drug resistance.

Influenza A virus (IAV) proteins interact with an array of cellular proteins and hijack host pathways which are at the helm of cellular responses to facilitate virus invasion. Nucleoprotein (NP) of IAV is a viral RNA genome-encapsulating structural protein that has been implicated in various other indispensable activities for virus replication and pathogenesis-like intracellular trafficking of the viral genome, viral RNA replication, virus assembly via its interaction with a plethora of cellular factors like cytoskeleton

scaffolding protein α-actinin-4, nuclear import receptor α importin, nuclear export receptor CRM1, DEAD-box helicase BAT1/UAP56 and cytoskeletal element F actin.

In the current study, using the yeast two-hybrid system (Y2H), we identified human RNF43, an E3 ubiquitin ligase protein as a novel interacting partner of influenza A virus NP. Ubiquitin Proteasomal Pathway (UPP) is a multi-enzyme cascade that involves the sequential action of three different enzymes, E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. With 600 putative E3 ligases encoded by the mammalian genome, the receptor specificity is maintained by these proteins and hence, are heavily exploited by different viruses. IAV has also been shown to alter UPP for immune evasion and efficient pathogenesis. However, there is a dearth of studies elucidating the role of NP in mediating that phenomenon.

To this end the present study was conducted with the following objectives:

Objective 1

To validate the interaction between Influenza A virus Nucleoprotein and host factor, E3 ubiquitin ligase RING finger protein 43(RNF43).

Objective 2

To study the implications of NP/RNF43 interaction in NP-driven p53 mediated cellular apoptosis.

Objective 3

To inspect the effect of RNF43 on influenza virus mediated cell cycle arrest in host.

CHAPTER 2
Review of Literature

For all their ubiquity and success, viruses are astonishingly simple. Yet they are capable of efficiently subverting the complexity of their hosts to produce viral components, assemble them into new viral particles and transmit from one host to the other. These biological entities have had a profound role to play in the evolution of the more complex organisms with at least 10% of the human genome being made up of virus origin particles.

2.1 ORTHOMYXOVIRUSES

Influenza viruses belong to the *Orthomyxoviridae* family (Palese and Shaw, 2006). Influenza viruses A, B, and C constitute three of the five genera of the family and the other two genera of the family are Thogotovirus, which includes Thogoto virus and Dhori virus; and Isavirus, that includes infectious salmon anemia virus (ISAV) (Kawaoka et al., 2005). Orthomyxoviruses are enveloped viruses with a segmented single-stranded (ssRNA) genome that has been termed negative stranded because the vRNA transcribes the mRNA which is defined as positive (Baltimore, 1971). Members of any of the three genera of this family can reassort and exchange genetic information, however, this kind of inter-generic reassortment has not yet been reported.

2.2 INFLUENZA A VIRUS

Influenza A viruses are distinguished from type B and C influenza viruses on the basis of antigenic differences between their nucleoprotein (NP) and matrix (M1) protein (Lamb et al., 2001).Unlike influenza B virus, known to infect only humans, influenza A virus has a vast range of hosts, including birds and mammals. Influenza B is an important cause of seasonal influenza but not pandemics like influenza A virus. Influenza A and B viruses contain 8 segments of ssRNA whereas influenza C contains seven segments since it lacks a neuraminidase gene (Webster et al., 1992).

There exist significant differences in evolutionary rates for influenza A, B, and C viruses. Influenza B viruses, but especially influenza C viruses, evolve at a slower rate than influenza A viruses. Type B and C viruses seem to be near or at an evolutionary equilibrium in humans as opposed to Influenza A viruses (Webster et al., 1992).

2.2.1 Classification and nomenclature

Subtyping of influenza A virus is done through different antigenic determinants to HA and NA surface glycoproteins of the virus. Currently, 17 HA subtypes (H1–H17) and 9 NA subtypes (N1–N9) are known (Jagger et al., 2012; Fouchier et al., 2005). HA is divided into two lineages or groups, group 1 consisting of H1a, H1b, and H9 clades while H3 and H7 subtypes constitute group 2. With an error-prone RNA polymerase, a continuous accumulation of point mutations in these genes, governed by immune selection pressure, gives way to a directional antigenic change. This equips the virus with a stronger immune system escaping mechanism. This ever going "antigenic drift" is the reason behind ever evolving seasonal influenza strains of a single subtype (Mc Caughey, 2010; Rambaut et al., 2008).

The present nomenclature system includes the type of virus, the host of origin (except for humans), the geographic site of isolation, the strain number, and the year of isolation, followed by the antigenic description of the HA and NA subtypes in parenthesis. For example, A/swine/Iowa/15/30 (H1N1) describes an influenza A virus isolated from a pig in Iowa in 1930 with a strain number of 15 and an H1N1 subtype. No antigenic subtypes have been yet identified for influenza B and C viruses. Interestingly, Thogoto virus, Dhori virus, and ISAV do not cross-react antigenically (Wright et al., 2007).

2.2.2 Epidemiology and past pandemics

Constant antigenic variations govern the epidemiology of influenza viruses. Aquatic birds are the natural reservoirs of IAV in which, it seems, the virus has attained evolutionary statis through penultimate adaptation. Orders, Anseriformes (ducks, geese, swan) and Charadriiformes (gulls, terns, surfbirds, sandpipers) are their native hosts with the highest HA (17) and NA (9) subtypes being reported to circulate in them (Wright et al., 2007). However, transmission of the virus to land-based poultry or mammals has led to their rapid evolution (Ludwig et al., 1995). Furthermore, the division of influenza A viruses, following their pathogenicity to chickens, into low pathogenic (LPAI) and highly pathogenic (HPAI) proves its exacerbation. However, a limited number of influenza subtypes are known to have established themselves in humans (H1N1, H3N2), pigs (H1N1, H1N2), horses (H3N8 and H7N7), and dogs (H3N8) (Peiris et al., 2007).

Swine has emerged as one of the major players in pandemic outbreaks due to the presence of both avian and human influenza virus receptors, α2, 3- sialic acid and α2, 6- sialic acid, respectively (Sharma et al., 2011). This receptor affinity makes them an efficient "mixing vessels", giving birth to a new reassortant virus and a possible dawn of another pandemic. Apart from reassortment, a continuous replication of avian viruses in pigs may form variants recognizing human type receptors. This mode of interspecies transfer makes humans vulnerable to influenza pandemics (Ito et al., 1998).

Influenza infection in humans is characterized by acute infections rather than latent or chronic ones. Annual influenza virus epidemics follow a seasonal pattern reaching a peak between January and April, in the northern hemisphere, when humidity is relatively low prolonging the virus survival in aerosols. In the southern hemisphere, influenza A virus

outbreaks arise from May to September. A rather persistent virus activity is detected in the tropical and subtropical regions (Wright et al., 2007).

Pandemics are the most calamitous manifestations of influenza virus infecting 20-40% of the world's population simultaneously. For centuries, influenza virus pandemics have played havoc with nature and been responsible for mass killings across continents. Influenza pandemics date back to 1918, when the deadly "Spanish Flu", H1N1 killed ~25 million people in 25 weeks (Wright et al., 2007). It is supposed to be a unique adaptation of a purely avian influenza virus which transmits efficiently to humans. Its symptoms included high fever, severe headache, myalgia, pharyngytis, cough and coryza and deaths occurred primarily due to pneumonia and respiratory failure. This remains till date, the most miasmatic devastation caused by influenza virus (Ito et al., 1998; Palese, 2004).

In February 1957, "Asian influenza", H2N2, originated in the southern Chinese province of Guizhou, and from there it spread to Hunan province in China, Singapore, and Hong Kong. H2N2 was a reassortant of human and avian viruses, having H2 HA, N2 NA and PB1 genes of the avian virus. Though, H2N2 virus was mildly pathogenic but deaths occurred due to the lack of immunity to new HA and NA surface glycoprotein (Scholtissek et al., 1978; Kawaoka et al., 1989).

Hongkong influenza virus 1968, H3N2, another pandemic strain , contained H3 HA , PB1 from an avian source and shared approx 30% sequence homology with the Asian influenza pandemic strain, H2N2 (Wright et al., 2007; Kawaoka et al., 1989).

H1N1 strain has been circulating and evolving in the mammalian and swine populations since its first pandemic in 1918. Fifty-nine years later in 1977, the virus re-emerged from the H1N1 strain circulating in humans and caused a mild epidemic in Russia. This influenza

outbreak was restricted to the population below 25 years of age suggesting pre- existing immunity in the older people (Palese, 2004).

The 2009 H1N1 "swine flu" strain emerged from the H1N1 circulating in pigs. Though evolved and diversified in pigs, it maintained a significant antigenic proximity with the original 1918 virus, hence, the latter is considered as its ancestor (Kasowski et al., 2011). 2009 H1N1, the first and till now the only pandemic of the 21st century, was a reassortant between Eurasian swine and North American triple reassortant swine H1N2 virus (Brockwell-Staats et al., 2009). Compared to its ancestor, it came across as a relatively less virulent strain but still managed to establish its pathogenesis throughout the world with estimated respiratory deaths around 201,200 including 80% of people > 65 years of age (Dawood et al., 2012).

2.2.3 Virion structure

The influenza A viruses are small pleomorphic particles that later become generally spherical. Both spherical and filamentous virions are produced by influenza infection, having a diameter of approx. 100nm and reaching up to as long as 20μm in length, respectively. This pleomorphism in morphology is considered as an adaptation of the virus to grow inside an egg (Scheiffele et al., 1999). The envelope of each virion consists of a lipid bilayer derived from the plasma membrane of the host cell with HA and NA protein on the surface, constituting 80% and 17% of the total protein content of the virus, respectively (Fig. 2.1). Host selection is determined by HA through its binding to receptors containing terminal α-2,6-linked or α-2,3-linked sialic acid (α-2,6-SA or α-2,3-SA) moieties, in birds and humans, respectively. NA is crucial for virions budding and release as it removes the SA receptors from the cell surface (Mc Caughey, 2010; Scheiffele et al., 1999; Das et al., 2010).

The third integral membrane protein of IAV is the M2 ion channel protein present only in a few copies. Underlying the membrane is the M1 matrix protein, the major structural component of the virion, thought to act as an adaptor between the lipid envelope and the internal RNP particles, and the probable driving force behind virus budding (Golmez-Puertas et al., 2000). Inside the M1 shell lies the RNPs comprising the genomic RNA segments associated with a trimeric RNA polymerase (PB1, PB2 and PA subunits) and nucleocapsid protein NP.

Overall the composition of virus particles is about 1% RNA, 5% to 8% carbohydrate, 20% lipid, and approximately 70% protein (Ada and Perry, 1954; Compans et al., 1974; Frommhagen et al., 1959).

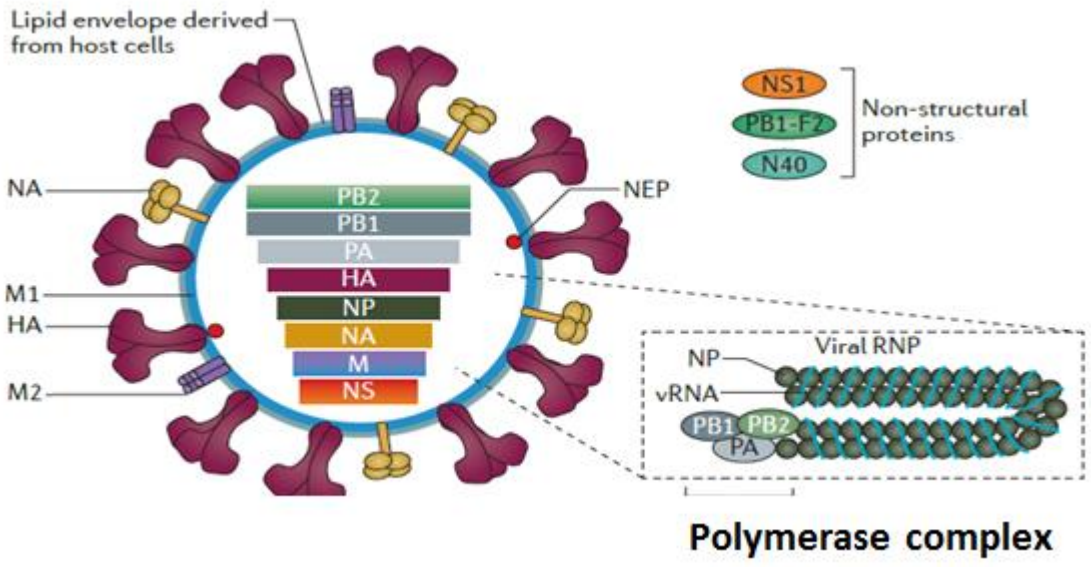

Fig. 2.1. The structure of influenza virus. (Adapted from Medina and García-Sastre, 2011)

2.2.4 Genome organization

The eight negatively stranded RNAs of influenza A virus, encode 10 major proteins responsible for virus replication, release and pathogenesis: Hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural (NS) proteins NS1 and NS2, the polymerases, the PB1 (polymerase basic 1), PB2, and PA (polymerase acidic) proteins, the nucleocapsid (Webster et al., 1992). Several other proteins have been discovered to be transcribed by different viral genome segments. PA-N155, PA- N182 and PA-X are expressed from IAV PA gene (Rodriguez et al., 2015). Influenza virus proteins, N40 and pro-apoptotic PB1-F2 protein transcribed from alternate reading frames of PB1 (Chen et al., 2001; Rodriguez et al., 2015).

2.2.4.1 The polymerase proteins

The three largest RNA segments code for three polymerase complex proteins PB1, PB2, and PA. PB1 containing the enzymatic motifs required for RNA polymerization activity is the most conserved of the polymerase subunit proteins and is the core of the polymerase complex. The PB2 subunit recognizes and binds with host 5' mRNA cap structures which act as the templates for viral mRNA transcription. Thus, PB2 has a key role in viral transcription (Blaas et al., 1982; Braam et al., 1983). A recently discovered pro-apoptotic PB1-F2 protein is transcribed from a second reading frame of PB1 gene. The PB1-F2 protein elicits both humoral and cell-mediated immune responses in the human hosts. The pro-apoptotic activity of PB1-F2 is mediated through the induction of alterations in mitochondrial membrane potential (Conenello and Palese, 2007).

Acidic protein PA has an endonucleolytic activity needed for the viral cap-snatching process (Sanz-Ezquerro et al., 1995). The crystal structures of the complete RNA polymerase complexes from influenza A and B viruses show that the three subunits have multiple interactions with each other and all of them participate in the binding of the two strands of the RNA promoter (Pflug et al., 2014; Reich et al., 2014; Krug, 2014). It is also well reported that the polymerase complex plays a decisive role in the efficient host switching wherein mutations increasing polymerase activity are a pre-requisite for host adaptation (Cauldwell et al., 2014).

2.2.4.2 Nucleoprotein (NP)

The nucleoprotein (NP) is the major structural protein encoded by the segment 5 of the viral genome. It is also one of the proteins essential for viral transcription and replication for molecules of NP encapsidate the vRNAs and cRNAs via its interaction with the acidic phosphate residues of RNA (Beaton et al., 1986; Biswas et al., 1998).

2.2.4.3 Hemagglutinin (HA)

Originally named because of the ability of the virus to agglutinate erythrocytes by attaching to specific sialic acid receptors. HA is encoded by RNA segment 4 and plays a decisive role in host adaptation which in turn is governed by receptor specificity of HA. Human viruses recognize SA linked to the galactose by α2, 6 linkages whereas avian viruses preferentially recognize α2, 3 SA receptors. Synthesized as a precursor protein HA0, it is cleaved post-translationally into two disulfide-linked subunits HA1 (MW~47,000) and HA2 (29,000). HA2 plays a prominent role in HA-mediated fusion of the endocytosed virus particle and the

endosome membrane, and ultimate release of viral nucleocapsids in the cytoplasm (Skehel et al., 2000; Harrison, 2008).

2.2.4.4 Neuraminidase (NA)

Encoded by the RNA segment 6 Neuraminidase is another subtype specific glycoprotein of influenza virus. A homotetramer containing 4 domains: a globular head, a stalk, a transmembrane region and a cytoplasmic domain (Verghese and Colman, 1991), NA catalyzes the cleavage of the α-ketosidic linkage between a terminal sialic acid and an adjacent D-galactose or D-galactosamine sugar moieties. NA facilitates the mobility and release of virions from cells through the removal of SA residues from cellular receptors. NA enzyme activity exists in the head domain, and mutations in the stalk domain have been associated with an altered ability to release virions from the cell surface (Palese et al., 2001; Palese and Compans, 1976).

2.2.4.5 Matrix proteins M1 and M2

Segment 7 generates two gene products: the matrix proteins M1 and M2. M1 protein that is a collinear transcription product is a structural component forming a layer between the viral membrane and the vRNPs. The NLS present at the N-terminus of M1 is considered to be important for nuclear export of vRNPs and membrane binding (Arzt et al., 2004). M2 encoded by the spliced mRNA of segment 7 is present under the viral lipid membrane in a very low abundance (16 to 20 molecules/virion) (Lamb et al., 1985). It plays an important role in the release of the viral ribonucleoprotein complex (vRNPs) through its ion channel activity, inside the cytoplasm after virus binding through HA to the sialic acid receptors on the host cell (Pinto et al., 1992; Wang et al., 1994).

2.2.4.6 Nonstructural proteins 1 and 2

RNA segment 8 of influenza virus is 890 nucleotides long and codes for two proteins: Nonstructural protein 1 (NS1) and Nonstructural protein 2 (NS2), the latter being the product of mRNA splicing. Originally thought to be nonstructural proteins, NS2 has now been shown to be the part of virions and also forms an association with the M1 protein (Yasuda et al., 1993; Ward et al., 1995).

NS1, on the other hand is one of the very well characterized proteins of the influenza virus. Influenza A NS1 is a 230 aa protein with two structural domains namely, RNA binding domain and an effector domain, which are joined by a flexible linker region. NS1 acts as an interferon antagonist in the infected host cells and also a regulator of both mRNA splicing and translation (Garcia-sastre et al., 1998; Marion et al., 1997; DeLa Luna et al., 1995; Enami et al., 1994).

2.2.5 Influenza A virus life cycle

2.2.5.1 Virus adsorption, entry, and uncoating

The life cycle of influenza virus is initiated with the binding of the virus to sialic acid receptors on the host cell through the distal tip of the HA molecules (Fig. 2.2). Different influenza viruses have different specificities for sialic acid linkages (α2, 3 or α2, 6) which in turn depends on the specific residues in the HA-receptor binding pocket (Coucerio et al., 1993; Matrosovich et al., 2004). The low affinity of the HA and sialic acid interaction is compensated by high avidity through multiple low affinity interactions (Weis et al., 1988).

Binding is followed by clathrin-dependent endocytosis of the virus which is a well-described model for influenza virus (Matlin et al., 1981) However, clathrin-independent internalization

mechanisms have also been identified for influenza virus (Conner and Schmid, 2003; Lakadamyali et al., 2004). Low pH of the endocytotic vesicle (around 5 to 6) facilitates the uncoating of the influenza virions through the following mechanisms:

1. Acidic endosomal pH causes structural changes in the HA molecule which is initiated with the cleavage of the precursor peptide HA0 into two subunits, HA1, and HA2. The conformational change exposes the fusion peptide at the N-terminus of the HA2 subunit, enabling it to interact with the membrane of the endosome. This opens up a pore that releases viral RNPs into the cytoplasm (Stegmann, 2000).
2. Low endosomal pH also activates M2 ion channel activity permitting the flow of the ions from the endosome to the inside of the virus particle. This ion flow inside the virus disrupts protein-protein interactions and ultimately releasing RNPs free of the M1 protein, which marks the completion of uncoating process (Matlin et al., 1981; Zhirnov and Grogoreiv, 1994).

2.2.5.2 Nuclear import of ribonucleoproteins

Transcription of the RNPs into mRNA occurs in the nucleus of the host cell. The eight influenza virus genome segments exist as naked RNA but are associated with four viral proteins to form viral ribonucleoprotein complexes (vRNPs) (Hsu et al., 1987). The viral RNPs are trafficked to the nucleus of the host cell for replication and transcription of the viral genome. Being large and bulky for passive diffusion, trafficking of the vRNPs rely on the nuclear localization signals (NLS) present on each subunit of the RNP complex (Jones et al., 1986; Mukaigawa and Nayak, 1991; Nath and Nayak, 1990; Nieto et al., 1994; Smith et al., 1987). The nuclear import is heavily dependent on the host and viral protein interactions and is reported to be initiated on recognition of an NLS-containing NP by

members of the karyopherin α (also called importin α) family (O'Neill et al., 1995; Wang et al., 1997). Also, another host factor RanBP5 is reported to interact with the PB1 subunit and facilitate the transport of vRNP (Deng *et al*, 2006).

2.2.5.3 Transcription and replication of viral genome

The incoming negative sense vRNA is transcribed as well as replicated within the nucleus (Herz *et al*, 1985; Jackson *et al*, 1982). The negative sense RNA is transcribed into the capped, polyadenylated messenger RNA via primer dependent mechanism. The transcription process requires the activity of cellular RNA polymerase II and begins with the 5' cap snatching from the host pre-mRNA transcripts. This process of cap-snatching is mediated by the cap-binding function of the PB2 protein and endonuclease function of the PB1 protein of the RNP complex (Krug, 1981). The PB2 polymerase subunit binds to the 5' cap of the host pre-mRNAs. The endonuclease activity of the PB1 protein leads to the cleavage of bound pre-mRNAs at approximately 10 to 13 nucleotides from the 5′ caps, usually after a purine residue (Beaton and Krug, 1981; Plotch et al., 1981). This is followed by RNA chain elongation catalyzed by the polymerase activity of PB1 protein by the addition of 'G' residue at the 3' end until it encounters a track of 5-7 uridine residues approximately 16 nucleotides before the 5′ end of the vRNA (Li and Palese, 1994; Luo et al., 1991; Robertson et al., 1981). Polyadenylation of the transcribed viral mRNA is dependent on the stretch of five to seven uracil residues in the vRNA and is catalyzed by the same polymerase to produce a poly A tail.

In contrast, the cRNA molecules are full-length copies of vRNAs and serve as templates for the synthesis of negative sense genomic vRNA. Although, the mechanism of synthesis remains primer independent but via terminal initiation instead of internal initiation and

realignment as in the case of cRNA synthesis. The switch between the two processes, i.e, transcription and replication is not entirely understood but is partially explained by the structural difference in transcription-competent polymerase and the replication-competent polymerase (Gonzalez and Ortin, 1999). Also, the cap-binding and endonuclease functions of PB2 and PB1 are not required when the polymerase is in replication mode. Unlike mRNAs, cRNAs and vRNAs are encapsidated.

2.2.5.4 Protein expression

Viral gene expression is temporally regulated which is primarily driven by the roles different proteins play at different points in the virus life cycle. Synthesis of NP and NS1 mRNAs and protein is favored at early stages of infection due to their roles in replication and in combating host interferon response respectively (Hatada et al., 1989).

In the late phase, NS1 levels go down, while HA, NA, and M1 mRNAs are preferentially expressed (Hay et al., 1977; Shapiro et al., 1987; Smith and Hay, 1982). The differential gene expression is also regulated at the level of vRNA promoter. The uracil to cytosine variation at position 4 in the 3′ vRNA end of the PB1, PB2, and PA RNA segments leads to a downregulation of transcription and upregulation in replication and ultimately resulting in lower polymerase mRNAs and proteins in infected cells (Hay et al., 1977). The translational regulation is achieved via both selective translation of viral mRNAs as well as inhibition of host mRNA processing.

2.2.5.5 Nuclear export of ribonucleoproteins

Once the viral mRNAs are synthesized they have to be exported to the cytoplasm of the infected cell for viral protein translation. The newly assembled RNP complexes are also exported from the nucleus to cytoplasm. The matrix protein (M1) and the nuclear export

protein (NEP/NS2) direct the nuclear export of newly assembled RNP complexes. M1 interacts with RNPs and NP, and also nucleosomes thereby leading to the dissociation of RNP from the nuclear matrix (Baudin et al., 2001; Ye et al., 1999).

NEP/NS2 has been shown to interact with the export receptor, Crm1 (Neumann et al., 2000), and several nucleoporins. Interaction of NEP/NS2 with M1 is also reported leading to the currently accepted model that an RNP-M1-NEP/NS2 complex is formed in the nucleus and that NEP/NS2 (Akarsu et al., 2003) is responsible for recruiting the export machinery and directing export of the complex (Yasuda et al., 1993).

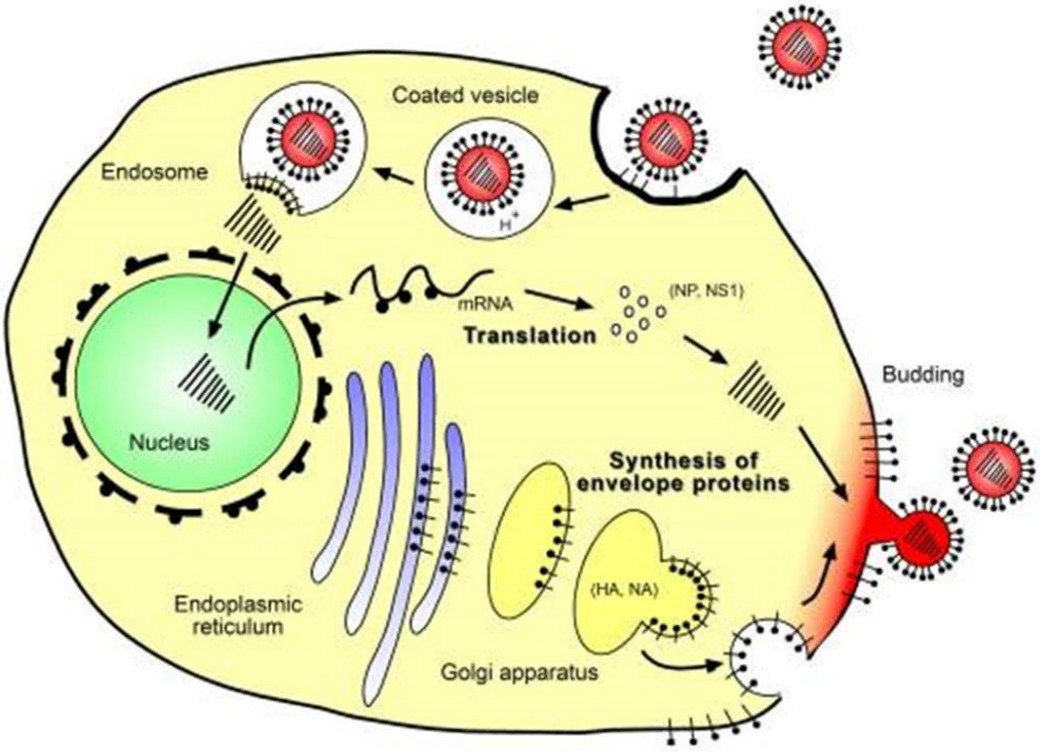

Fig. 2.2. Influenza virus life cycle. (Adapted from Behrens and Stoll, 2006)

Regulation of RNP export prevents premature exit of RNPs as well as the re-entry of RNPs into the nucleus following export. Mapping of NEP/NS2 binding site on M1 to M1 NLS suggested that it may act to keep the RNPs from re-entering the nucleus (Akarsu et al., 2003). Cytoplasmic anchoring of RNPs is also facilitated by NP through its binding to filamentous actin (Digard et al., 1999).

2.2.5.6 Virus assembly and budding

Correct assembly and packaging are pre-requisites for fully infectious virion. Virion components are assembled at the last stage of the viral life cycle. HA, NA, and M2 via their apical sorting signals are directed towards the apical plasma membrane, the virus assembly site. Thus, they are proposed to utilize lipid rafts for cell surface transport (Nayak et al., 2004). M1 protein plays an indispensable role in virus budding and is reported to be transported through either existing as M1-vRNP complex (Watanabe et al, 2010) or by associating with envelope glycoproteins (HA and NA) during exocytic transport (Ali et al., 2000).

Virus genome packaging is a debatable topic with two contrasting theories of random incorporation model and selective incorporation model. The first one describes the random packaging of the viral genome and is supported by the evidence that virions may possess more than eight vRNPs, to assure the presence of a full complement of eight vRNPs in virus particles (Bancroft and Parslow, 2002). The second model suggests that each vRNA segment contains a unique “packaging signal” and that every virion possesses a full complement of the eight vRNP segments. Packaging signals have been found to exist in the coding region at both the 5′ and 3′ ends of the genomic RNA (Enami et al., 1991). The selective incorporation model relies upon various RNA-RNA and RNA-protein interactions.

Accumulation of M1 at the lipid bilayer stimulates the bud formation by an outward curvature of plasma membrane. The budding process completes when the membranes fuse followed by fission and ultimately the release of viral particle. Sialidase activity of NA aids virus budding by cleaving the association of virions and the sialic acids on cellular surface (Matrosovich et al., 2004).

2.3 IAV and host interaction for efficient virus infection and pathogenesis

For an efficient replication and pathogenesis, IAV is known to collaborate with different cellular factors. It greatly depends on the host machinery at every step of the virus life cycle (Fig. 2.3). The most important aspect of understanding the virus- host interactions is to identify potential targets for anti-influenza therapy. The segmented genome of IAV has bestowed the virus with evolutionary advantages. It allows for a frequent generation of new viruses during replication of two different IAV subtypes in the same cell leading to reassortment of parental segments. Also, the current antivirals targeting the viral proteins seem to be inefficient in combating these new viral strains which harbor mutations due to an error-prone replication by viral RNA polymerase. These mutations confer resistance towards the currently available drugs. The Adamantane drugs, Amantadine, and Rimantadine, are 2 of the antivirals that target the proton flow through the M2 ion channel by binding near serine-31 clusters, hence, blocking the release of viral ribonucleoprotein complexes in the cell (Das et al., 2010). S31N mutation is the most prevalent mutation conferring adamantane resistance found in the recent epidemic causing H7N9 virus (Chen et al., 2013; Kageyama et al., 2013).

The imperative role of each host-virus interaction makes it an attractive target for developing a broad spectrum antiviral to contain the ever evolving IAV. Moreover, in contrast to viral proteins, host factors are less susceptible to mutations. Several genomic, proteomic and yeast two-hybrid screenings have been undertaken to identify the cellular factors recruited by the virus throughout infection (Bredel-Tretheway, 2011; Mayer et al., 2007; Shaw, 2011). A yeast two-hybrid and proteomic-based screening done by Shapira et al revealed an interactome of virus and host proteins and it was inferred from the study that the intra-viral network is extremely interconnected. When compared to other viruses, IAV interacts with greater number of human proteins. Also, a variability in interaction specificity was seen with 24 human proteins interacting with at least two flu proteins (Shapira et al., 2009). Genome-wide RNAi screens also prove to be an excellent approach to search for host factors. A Drosophila RNAi technology used by Hao et al. tested against 13,071 genes identified over 100 genes in drosophila which on suppression resulted in decreased influenza virus infection (Hao et al., 2008).

Although some of these host factors are not crucial for normal cellular metabolism, they have been reported to be indispensable for virus replication. Inhibitors of these factors could halt progression of IAV inside the host cell at nontoxic concentrations. Inhibitors of cellular proteins required for endocytosis- like dynamin, ENac and viral replication such as PolII, CAMK2B, DHODH and CLK1 (Hoffmann et al., 2011; Karlas et al., 2010; Konig et al., 2010), were able to block virus infection by blocking their respective functions. Thus, discovery of novel antiviral targets through *in vitro* and in silico screening of chemical compounds is a novel strategy and a source of antiviral targets. A virtual ligand screen of the NCI library with around 275,000 compounds against importin α5 using the molecular

modeling program Q-MOL, revealed various compounds as potent antiviral hits (Müller et al., 2012). Among the identified hits were FDA- approved drugs belonging to class of triazoles and bis-triazoles including NSC331942 (Devries et al., 2011; Eierhoff et al., 2010).

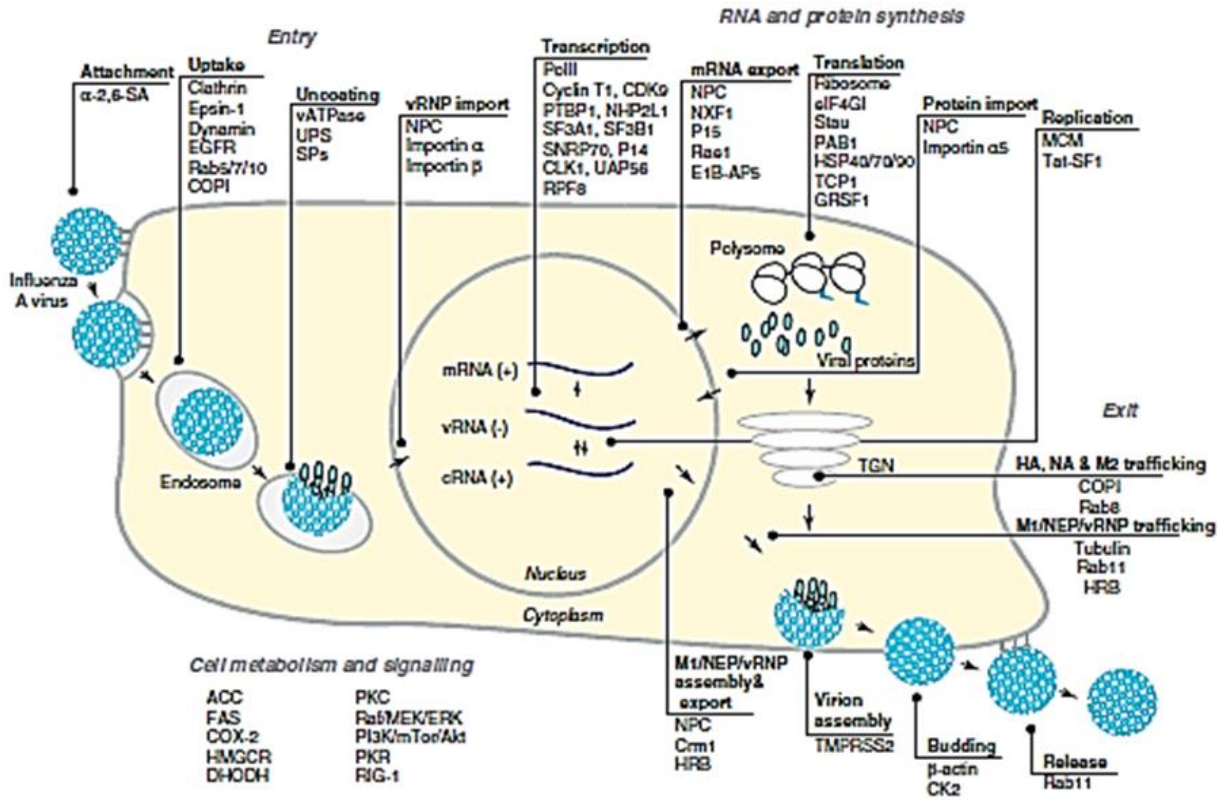

Fig2.3. Overview of host factors involved in influenza virus life cycle. (Adapted from Müller et al., 2012)

2.4 Influenza A virus Nucleoprotein

All viruses with negative sense RNA genomes encode single strand RNA (ssRNA) binding nucleoprotein that encapsidates the virus genome to form a ribonucleoprotein complex (Tordo e al., 1992). NP, encoded by RNA segment 5, is a 498 amino acids long polypeptide. Structurally, NP consists of a head and a body domain folding into a crescent-shaped structure. The head domain is the most conserved part followed by tail/linker and body regions. Inserting into a neighboring nucleoprotein molecule for oligomerization is a tail loop. The oligomerization is an essential requirement for vRNP formation. It plays a major role in maintaining RNP structure. Deletion mutant studies identified two regions, NP1 and NP2 (Fig. 2.4) that were capable of forming NP-NP contacts. At the same time, 23 residues at the c-terminal were found to inhibit oligomerization (Elton et al., 1999).

NP has highly helical secondary structure with 19 α helices and 8 β strands, while the rest are loops. The polypeptide has a predicted pI of 9.3 due to the predominance of basic amino acids, the 30 residues at the c-terminal are acidic with a pI of 3.7 (Arranz et al., 2012). NP gene is relatively well conserved among virus strains isolated from different hosts (Shu et al., 1993). Additionally, NP is modified through phosphorylation that has a potential role in RNP trafficking.

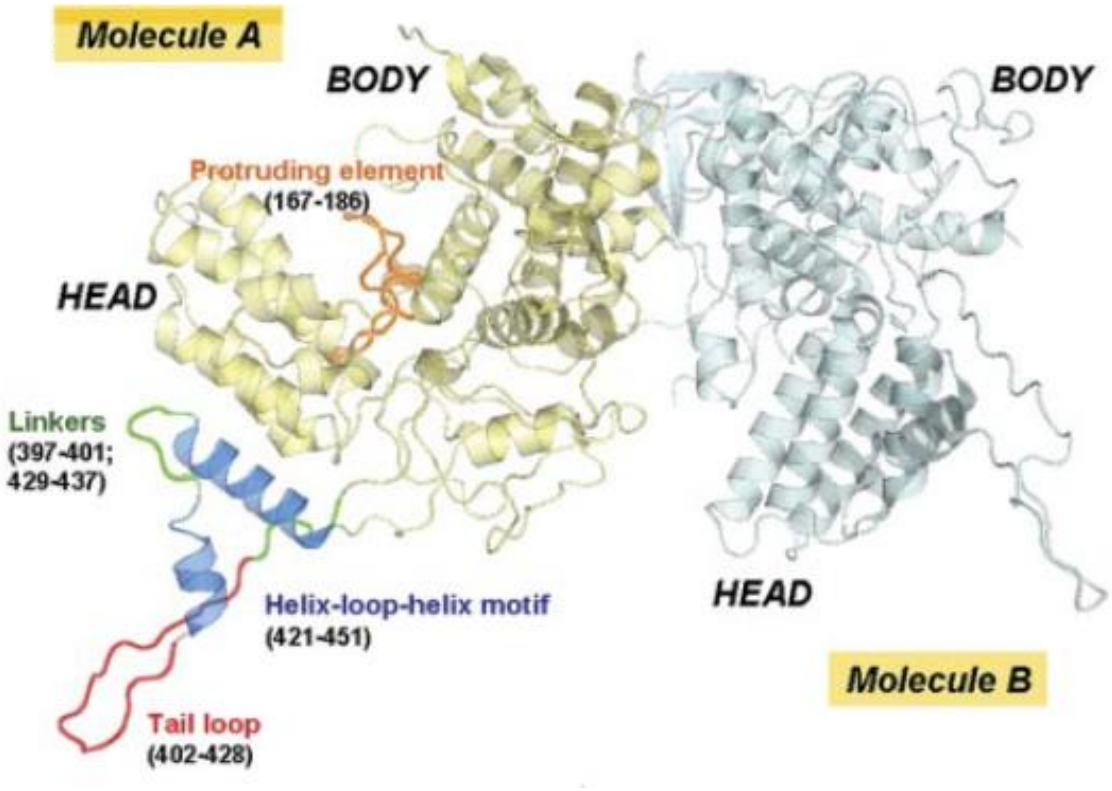

Fig2.4. Crystal structure of NP dimer. (Adapted from Ng et al., 2008)

RNA binding activity of NP is a well-studied aspect of NP. The RNA binding groove is located on the exterior between the head and body domains. The binding is characterized with a high affinity (k_d~20 nM) but little or no sequence specificity (Scholtissek and Becht, 1971; Kingsbury et al., 1987; Yamanaka et al., 1990; Baudin et al., 1994; Digard et al., 1999). Most importantly, the RNA binding region was mapped to the N-terminal of NP which was later postulated to work in coherence with other sequences for the high-affinity RNA binding (Albo et al., 1995). EM analysis by two separate groups revealed that native RNPs are double helical structures with two NP strands of opposite polarity that facilitates the association between the two. However, while one study proposed that the stem region

has a rise of 32.6 A° between two neighboring NPs with 4.9 NP molecules per turn (Moeller et al., 2013), the other presented a reconstruction of the native RNPs from influenza virions (Arranz et al., 2012) showing that the rise step per monomer was constant at 28.4 A° and the rotation angle between monomers ranged from –578 to –648, forming helices with approximately 12 NP monomers per turn. Recent evidence proves that NP is required for viral replication wherein it mediates the switch from capped- primed viral mRNA synthesis to unprimed viral RNA replication. The function is considered to be the result of its interaction with the polymerases.

Apart from being the structural component of the virus, NP performs various essential functions via its interaction with a plethora of cellular factors like cytoskeleton scaffolding protein α-actinin-4 (Sharma et al., 2014), nuclear import receptor α importin (Elton et al., 2001), nuclear export receptor CRM1 (Momose et al., 2001), DEAD-box helicase BAT1/UAP56 and cytoskeletal element F-actin (Digard et al., 1999). Recently it was reported that NP induces apoptosis through its interaction with host anti-apoptotic host protein Clusterin (Tripathi et al., 2013). Considering its critical importance in IAV infection and pathogenesis, it is speculated to utilize other cellular pathways to accomplish its pro-apoptotic function, including the much-conserved ubiquitin proteasomal pathway (UPP).

2.5 Ubiquitin proteasomal pathway

Ubiquitin is a small molecule of 76 residues found to covalently attach to proteins to target them for degradation via 26S proteasome. The process is comprehensively known as the ubiquitin proteasomal pathway (UPP). Ubiquitin is expressed from polyubiquitin gene carrying tandem identical ubiquitin repeats and two N-terminal ubiquitin moieties. The mature 76 amino acid protein carrying the characteristic C-terminal di-Gly motif, is released

following the cleavage of the blocked C-termini of the precursor through specific endopeptidase (Mukhopadhyay and Riezman, 2007).

Ubiquitylation is a cumulative effect of the sequential action of 3 enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligase enzymes (Fig. 2.5). The three step conjugation reaction is initiated with an ATP-dependent thiol ester formation with the C-terminal Gly of ubiquitin by the E1 enzyme. This activates the ubiquitin for nucleophilic attack, which is then transferred by transesterification reaction onto a ubiquitin conjugating enzyme (E2). Finally, the attachment is catalyzed by ubiquitin ligase E3 through an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of a lysyl residue in a target protein. However, E2 ubiquitin conjugating enzyme may play a role in determining linkage specificity alone or in conjunction with E2-associated proteins. When there is an attachment of more than a single Ub, different chain types ensue, depending on whether the N-terminal Meth or one of the seven Lys residues of the acceptor ubiquitin reacts with the incoming E2-bound donor ubiquitin. Depending upon the type of ubiquitination chains, varying from monoubiquitination to poly or multi-ubiquitination, and the Lysine residue involved, the physiological function may vary. Lys 48 and Lys 63 are the best-characterized chains marking proteins for proteasomal degradation and signaling process respectively. similarly, Lys 11 chains can control cell cycle progression and Met 1 chains (also known as linear chains) contribute to NF-κB signaling (Mukhopadhyay and Riezman, 2007; Randow and Lehner, 2009).

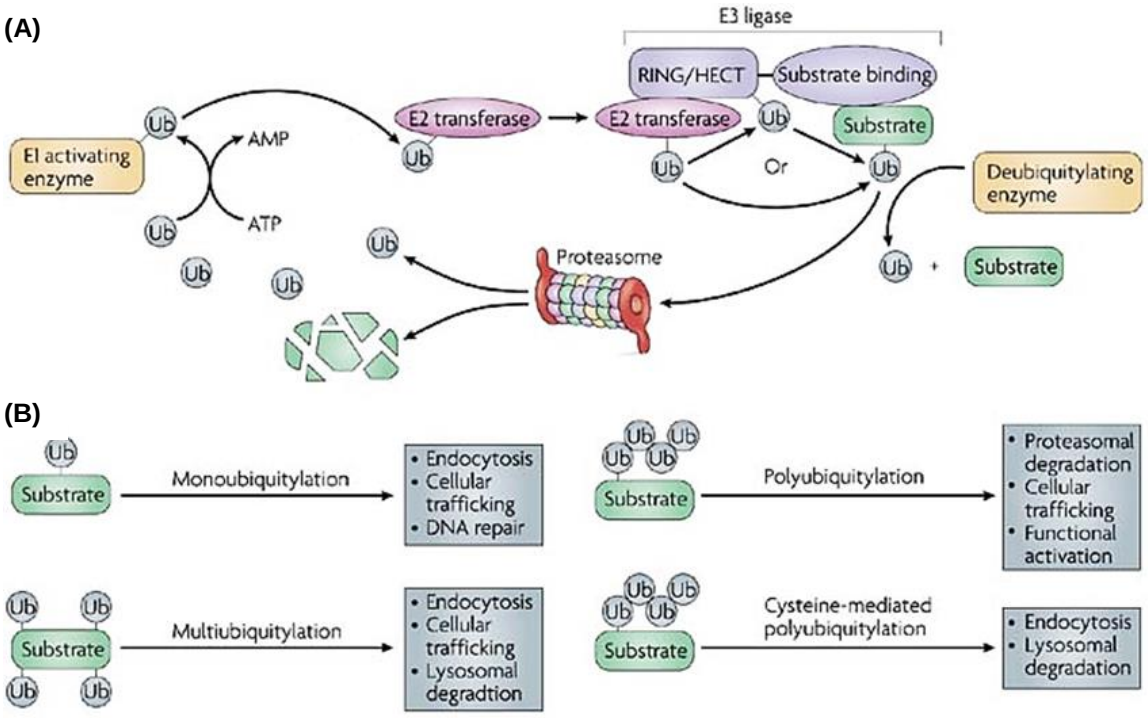

Fig 2.5. The ubiquitin chain formation. (A) The ubiquitin conjugation reaction. (B) Regulation by different ubiquitination chain. (Adapted from Hoeller et al., 2006)

A completely different class of proteins, known as deubiquitinases, reverses this process by removing the Ub molecules from target proteins. These enzymes release ubiquitin from its precursor forms by hydrolysing peptide bonds, however, their specificity is also directed towards a specific ubiquitin chain linkage, such as Lys 63 or Lys 48 chains (Lindner, 2007).

2.6 Viral exploitation of the ubiquitin system

Ubiquitination is a key regulatory mechanism in the orchestration of various cellular activities including signal transduction, transcription, membrane protein trafficking, apoptosis, autophagy and immune responses (Mukhopadhyay and Riezman, 2007; Welchman et al., 2005; Pickart, 2004). Hence, it has become increasingly evident that the comprehensive ubiquitinylation process remains a prime target of a broad range of pathogens including viruses (Randow and Lehner, 2009; Lindner, 2007). Viruses may employ various strategy to exploit this multifaceted process. Viruses may encode their own ubiquitin, for eg. Baculoviruses encoded ubiquitin that is clearly not essential for virus replication but contains 58 conserved residues and hence is presumed to selectively perform some of the cellular ubiquitin functions (Reilly and Guarino, 1996). Besides ubiquitins, viruses encode E3 ligases or deubiquitinases as well as adaptor proteins to modulate the cellular E3s and deubiquitinases to modulate the ubiquitination status of substrates of choice.

2.6.1 E3 ligases as viral targets

With 600 putative E3 ligases encoded by the mammalian genome, the receptor specificity is maintained by E3 ligase and hence these proteins are heavily exploited by viruses. The most versatile and largest of the three known classes of E3 ligases, is the RING (Really Interesting New Gene) E3 ligase, as compared to the other two, HECT (homology to E6AP carboxyl terminus) and U-box ligase (Randow and Lehner, 2009). A small proportion of RING E3 ligases has transmembrane domains, enabling their insertion into cellular membranes.

Currently, the only known viral E3 ligases are RING-type, including the RING-CH family and the ICP0 family. Infected cell protein 0 (ICP0) of Herpes Simplex Viruses-1 (HSV-1)

induces ubiquitylation and proteasomal degradation of PML and Sp100 (Boutell et al., 2002). P53 and USP7 are other substrates of ICP0 wherein its binding with the latter prevents auto ubiquitination of ICP0 and suppress NF-kB signaling (Boutell Evereit, 2003; Canning et al., 2004). RING-CH E3s have been identified in viruses like murine and human γ-herpesviruses, where the key function varies from ubiquitination mediated down regulation or proteasomal degradation of various receptors to promote viral immune evasion (Stevenson et al., 2000; Coscoy and Ganem, 2000; Ishido et al., 2000; Haque et al., 2001).

To further redirect ubiquitylation, viruses encode adaptors that recruit the cellular E3 ligases and modulate the ubiquitination status of the desired substrates. p53 remains a prime target of viruses to subvert cellular apoptosis including adenoviruses. The E6 protein of Human oncogenic papilloma virus (HPV) 16 and 18, induces degradation of p53 by forming a complex with cellular HECT E3 ligase, E6AP (Scheffner et al., 1990).

Some paramyxoviruses, including mumps virus, human parainfluenza virus type 2 (HPIV2) and simian virus 5 (SV5), through their highly conserved V proteins, target STATs ubiquitylation and degradation. It is reported to be mediated through DDB1, a substrate adaptor of the Cul4A E3 ligase and ultimately results in limiting the activity of interferons (Fontana et al., 2008; Li et al., 2006; Parisien et al., 2002).

2.6.2 Influenza virus and UPP

Previous reports have shown that UPP has an important function in early steps of influenza virus infection. Treatment of host cells with proteasome inhibitor MG132 or lactacystin affected the early stages of virus replication. The confocal laser scanning analysis showed that influenza virus selectively employs the ubiquitin/vacuolar protein-sorting pathway for

entry into host cells (Khor et al., 2003). Recently, two complementary mass spectrometry based comprehensive proteomic analysis of purified influenza virus particles revealed that Ubiquitin is among the 36 host-encoded proteins in influenza virus particles (Shaw et al., 2008).

IAV protein NS1 is reported to target the ubiquitin ligase TRIM 25 to escape RIGI recognition. The NS1 protein is known to inhibit type-I interferon (IFN) production and part of this function is achieved by blocking the Lys63 linked RIG-I ubiquitination by E3 ligase TRIM25 (Gack et al., 2009). TRIM25 mediated ubiquitination is critical in RIG-I downstream signaling, and NS1 encoded by human (Cal04), avian (HK156), swine (SwTx98), and mouse-adapted (PR8) influenza viruses binds to human TRIM25 (Rajsbaum et al., 2012). M1 protein reportedly interacts with the cellular E3 ligase, Itch. Identified through genome-wide pooled shRNA screen, Itch ligase reportedly ubiquitinates M1 protein and mediates IAV release from the endosome (Su et al., 2013).

It was shown that another cellular E3 ligase, NEDD4 indirectly promotes cellular entry of influenza virus by depleting Interferon (IFN)-induced transmembrane protein 3 (IFITM3) levels through ubiquitination. IFITM3 is known to restrict cellular infection by influenza virus in humans and mice, thus, NEDD4 is proposed as a novel drug target for developing influenza other IFITM3-sensitive virus antivirals (Chesarino et al., 2015).

Cellular deubiquitinating enzyme USP11 down regulation led to an enhanced influenza virus replication which was further proved to interact with the components of the viral polymerase complex, PA, PB2, and NP. Also, USP11 deubiquitinates NP which was found to be monoubiquitinated at K184 residue (Liao et al., 2010). Moreover, it has been discovered recently that NP itself acts as an adaptor protein wherein it interacts with the ubiquitin ligase

MDM2 to stabilize the tumor suppressor protein, p53 through its decreased ubiquitination (Wang et al., 2012).

2.7 RING Finger 43 (RNF43)

RING Finger 43 protein (RNF43) is a recently identified PA-TM-RING finger ubiquitin ligase that is implicated to be over-expressed in human colorectal cancers and hepatocellular carcinomas with anti-apoptotic and growth promoting effects (Sugiura et al., 2008; Koo et al., 2012; Xing et al., 2013).

2.7.1 RNF43 Structure

RNF43 is a 783 amino acid long single pass transmembrane protein, containing an extracellular protease-associated (PA) domain, and a cytoplasmic RING domain (Fig. 2.6) (Chen et al., 2013). It derives its name from the RING domain (272-313aa) typically responsible for the ubiquitin ligase activity. The Ring finger protein sequence was first identified in the protein product of the human gene RING1-Really Interesting New Gene 1 (Hao et al., 2012). The RING finger motif is a unique linear series of conserved cysteine and histidine residues, Cys–X_2–Cys–$X_{9–39}$–Cys–$X_{1–3}$–His–$X_{2–3}$–Cys/His–X_2–Cys–$X_{4–48}$–Cys–X_2–Cys, where X can be any amino acid, but there are distinct preferences for particular types of amino acid at particular positions. The structural and biophysical analysis confirmed that the RING finger binds two zinc atoms in an interleaved arrangement.

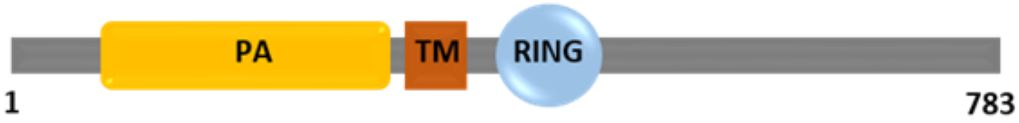

Fig2.6. Domain organization of human RNF43.

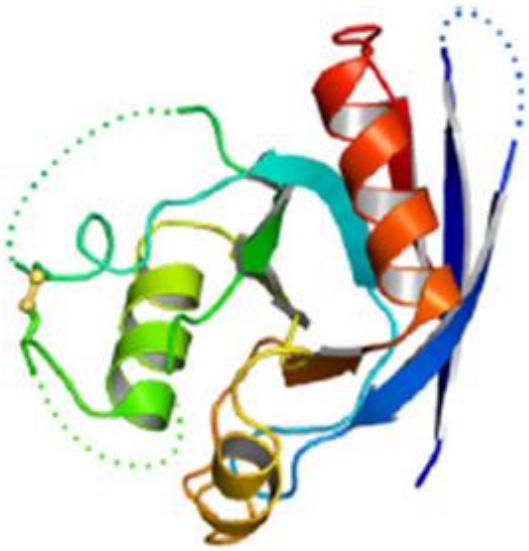

Fig 2.7. Crystal structure of the PA domain of RNF43. (Adapted from Zebisch and Jones, 2015)

PA domains play regulatory roles by functioning as ligand recognition motifs. These are commonly found in proteases as well as receptors such as transferrin receptors and plant-sorting receptors (Luo and Hofmann, 2001). The PA domain of RNF43 consists of seven β strands that form a twisted β sheet and three peripheral α helices (Fig. 2.7) (Chen et al., 2013; Zebisch et al., 2013; Peng et al., 2013).

2.7.2 Cellular Functions of RNF43

RNF43 was identified to be up-regulated in colorectal carcinomas (CRCs) after a genome-wide cDNA microarray analysis. RNF43 expresses at almost undetectable level in fetal kidney and lung but low-level expression in adult tissues. The primary biological functions of RING finger proteins is to catalyze ubiquitylation as a ubiquitin isopeptide ligase. Even though the presence of the RING finger domain implies the relationship of RNF43 with the ubiquitylation pathway, the biochemical characterization of RNF43 came later. RNF43 was found to reside mainly in the endoplasmic reticulum but also partially in the inner nuclear membrane (Sugiura et al., 2008). Ectopic expression of RNF43 in COS7 and NIH3T3 cells conferred growth promoting effects. RNF43 was identified as immunogenic and proposed to be a putative tumor associated antigen (TAA) of colorectal cancers as well as for lung, gastric, and liver cancers. This finding led to the development of the epitope peptides derived from RNF43 for peptide vaccination for various cancers (Yagyu et al., 2004). At the same time, anti-apoptotic role of RNF43 in hepatocellular carcinomas (HCCs) was elucidated by RNF43 knockdown which induced apoptosis and inhibited proliferation, invasion, colony formation and xenograft. In the same study, microarray-based gene profiling after RNF43 knockdown revealed 229 differentially expressed genes with most of them implicated in

oncogenic processes like cell proliferation, cell motility, cell adhesion, cell death and DNA repair (Xing et al., 2013).

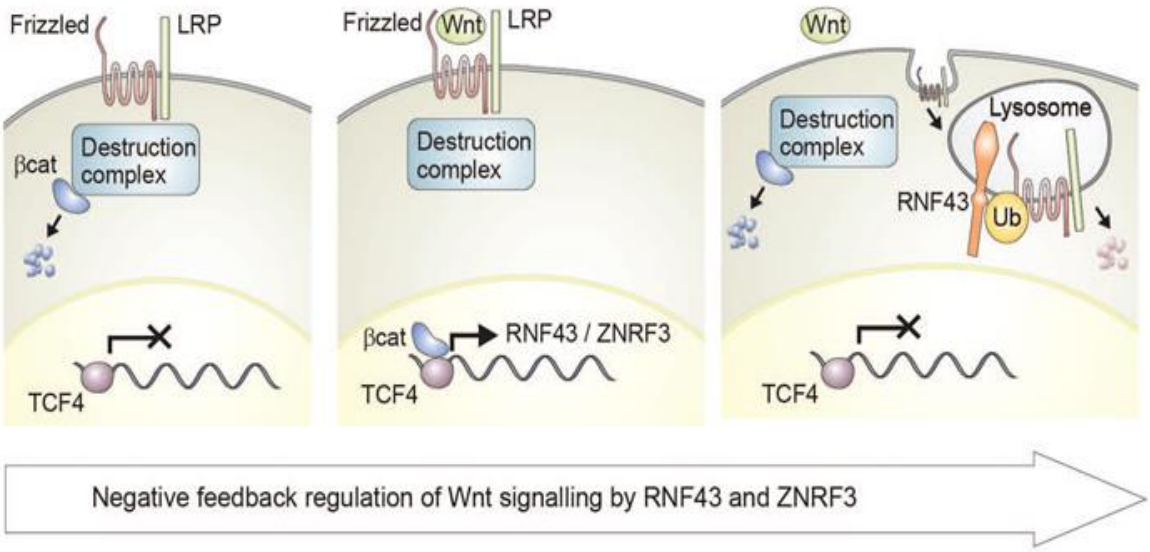

Fig.2.8 Negative Feedback regulation of Wnt signaling by RNF43. (Adapted from Koo et al., 2012)

One more evidence of anti-apoptotic activity of RNF43 came from the study where it was shown to interact with NEDL1 and that overexpression of RNF43 suppressed the transcriptional activity of p53 and UV-induced apoptosis. This provided a direct evidence of RNF43's association with p53-associated apoptotic cell death in colorectal carcinogenesis via interaction with NEDL1 (Shinada et al., 2011).

The growth-promoting effect of RNF43 in CRC is driven by the negative feedback regulation of Wnt signaling by the E3 ligase (Fig. 2.8). RNF43 along with ZNRF3 ubiquitinates Frizzled receptors leading to its endocytosis and lysosomal degradation. The

PA domain of the ZNRF3/RNF43 ectodomain interacts with the N-terminal cysteine–rich domain of the Frizzled. The interaction could either be direct or in combination with other components. The ubiquitination results in the removal of the receptors from the cell surface and down-regulation of the Wnt signaling (Koo et al., 2012).

CHAPTER 3
Materials and Methods

3.1 MATERIALS

3.1.1 Cell lines and strains

Bacterial strains	
Escherichia coli DH5α [*supE*44 Δ*lac* U169 (Φ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1*relA*1]	Invitrogen Life Technologies, USA
Yeast strains	
L40 (*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS2 (*4lexAop-*HIS3) URA3:* (8lexAop-*lacZ*) *GAL4)*	Invitrogen, USA
Mammalian cell lines	
HEK293, HEK293T, A549, Huh7	ATCC, USA
HCT116 $p53^{-/-}$	Kind gift from Dr. Bert Vogelstein

Table 3.1. Bacterial, yeast strains and cell lines used in the study.

3.1.2 Reagents

Molecular weight markers	
100 bp ladder	Fermentas, Canada
λLadder (EcorI + HindIII)	Fermentas, Canada
Prestained protein marker	Fermentas, Canada

Antibodies	
α-RNF43 (sc-165398), α -M2, α -p53, α -p21, α -HA, α –GAPDH, α - GFP, Goat α-mouse-HRP, Goat α-rabbit-HRP, α-Rb, α-CDK4	Santa Cruz Biotechnology, CA, USA
α -RNF43 (SAB2102033) and α -β-actin	Sigma-Aldrich, St. Louis, MO, USA
anti-E2F1, α -Bax and α -Puma	Cell Signaling, Boston, MA, USA
α-acetyl p53 (Lys-382)	Biolegend, San Diego, CA, USA
α-rabbit alexa 594, α-mouse alexa 488, DAPI (Molecular Probes, USA),	Molecular Probes, USA
α-NP	Immunology and Pathogenesis Branch, Influenza Division, Centres for Disease Control and Prevention, Atlanta, GA, USA.
Drugs	
Cycloheximide (CHX)	Sigma Aldrich, St. Louis, MO, USA
MG132	Calbiochem, EMD Biosciences, Billerica, MA, USA
Kits	
Dual Luciferase Reporter Assay System	Promega Life Science, Madison, WI, USA
Mini/ Midi prep plasmid kit, PCR/gel extraction kit	Qiagen, Germany

Annexin V FITC Assay kit	Cayman Chemicals, Ann Arbor, MI, USA
5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I	Sigma Aldrich, St. Louis, MO, USA
Membranes and filter papers	
Whatman no.1 paper	Whatman Co., NJ, USA
Nitrocellulase membrane	Amersham biosciences, UK
Restriction endonucleases and DNA modifying enzymes	
Restriction endonucleases	Fermentas, Canada
T4 DNA Ligase	NEB, MA, USA
Pfu Polymerase, Phusion polymerase	Finnzymes, Finland
Reverse transcriptase (M-MuLV)	Invitrogen, Carlsbad, CA, USA
Bacterial culture components	
Yeast Extract, Tryptone, Agar	Becton Dickinson Company, Maryland, USA
Sodium chloride	Merck, NJ, USA
Tissue culture reagents	

DMEM, Penicillin-streptomycin, Trypsin	GIBCO-Invitrogen, NY, USA
Sodium biocarbonate, HEPES, Glucose	Sigma Chemicals, St. Louis, MO, USA
Lipofectamine 2000, Opti-MEM	Invitrogen, Carlsbad, CA, USA
Yeast culture components	
Yeast nitrogen base, Peptone	Becton Dickinson Company, Maryland, USA
Amino acids, X-Gal	Sigma Chemical, St. Louis, MO, USA

Table 3.2. Reagents used in the study.

3.1.3 Viral strains and plasmid constructs

No.	IAV Strains	Genbank ID
1	A/Puerto Rico/8/34 (PR8) H1N1	CY033577.1
2	A/Aichi/2/1968 (X-31) H3N2	CY044304.1

Table 3.3. Influenza A viruses used in the study.

No.	DNA constructs	Cloning Details
1	pHLZ-NP	NP gene of A/Hatay/2004(H5N1) IAV (AJ867076.1) cloned into pHLZ (Invitrogen). (Sharma et al., 2011)
2	NP- Myc	NP gene of A/Hatay/2004 (H5N1) IAV (AJ867076.1) cloned into pcDNA3.1MycHis (Invitrogen). (Sharma et al., 2011)
3	NP- GFP	NP gene of A/Hatay/2004 (H5N1) IAV (AJ867076.1) cloned into pEGFPN1. (Sharma et al., 2011)
4	RNF43-Flag/HA	Homo sapiens RING Finger protein 43 (RNF43) cloned into pcDNA3.1Zeo. (Koo et al., 2012)
5	pYestrp2- RNF43	Homo sapiens RING Finger protein 43 (RNF43) cloned into pYESTrp2 at EcoRI and Sph1.

Table 3.4. Plasmid constructs used in the study.

3.1.4 Primers and oligo sequences

Real time primers	Sequence [5'-3']	
RNF43 mRNA	Fwd	GCAGGAGCTACGGGTCATTTC
	Rev	GATGCTGATGTAACCAGGGGT
NP mRNA	Fwd	CTCGTCGCTTATGACAAAGAAG
	Rev	AGATCATCATGTGAGTCAGAC
P21 mRNA	Fwd	GTCAGTTCCTTGTGGAGCCG
	Rev	CTCCAGTGGTGTCTCGGTG
P53 mRNA	Fwd	GTTTCCGTCTGGGCTTCTTGC
	Rev	ACGCAAATTTCCTTCCACTCGG
GAPDH mRNA	Fwd	TCACTGCCACCCAGAAGACTG
	Rev	GGATGACCTTGCCCACAGC

Table 3.5. Real time primers used in the study.

Construct	Sequence [5'-3']
pYestrp2-RNF43	Fwd AGAATTCTGGAAATGGTTATGAGTGGTGGCCACCAGCTG
	Rev GAAGCTTAAGCATGCTCACAGCCTGTTCACACAGCTC

Table 3.6. Primers and oligo sequence used in the study.

3.2 COMPOSITION OF REAGENTS AND MEDIA

Reagents/Buffer/Media Composition	
1X Protease inhibitor cocktail	16 mg/ml benzamidine 10 mg/ml aprotinin 10 mg/ml leupeptin 10 mg/ml pepstatin A 1 mM PMSF 10 mg/ml phenanthroline
2X SDS-PAGE buffer	100 mM Tris-HCl (pH 6.8) 4% SDS 20% glycerol 4% β-mercaptoethanol 0.01% bromophenol blue
LB (Luria Bertani) medium (1 liter, pH 7.5)	10 g Bacto-tryptone 5 g Bacto yeast extract 10 g NaCl
YPD (Yeast Peptone Dextrose) (1 liter, pH 7.5)	20 g Bacto-peptone 10 g Bacto-yeast extract 20 g dextrose
1X PBS (Phosphate Buffer Saline) (pH 7.4)	150 mM NaCl 0.27 mM KCl 10 mM disodium hydrogen phosphate (Na2HPO4) 0.2 mM sodium di-hydrogen phosphate (NaH2PO4)
1X PBST	0.05% (v/v) Tween-20 in 1X Phosphate buffer saline (PBS)
Western Blocking buffer	5% (w/v) Non-fat dry milk (Biorad) in 1X PBST
1X Cell Lysis buffer	50 mM Tris-Cl (pH7.5) 150 mM NaCl 1mM EDTA 1% triton X-100 1X protease inhibitor (prior to use).

Immuno-precipiration (IP) buffer	50 mM Tris, pH 7.5 150 mM NaCl 1 mM EDTA 0.1% NP-40
Ubiquitination assay lysis buffer	6M guanidinium-HCl 0.1 M Na_2HPO_4/NaH_2PO_4 0.01 M Tris/HCl (pH 8.0) 5mM Imidazole 10mM β-mercaptoethanol
1X LiAc buffer	10 mM Tris-HCl (pH 8.0) 1 mM EDTA 100 mM LiAc
Buffer Z	0.1 M Na2PO4 buffer (pH 7.0) 10 mM KCl 1 mM MgSO4
PEG/LiAc solution	10 mM Tris-HCl (pH 8.0) 1 mM EDTA 100 mM LiAc 40% PEG 3350
Tris-EDTA Buffer (TE)	10 mM Tris 1 mM EDTA (pH 8.0)
Tris-Borate Buffer (TBE)	88 mM Tris-Borate 89 mM Boric acid 2mM EDTA
Solution I, plasmid prep	50 mM Tris-HCl (pH 8.0) 10 mM EDTA 100 ug/ml RNase A Glucose
Solution II, plasmid prep	200 mM NaOH 1% SDS

Solution III, plasmid prep	3M potassium acetate (pH 5.5)
Ampicillin	Stock of 100 mg/ml in 50% absolute alcohol
Kanamycin	Stock of 50 mg/ml in autoclaved MQ-water

Table 3.7. Primers and oligo sequence used in the study.

3.3 METHODS

3.3.1 Preparation of competent cells

A single colony of *E. coli* DH5α strain was inoculated into 5 ml LB medium and was grown for 12-16 h at 37°C with shaking at 200 rpm. Next day, 100 ml of fresh LB medium was inoculated using 1% inoculum of the overnight grown primary culture and grown till A_{600} 0.5 to 0.6 was reached. The cell culture was incubated at 4°C for 30 min and pelleted down at 4,000 rpm at 4°C for 10 min. The cell pellet was re suspended in 10 ml of 100 mM $CaCl_2$ and kept at 4°C for 1 hour. The cells were then pelleted down and resuspended in 1.6 ml of 100 mM $CaCl_2$and incubated overnight at 0°C. The cells were finally re-suspended in glycerol with working concentration as 20% following which aliquots were made in sterile microfuge tubes and stored at 70°C for future use.

3.3.2 Transformation

The competent cells were used for transformation and propagation of various DNA constructs. For transformation, about 50-200 ng of DNA or ligation mixture in a volume of ranging from 10-25 µl was added into 100 µl of competent cells in sterile 1.5 ml microfuge tubes and incubated on ice for 30 min. The tubes were incubated at 42°C in a water bath for 1.5 min followed by incubation on ice for 2-3 minutes. To this, 1ml of sterile LB broth was added and incubated at 37°C with shaking at 220 rpm for 1 h. The cells were then harvested, re-suspended in 100 µl of LB medium and plated on LB agar plates containing the appropriate antibiotic. Plates were incubated for 12-16 h at 37°C for colonies to appear.

3.3.3 DNA isolation

DNA mini-preparations were carried out using 25 ml culture following alkaline lysis method, as described in Sambrook *et* al. or using Qiagen columns as per manufacturer's instructions. Large scale preparation of DNA was done by PEG.

3.3.4 Estimation of nucleic acid

Estimation of DNA and RNA was carried out spectrophotometrically. While the quantity of nucleic acid was estimated by measuring A_{260}, the purity of nucleic acids in solution was determined by measuring the ratio A_{260}/A_{280}. The concentration of nucleic acids was calculated by taking 1 A_{260} = 50 µg/ml for DNA, 40 µg/ml for RNA and 33 µg/ml for single stranded oligonucleotides. The purity of nucleic acids was checked by their A_{260}/A_{280}ratio.

3.3.5 Preparation of *S. cerevisiae* competent cells

A 5 ml preculture of *S. cerevisiae* cells was prepared by growing a single yeast colony overnight in YPD medium (Yeast extract Peptone Dextrose medium: 1% Yeast extract, 2% Peptone, and 2% Dextrose). 400-800 µl of the preculture was transferred to 100 ml YPD

medium in a baffled flask. The cells were grown overnight with shaking (200 rpm) at 30ºC. When the cell density approximated 1-4 x 107 (*A*600 1.00), the culture was transferred to a sterile polypropylene tube and centrifuged at 3,000 rpm for 2 min. The yeast pellet was washed once with 10 ml lithium acetate solution (0.1M Lithium acetate, 10 mM Tris-Cl (pH-8.0), and 1 mM EDTA) and finally resuspended in 1 ml of this solution. The cells were stored on ice until transformation.

3.3.6 Transformation of yeast plasmid

DNA (5 µg) mixed with 4 µl denatured salmon sperm DNA (10 mg/ml) was added to 100 µl of competent cells and kept at room temperature for 5 min. 700 µl of 40% PEG 3350 in lithium acetate solution was then added to the tube and mixed by inverting 4-6 times. The tube was incubated at 30°C for 45 min after which 50 µl of DMSO was added to the tube. Cells were given heat shock at 42°C for 5 min followed by 2 min on ice. The cells were washed once with sterile water and then plated on appropriate YC media. The plates were incubated at 30°C.

3.3.7 Cell culture

HEK293, HEK293T, A549 and Huh7 were purchased from American Type Culture Collection (ATCC). HCT116 $p53^{-/-}$ cell line was a kind gift from Dr. Bert Vogelstein. All the cell lines were grown and maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), Penicillin Streptomycin solution (100 units/ml) in 5% CO2 containing environment. The cell lines were sub-cultured on reaching 90% confluency.

3.3.8 DNA transfection

Cells were seeded on 60 mm dishes at around 50-60% confluency followed by DNA transfection the next day. DNA transfections were done with either Lipofectamine 2000 or Lipofectamine 3000 in NANS medium. The Lipofectamine and DNA mix in NANS was kept for 20min. and then added to the cells. After 6hours of incubation, NANS was replaced with 10% FBS containing DMEM. 48 h post transfection, the cells were harvested and analyzed as needed.

3.3.9 Preparation of cell lysates

At the indicated time post transfection, cells were scraped either in SDS-PAGE gel loading buffer or lysed in 1X cell lysis buffer containing the protease inhibitor cocktail (Roche). Cell lysis was carried out for 45 min at 4^{o}C with gentle vortexing every 15 minutes. The lysate was clarified by centrifugation at 13000rpm for 10 min at 4^{o}C. The protein concentration of the lysate was estimated using the Bradford reagent.

3.3.10 Immunoprecipitation

Cell lysates were prepared by incubating cells in 1X cell lysis buffer for 45 minutes with intermittent brief vortexing following which lysates were cleared by centrifugation at maximum RCF for 10 minutes at 4^{o}C. Protein estimation was carried out for cleared lysates using Bradford's reagent. Equal amounts of protein diluted to 500µL volume with 1X cell lysis buffer were incubated with 1 µg of antibody overnight at 4^{o}C. 50 µL of bead volume of A or protein G sepharose beads were incubated for 90 minutes with protein. Beads were then washed thrice with chilled PBS. Immunoprecipitates were eluted through boiling the beads in SDS- PAGE sample buffer for 10 minutes, subjected to SDS-PAGE followed by western blot analysis.

3.3.11 SDS- Polyacrylamide gel electrophoresis of proteins

Protein samples were prepared by mixing with equal volume of 2X SDS-PAGE loading buffer. Protein concentrations of the samples were measured using Bradford reagent. Samples were denatured in boiling water for 5 min, centrifuged briefly and loaded on the gel. Gels were run at 60 V till the proteins stacked properly and thereafter gels were run at a constant voltage of 100 V. Following the run, gels were transferred onto a nitrocellulose membrane.

3.3.12 Western blotting

For western blotting, proteins resolved with SDS-PAGE were transferred to a nitrocellulose membrane in transfer buffer at a constant voltage of 60 V for 1 h. Membrane was Ponceau stained to check the quality of transfer and later agitated in 1X PBST till the dye was completely washed off. To reduce non-specific binding, the membrane was blocked with 5% non-fat dry milk or Bovine Serum Albumin (BSA), prepared in 1X PBST, for 1–2 h at room temperature or overnight at 4°C. After blocking, the membrane was washed with PBST and incubated with appropriate primary antibody diluted in PBST containing 2-3% milk or BSA for 1 h at room temperature or overnight at 4°C. The blot was then washed three times for 10 min each with PBST and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG diluted in PBST containing 2-3% milk or BSA for 1-2 h at room temperature. Chemiluminescent detection of proteins was carried out using the Santa Cruz Western blot detection system, according to the supplier's protocol. For re-probing the same blot with other antibodies, the membrane was stripped with stripping buffer and used as described in the Cell Signaling Technology protocol.

3.3.13 Immunofluorescence assay

Cells were grown on coverslips to 40-50% confluency and DNA was transfected using lipofectamine in antibiotic-free and serum-free DMEM. 4-6 hours post-transfection, the medium was removed and replaced with complete DMEM containing 10% FBS. Around 48 h post-transfection, the cells were washed with PBS and were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.4% triton X-100 in PBS for 20 min at room temperature. Similarly for virus infection studies, cells were grown on coverslips to 80% confluence and infected with Influenza A virus A/Puerto Rico/8/34 H1N1 (PR8) virus, wild-type (WT), at 5 MOI in DMEM containing 0.3% BSA. At 0, 4 h and 8 h post-infection, the medium was removed and the cells were washed with PBS and fixed with 2% paraformaldehyde for 10-15 min at room temperature. Fixed cells were then washed twice with PBST (0.2% Tween 20), blocked with 2% bovine serum albumin for 1hour at room temp and left at 4^{o}C for overnight immuno staining with the specific primary antibody in 1:1000 dilution in 0.5% BSA. Later, cells were washed with PBST, incubated with secondary alexa flour antibodies' (594, 488) (Invitrogen) solution in 1:1000 dilution in 0.5% BSA for 1hour and washed with PBST. Slides were prepared by mounting the cover slips with Prolong Gold antifade medium with nuclei staining dye, DAPI (Invitrogen) and sealing the sides of the cover slips synthetic rubber-based adhesive (FeviBond). Cell images were taken under X60 objective lens of Leica DM6000B confocal microscope. Images were processed using NIS Elements AR 3.0 software (Nikon, Melville, NY, USA).

3.3.14 Luciferase reporter assay

A549 lung epithelial cells were pre-treated with NT siRNA and RNF43 siRNA. Components of influenza polymerase PB2, PB1, PA and NP were transfected along with luciferase

reporter plasmid which contains noncoding sequences from the NS1 segment of influenza A virus and the luciferase gene driven by Pol I, 24hr post-transfection of siRNA (Chen et al., 2008). Firefly luciferase plasmid pGL3 was used as an internal control. Similarly, A549 cells were transiently co transfected with p21 luciferase reporter plasmid containing the p53 binding site on its promoter together with different combinations of plasmids and a control plasmid, Renilla luciferase pRL-TK. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions (Promega). The firefly luciferase activity of each sample was normalized to constitutively expressed Renilla luciferase plasmid, pRL-TK.

3.3.15 Influenza virus infection

For virus infections, DMEM supplemented with 0.3% bovine serum albumin was used. Cells were washed with phosphate-buffered saline (PBS) followed by virus infection and incubated for virus adsorption at 37 °C. One hour later the virus was removed and cells were incubated with DMEM containing 0.3% BSA. In mock-infected cells, virus was replaced with PBS.

3.3.16 RNA isolation

Total RNA was isolated from cells using TRIzol reagent as per the supplier's instructions (Invitrogen). Briefly, to a 60 mm culture dish containing $1x10^6$ uninfected/ infected cells, 1 ml of Trizol was added and the cells were lysed by repeated pipetting. 200 µl chloroform per 1ml of trizol was added and incubated for 5 min at room temperature after a vigorous shaking. The aqueous phase was separated by centrifuging at 12,000g for 15 min at 4°C. RNA was precipitated by adding 0.5 ml of isopropanol to the aqueous phase obtained and incubating for 15min at room temp followed by centrifugation at 12,000g for 10 min at 4°C.

The RNA pellet was washed once with 75% EtOH at 7500g for 5min and air dried. The RNA was dissolved in 50µl of DEPC treated water. The RNA concentration was checked by spectrophotometer.

3.3.17 First strand cDNA synthesis

The first strand cDNA synthesis was primed by oligo dT using MMLV reverse transcriptase and starting with 5-10 µg of total RNA isolated from transfected/infected cells as per supplier's instructions. Synthesis of cDNA was carried out at 42°C for 1 h in 50 µl final volume containing 5 µg of total RNA, 1X reverse transcription buffer, 1 mM of each dents, 40 U/µl recombinant RNasin® (ribonuclease inhibitor), 10 U/µg (total RNA) MMLV reverse transcriptase and 0.5 µg oligo dT primer. Reverse transcribed samples containing first strand cDNAs were directly used for PCR. The PCR reaction volume of 50 µl consisted of 5 µl of reverse transcribed sample, 1X Taq DNA polymerase reaction buffer, 200 µM of each dNTP, 2.5U Taq or Phusion DNA polymerase and 10 pmole each of forward and reverse primers. PCR amplification was performed for 25-30 cycles with denaturation at 95°C for 60 sec, annealing at optimal temperature for 45-60 sec and extension at 72°C for 60-75 sec. Optimal number of PCR cycles for linear amplification of the different transcripts was defined. The human GAPDH specific primers were used in control reactions in all RT-PCR analyses. PCR products were electrophoretically fractionated on a 1% agarose gel containing ethidium bromide.

3.3.18 Cycloheximide assay

Cycloheximide (CHX), a protein translation inhibitor, was added to the media at a concentration of 50µg/ml, 44 h post transfection in A549 cells. Cells were incubated for

indicated time periods at 37^0C and harvested in SDS-PAGE sample buffer and subjected to SDS-PAGE followed by western blot analysis.

3.3.19 Ubiquitination assay and purification of His –tagged ubiquitin conjugates

Proteasome inhibitor, MG132 was used to determine the ubiquitination status of p53 protein. HEK293T cells grown in 60mm cell culture dishes were transiently co-transfected with the indicated plasmids and treated with 20μM MG132 for 5 hours before harvesting. Cells were lysed in triton X-100 cell lysis buffer and mixed with SDS PAGE sample buffer followed by western blot analysis with anti p53 antibody. For purification of His tagged conjugates, HEK293T cells were treated similarly and were processed as described previously (Xirodimas et al., 2001). Concisely, the cells were lysed in a lysis buffer (6 M guanidinium-HCl, 0.1 M Na2HPO4-NaH2PO4, 0.01 M Tris-HCl pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol) followed by an incubation of 12 h with Ni2+-NTA beads (Qiagen, Hilden, Germany) at 4 °C on a rotating wheel. The beads were then washed with wash buffer for 5 minutes at room temperature as explained previously and incubated with 75 μl of elution buffer (200 mM imidazole, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol and 5% SDS) for 20 min at room temperature. The eluates were mixed with SDS-PAGE sample buffer and analyzed through western blotting with required antibodies.

3.3.20 Flow cytometry

Annexin V staining of cells was done with Annexin V FITC Assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. A549 and HCT116 $p53^{-/-}$ cells were seeded in 12 well plates and were processed for either only transfection or transfection followed by IAV infection. 48 hours post transfection, cells were collected after

washing with PBS and centrifuging at 5000 rpm for 5 min at 4°C. Cell pellet obtained was resuspended in 0.200 ml of 1X binding buffer. Following centrifugation at 5000 rpm for 5 min, cells were mixed with 50 µl of Annexin V FITC/ Propidium iodide staining solution and incubated for 10 min in dark. Cell suspension was diluted by adding 150 µl of 1X binding buffer and were acquired on BD FACS Calibur Flow cytometer (BD Biosciences) and analyzed using Flowjo version 9.3.3 software (Tree Star Inc., Ashland, OR, USA).

3.3.21 5-Bromo-2' deoxy-uridine labelling

HEK293 cells grown on coverslips were labelled using 5-Bromo-2'-deoxy-uridine (BrdU) labeling and detection kit I (Roche) as per supplier's instructions. Cells were incubated with 10µM BrdU for 1 hour before harvesting. Cells were washed thrice with washing buffer and fixed with 4% paraformaldehyde for 20 min at room temperature followed by pemeabilization with ethanol based fixative at -15 to -25 °C for 20 min. Cells were covered with anti-BrdU antibody and anti-myc antibody for 1 hour at room temperature. Later, cells were washed thrice with wash buffer and incubated with anti- mouse Ig- fluorescein and anti-rabbit alexa 594 for 1 hour and washed with wash buffer. Slides were prepared by mounting the cover slips with Prolong Gold antifade medium with nuclei staining dye, DAPI and sealing the sides of the cover slips synthetic rubber-based adhesive (FeviBond).

3.3.22 Double thymidine block

48 h after transfection, cells were incubated in complete medium containing 2 mM thymidine for 10–12 h following which cells were washed twice with 1× PBS and incubated in complete medium for 12 h. Subsequently, for second block, cells were again incubated in complete medium containing 2 mM thymidine for 10–12 h. Later, cells were washed twice

with 1× PBS and induced to enter S-phase by incubating in complete medium. Harvesting of cells was carried out at the indicated time points.

3.3.23 Statistical analysis

Data are expressed as mean ± S.E. Means were compared by one-factor analysis of variance followed by Fisher protected least significant difference to assess specific group differences. Differences were considered significant at $p < 0.05$.

CHAPTER 4
Results and Discussion I

4.1 TO VALIDATE THE INTERACTION BETWEEN INFLUENZA A VIRUS NUCLEOPROTEIN AND HOST UBIQUITIN LIGASE RING FINGER PROTEIN 43 (RNF43).

4.1.1 Analysis of pYestrp2-RNF43 clone

A Yeast two-hybrid screening of a human c-DNA lung library was conducted previously in the lab to identify potential interacting partners of Nucleoprotein (NP) of IAV. Screening of a human lung cDNA library was done using NP from highly pathogenic A/Chicken/Hatay/2004 (H5N1), cloned into plasmid pHybLexA/Zeo, as the bait (Sharma et al., 2011). NP was expressed in fusion to the LexA DNA-binding domain (DNA-BD) of *pHybLex/Zeo*. A human lung cDNA B42 activation domain (AD) fusion library was constructed in *pYESTrp2*. Briefly, the screening was done by co-transforming the *pHybLex/Zeo-NP* sequentially with the AD fusion library (*pYESTrp2-LUL*). The selection of co-transformants was done on the synthetic dropout (SD) medium constituted of a nitrogen base, a carbon source (glucose) and amino acid dropout supplement lacking tryptophan and consisting of Zeocin ($SD^{-}W^{-}Zeo^{+}$) to select *pYESTrp2-LUL* and *pHyb-Lex/Zeo-NP* respectively. The positive colonies obtained were further selected on $SD^{-}W^{-}Zeo^{+}H$ medium to confirm histidine prototrophy. His^{+} clones were further tested for β-galactosidase activity, attained by LacZ+ phenotype, through filter-lift colony assay followed by E.coli transformation of positive clones (Sharma et al., 2014).

A clone encoding 1071-1622 nucleotide region of RNF43 was obtained as the positive interactor in the Y2H screening which was further confirmed by BLAST analysis (Fig. 4.1.1). Full-length RNF43 cDNA was cloned in-frame with the activation domain pYESTrp2

and both NP-pHLZ and RNF43–pYESTrp2 were co-transformed in the L-40 strain of *S. cerevisiae*. It tested positive for histidine prototrophy and β-galactosidase activity confirming the interaction of full-length RNF43 with NP (Fig. 4.1.2).

Bait	Prey	Growth on His-deficient medium	β-galactosidase assay
pHybLexA/ Zeo	pYestrp2	–	–
pHybLexA/ Zeo-**NP**	pYestrp2	–	–
pHybLexA/ Zeo	pYestrp2-**Clone 13-4**	–	–
pHybLexA/ Zeo-**NP**	pYestrp2-**Clone 13-4**	+	+
pHybLexA/ Zeo-Fos	pYestrp2-Jun	+	+

Fig. 4.1.1. Tabular representation of the yeast-two hybrid screening of lung cDNA library using NP as the bait protein. The filter β-gal assay for the clone 13-4 is shown in the last column of the table.

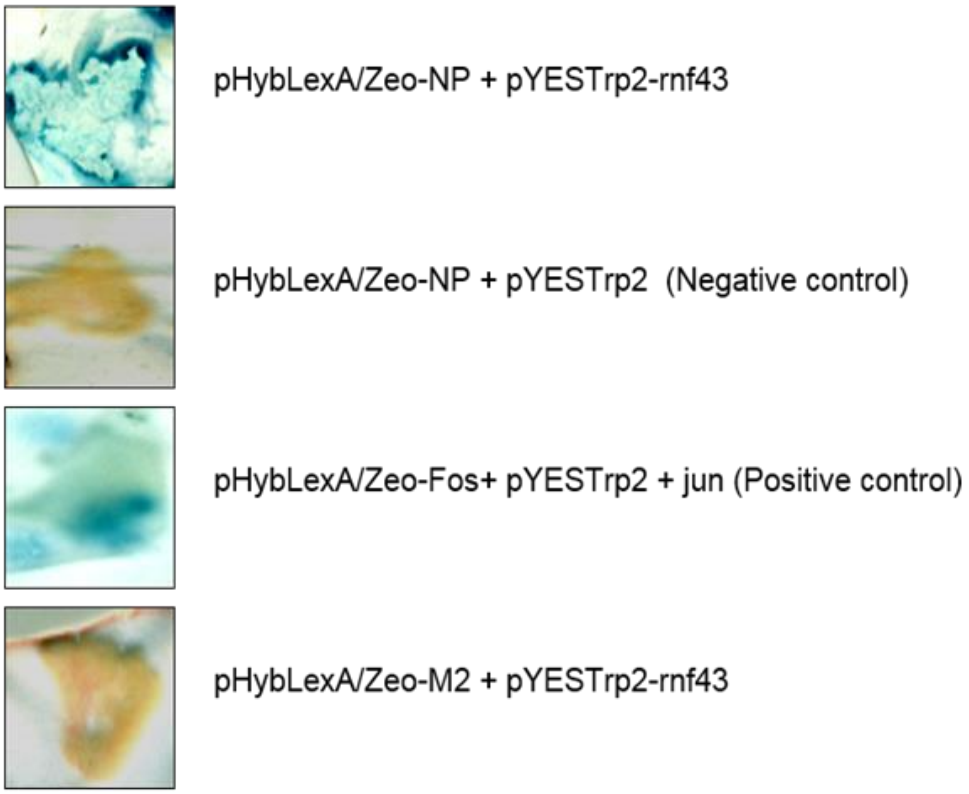

Fig. 4.1.2. Full-length RNF43 showed histidine prototrophy. pHybLexA/Zeo-NP and pYESTrp2-RNF43 were co-transformed in L-40 yeast strain and their interaction was verified by β-galactosidase assay. pHLZ-Fos and pYESTrp2-Jun acted as a positive control and empty pHLZ and pYESTrp2 as a negative control. pHLZ-M2 and pYESTrp2- rnf43 were also used as a negative control.

4.1.2 Nucleoprotein of influenza A virus interacts with human ubiquitin ligase protein RNF43 in a conserved manner.

After confirming the direct interaction of NP with the host factor, RNF43 in the yeast system, we proceeded to study this interaction in the mammalian system. The NP-RNF43 interaction was validated by performing co-immunoprecipitation (co-IP) assays in HEK293T cells. HEK293T cells were transfected with pCDNA3.1-Myc-NP plasmid construct. Myc-tagged NP expression was confirmed using western blotting with anti-myc antibody as shown in Fig. 4.1.3A (panel 3). Immunoprecipitation using anti-RNF43 antibody followed by immunoblotting with anti-myc antibody confirmed the interaction of RNF43 with NP (panel 1, Fig. 4.1.3A). The immunoprecipitation of RNF43 was confirmed by doing immunoblotting with anti RNF43 (panel 2, Fig. 4.1.3A).

To validate the existence of the said interaction during IAV infection, mammalian lung epithelial A549 cells were infected with A/Puerto Rico/8/34 virus (H1N1; PR8) and A/ Aichi/ 2/ 1968 virus (H3N2; X31) at 1 MOI. The cell lysates were harvested 24 hours post-infection (24 h.p.i) and subjected to co-immunoprecipitation with anti-RNF43 antibody. NP protein immunoprecipitated with RNF43 antibody thus confirming that NP-RNF43 interaction was conserved between both the virus isolates (Fig. 4.1.3B, panel1). Also, co-IP with another IAV protein, M2 (Fig. 4.1.3B, panel 3), showed that RNF43 antibody was unable to bind the M2 protein in the IAV infected lysate, proving that RNF43 interacts exclusively with NP of IAV.

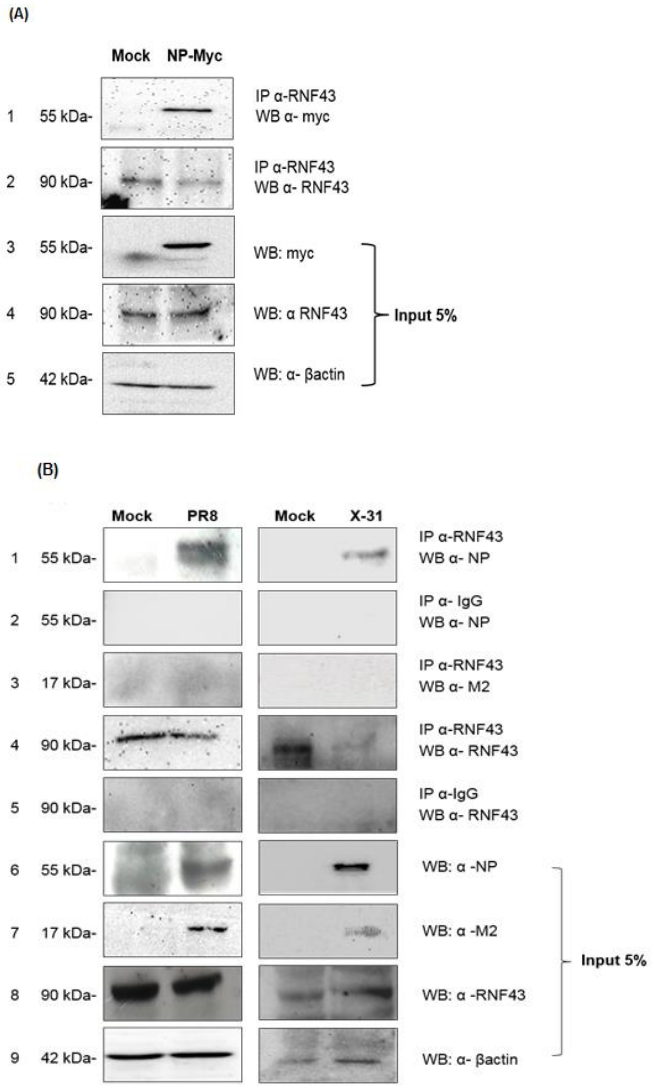

Fig. 4.1.3. NP interacts with RNF43. (A) HEK293T cells were transfected with either empty vector pCDNA3.1 (Mock) or pCDNA3.1-Myc-NP (NP-Myc). 48 hours post-transfection, cells were lysed and the lysates were subjected to co-immunoprecipitation with anti–RNF43 antibody followed by western blotting with anti-Myc antibody. (B) Lung epithelial A549 cells were infected with PR8 virus and X-31 virus at 1 MOI for 24 hrs and the whole cell lysates were used for co-immunoprecipitation assays. 5% lysates from the same experiment were subjected to western blotting with anti-NP (panel 6), anti-M2 (panel 7), anti-RNF43 (panel 8) and anti-βactin (panel 9) antibodies to show the cellular levels of these proteins.

These results clearly demonstrate that NP indeed interacts with RNF43 in the mammalian system as well as the interaction is conserved among the NP of various viral strains. NP isolated from A/Chicken/Hatay/2004 (H5N1) was shown to be interacting with RNF43 in transfection microenvironment whereas NP from A/Puerto Rico/8/34 (PR8) and A/Aichi/2/1968 (X-31) strains of IAV was apparently interacting with RNF43 in the virus-infected host system.

4.1.3 RNF43 co-localizes with NP in the nucleus.

We sought to ascertain the kinetics and site of interaction of NP and RNF43 after confirming the interaction of these two proteins. A549 cells were transfected with control plasmid pEGFPN1 or pEGFP-NP followed by fixing cells 48 hours post-transfection and further processing for immunofluorescence analysis. RNF43 was seen to have ubiquitous nucleo-cytoplasmic localization in mock-transfected cells (Fig. 4.1.4A). However, GFP-tagged NP localized in the nucleus and co-localized with RNF43 majorly inside the nucleus.

Similarly, A549 cells were infected with PR8 at an MOI of 5 and were fixed at 0, 4 and 8 h.p.i (Fig. 4.1.4B). At 4 h.p.i, NP has been observed to localize to the nucleus. At later stages of infection, it is reported to move into the cytoplasm (8 h.p.i) (Portella and Digard, 2002). RNF43 had nucleo-cytoplasmic localization but was noted to concentrate inside the nucleus on virus infection (4h.p.i, Fig. 4.1.4B). At 8 h.p.i, the two proteins were seen to co- localize inside the nucleus. Thus, it is inferred that the primary site of interaction for the IAV NP and RNF43 is the nucleus of the host cell.

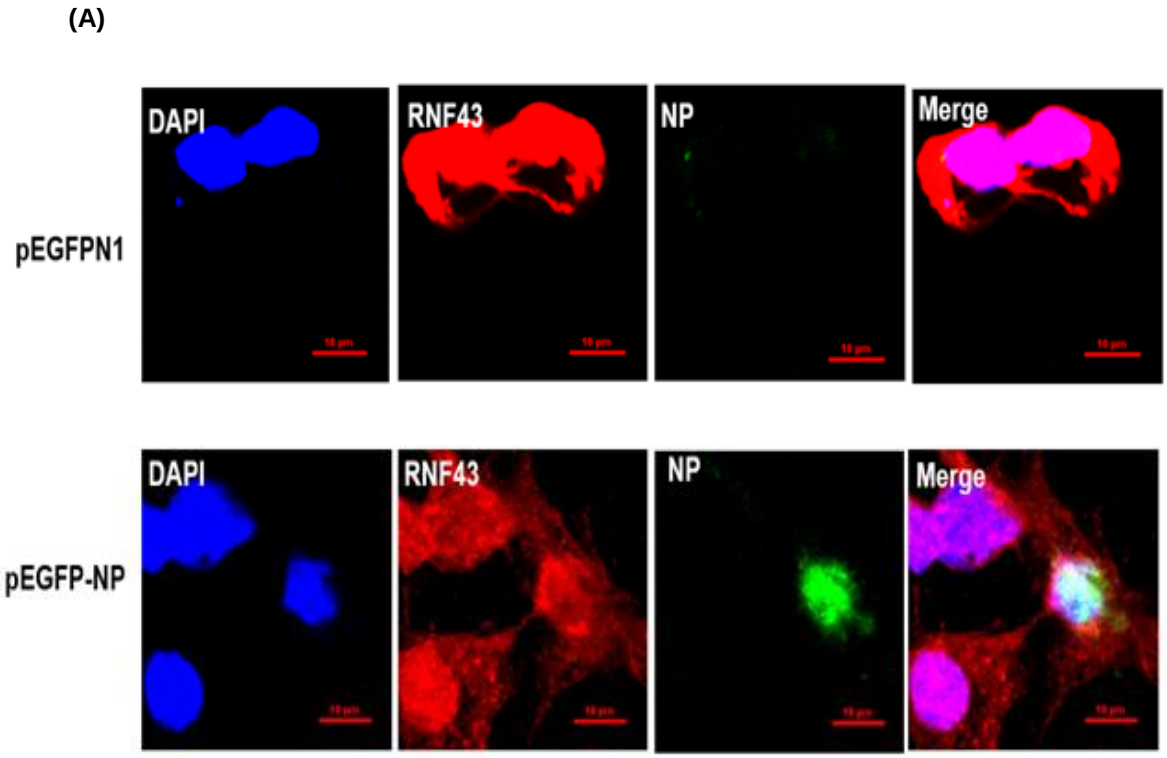

Fig. 4.1.4. IAV NP and RNF43 co-localize in the nucleus of mammalian cells. (A) A549 cells were transfected with pEGFPN1 control plasmid or pEGFP-NP. Cells were fixed after 48 hours and stained with DAPI for nucleus and anti-goat secondary antibody conjugated to Alexa-594 for RNF43 (red) and observed under confocal microscope. GFP-tagged NP is shown in green. Panels are labeled for their respective staining, RNF43 (red), NP (green), nucleus (blue).

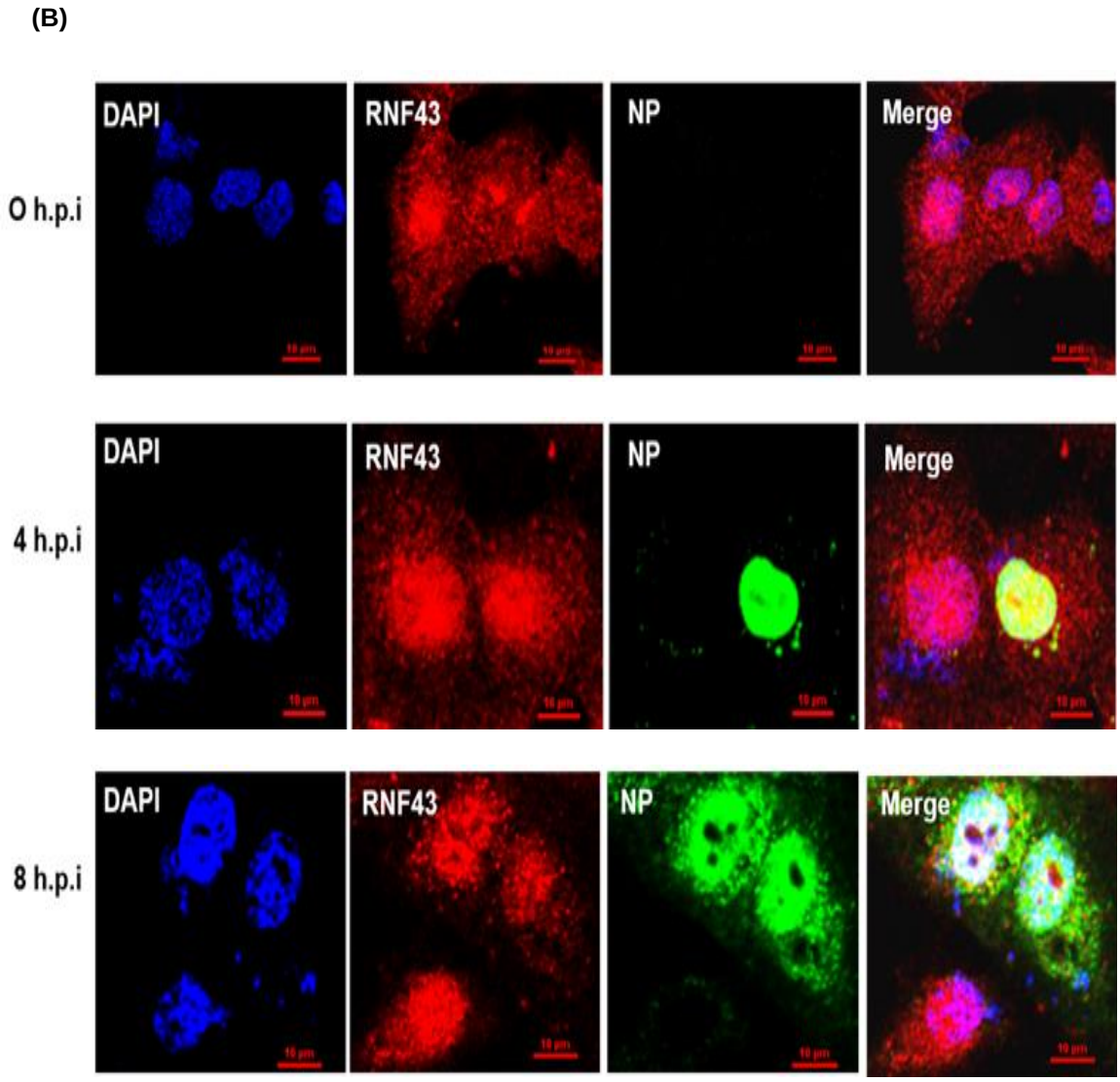

Fig. 4.1.4. IAV NP and RNF43 co-localize in the nucleus of mammalian cells. (B) A549 cells were infected with PR8 IAV at 5MOI and were fixed at the indicated time points. NP was stained using anti-NP monoclonal primary antibody and anti-mouse Alexa488 conjugated secondary antibody (green). RNF43 was stained using specific primary antibody and anti-rabbit Alexa-594 conjugated secondary antibody (red). Panels are labeled for their respective staining, RNF43 (red), NP (green), nucleus (blue).

4.1.4 Influenza infection decreases RNF43 mRNA and protein expression.

Viruses often target host factors through altering their abundance in the host. This is an important aspect of host-virus interaction and to elucidate the relevance of any interaction. Hence, to determine the physiological relevance of NP/RNF43 interaction in the influenza virus life-cycle, we aimed to study the mRNA and protein levels of RNF43 in IAV infected cells.

Western blot analysis of the PR8 infected A549 cells harvested at different time points post-infection was done (Fig. 4.1.5A) and it showed a gradual decline in RNF43 protein expression with the progression of infection reaching to its lowest levels at 48 h.p.i in the infected cells. We further checked the effect of virus doses on RNF43 expression. A549 cells were transfected with increasing MOI of PR8 virus and protein expression of RNF43 was evaluated using western blotting (Fig. 4.1.5B). A significant decrease in RNF43 protein levels with increasing virus MOI was in agreement with our previous observation. Protein levels were quantified using densitometric analysis on Image J software.

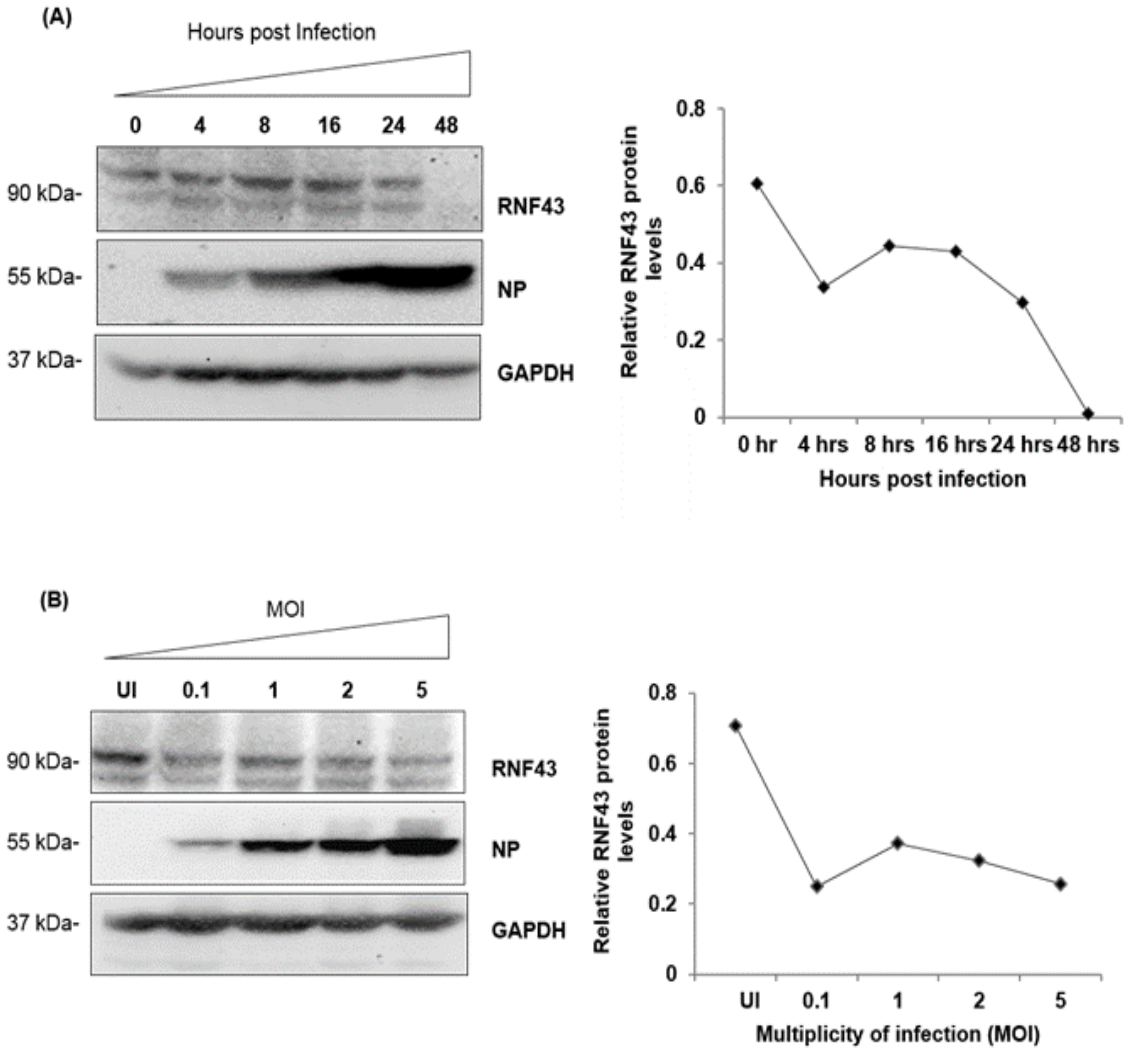

Fig. 4.1.5. IAV infection decreases the abundance of RNF43 in the infected cells. (A) Lung epithelial A549 cells were infected with PR8 virus at an MOI of 1 and cells were harvested at indicated time intervals post infection and the whole cell lysate was resolved on SDS-PAGE for western blot analysis of RNF43, NP, and GAPDH. (B) A549 cells were infected with PR8 virus at indicated MOIs and harvested at 24 hours post infection for Western blot analysis of RNF43, NP, and GAPDH. Quantitative representation of the immunoblots of both the experiments is shown in the line diagram after normalization with GAPDH (extreme right).

Subsequently, we studied the mRNA levels of RNF43 after IAV infection. Total mRNA of PR8 infected A549 cells was extracted 24h.p.i and quantified with quantitative real-time PCR. A remarkable 0.5 fold decrease in RNF43 mRNA levels was clearly observed as compared to the uninfected sample (Figure 4.1.5C).

These contemporaneous regulations of RNF43 transcripts and protein expression levels are indicative of an antagonistic interaction between NP and RNF43 where RNF43 is being attenuated with the progression of virus pathogenesis.

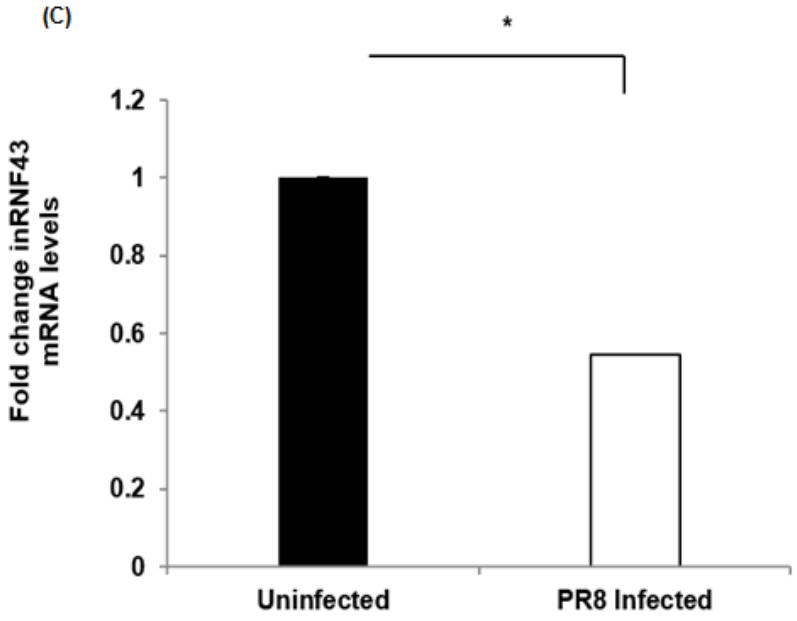

Fig. 4.1.5. IAV infection decreases the abundance of RNF43 in the infected cells. (C), A549 cells were mock infected or infected with PR8 virus for 24 hrs and total RNA was extracted followed by rnf43 mRNA estimation with qRT-PCR. Results are shown as mean of ± SD of three independent experiments. * indicates statistically significant difference at $p < 0.05$.

4.1.5 RNF43 acts an anti-viral protein to subside viral replication.

Increasing their efficiency of replication remains the prime motive of viruses to increase their infectivity in infected cells (Sharma et al., 2014). In order to investigate this, we monitored viral replication after transiently transfecting with pCDNA3.1-RNF43-Flag-HA plasmid at increasing concentrations of 0 (mock), 1µg and 2µg in HEK293T cells (Figure 4.1.6 A-C). Empty vector pCDNA3.1 was used as a negative control. After 24 hours' of incubation, cells were infected with PR8 virus and harvested at 24 hours. Quantification of mRNA and vRNA levels of NP was performed through real-time PCR after RNA isolation. RNF43 over-expression resulted in a gradient and a considerable decrease in NP mRNA and vRNA levels with 0.3 fold and 0.06 fold being the maximum recorded, respectively (Fig. 4.1.6A&B). The RNF43 overexpression was confirmed by RT-PCR analysis of RNF43 mRNA levels (Fig. 4.1.6C). This result very eloquently proves the anti-viral role of RNF43 in IAV infected cells whose ectopic expression resulted in not just a decrease in the levels of NP mRNA species that are resulted through viral mRNA transcription as well as NP vRNA species formed after viral RNA replication.

Next, we decided to study the effect of knockdown of RNF43 on NP mRNA levels. RNF43 was silenced using siRNA against RNF43 in HEK293T cells. An approximate 8 fold increase in NP mRNA levels with ~98% silencing of RNF43 (Fig. 4.1.6D) was observed. This drastic alteration in NP mRNA, as well as vRNA levels, exhibits a convincing antiviral role for RNF43.

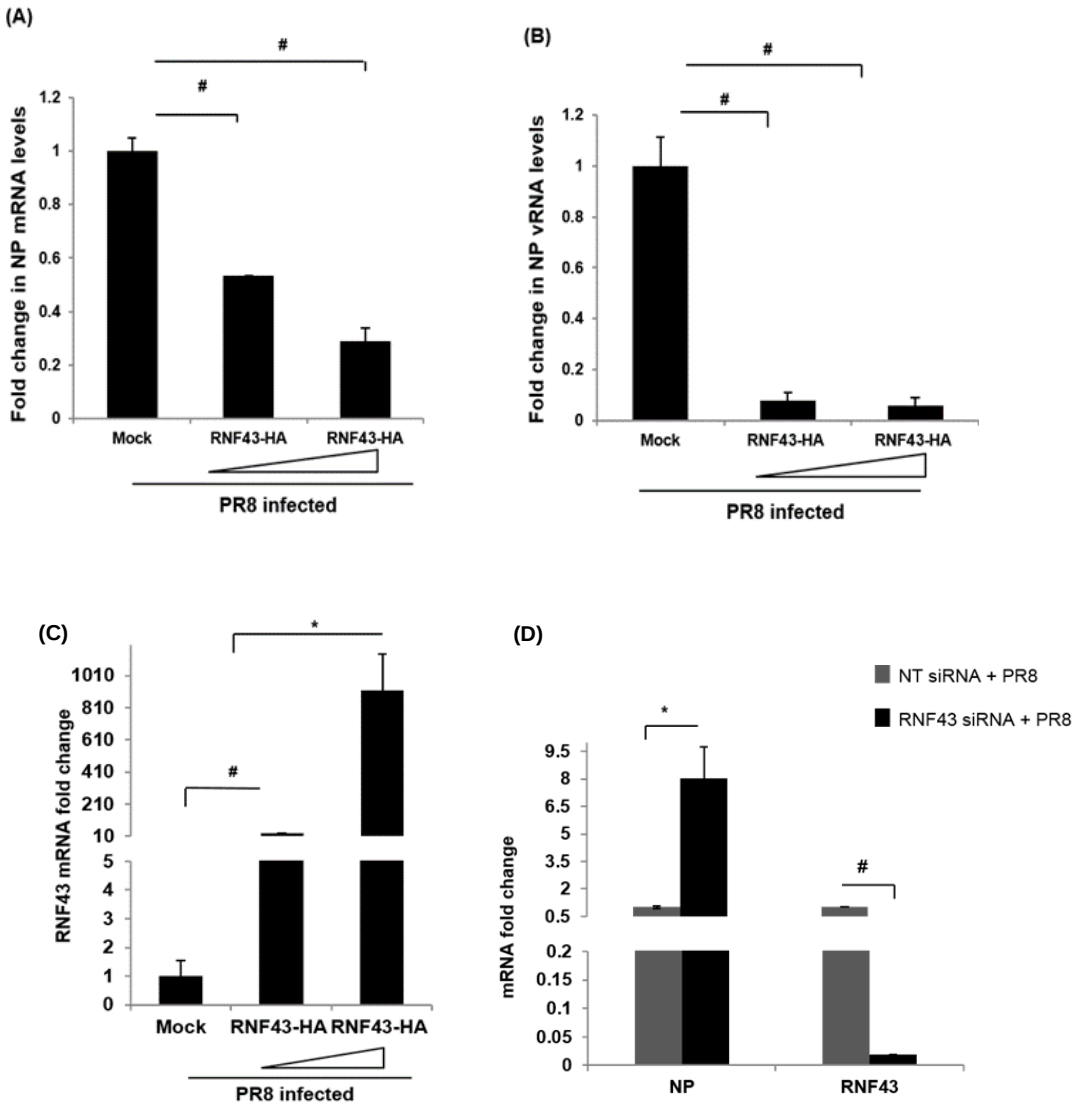

Fig. 4.1.6. Host factor RNF43 decreases IAV replication. (A-C) HEK293T cells were transiently transfected with plasmid pCDNA3.1 (Mock) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA) (1µg and 2µg) followed by PR8 infection and incubated for 24 hours. Total RNA was isolated and NP mRNA (A), NP vRNA (B) and RNF43 mRNA(C) levels were estimated through qRT PCR. (D) HEK293T cells were treated with NT siRNA or RNF43 siRNA for 24 h followed by PR8 IAV infection for next 24 h. Cells were processed for qRT-PCR analysis of mRNA levels of NP and RNF43. Results are shown as mean ± SD of three independent experiments. * and # indicate statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively.

In the view of above results, we decided to check the effect of RNF43 on influenza virus replicase activity. A549 cells were treated with either non-targeting siRNA (NT siRNA) or RNF43 siRNA followed by co-transfection with plasmids encoding the PR8 polymerase complex genes PB2, PB1, PA, and NP in conjunction with a reporter plasmid containing untranslated region (UTR) of NS1 segment upstream of the luciferase gene driven by the human RNA pol I promoter. Fig. 4.1.6E shows that there was a 6 fold increase in replicase activity in RNF43 siRNA-treated cells as compared to the NT siRNA treated cells. These findings strongly suggest that RNF43 is a strong inhibitor of virus replication and has antiviral properties.

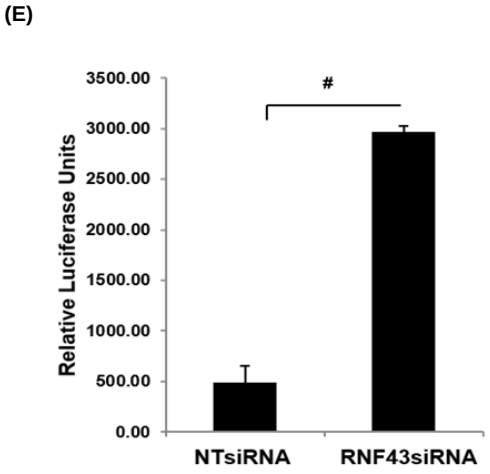

Fig. 4.1.6. Host factor RNF43 decreases IAV replication. (E) A549 cells, pretreated with either NT or RNF43 siRNA were transfected with plasmids encoding polymerase complex components (PA, PB1, PB2, NP) derived from PR8 (H1N1 virus) along with a reporter plasmid containing noncoding sequence from the NS1 segment of influenza A virus and luciferase gene driven by the Pol 1 promoter. The relative luciferase units were calculated after normalization with plasmid pGL-3 (Promega), which expresses Firefly luciferase that was transfected along. Results are shown as mean ± SD of three independent experiments. # indicates statistically significant difference at $p < 0.01$.

4.2 TO STUDY THE IMPLICATIONS OF NP/RNF43 INTERACTION IN NP DRIVEN P53 MEDIATED CELLULAR APOPTOSIS.

4.2.1 NP causes p53 stabilization through decreasing p53 ubiquitination by RNF43 ubiquitin ligase.

The anti-apoptotic characteristics of RNF43 protein gave us insights to ascertain the importance of the NP-RNF43 interaction in p53 stabilization and signaling. To assess p53 protein stability, 50µg/ml CHX, an inhibitor of protein synthesis in eukaryotic cells, was administered to A549 cells which were transiently transfected with different expression plasmids, pEGFPN1, pEGFP-NP with or without pCDNA3.1-RNF43-Flag-HA. The half-life of p53 protein was deduced by monitoring its decay over a period of 3 hours by western blot analysis. The half-life of p53 in the NP-GFP and RNF43-Flag-HA co-transfected cells was reduced to 60 min as compared to 120 min in the case of only NP-GFP transfected cells (Fig. 4.2.1A&B). This data clearly proves that p53 is getting stabilized in the NP transfected cells through the latter's interaction with RNF43 suggesting that NP-RNF43 interaction primarily leads to an enhanced p53 stabilization and accumulation.

Post-translational regulation of p53 via UPP mediated by different E3 ligases is a well-established phenomenon (Giaccia et al., 1998; Lee and Gu, 2010). Because of RNF43 protein's E3 ligase activity and a RING domain in its structure we were keen to look at its role in post-translational modification of p53 through ubiquitination and explore the possibility of p53 being a substrate of its ligase activity. To this end, we first checked p53 transcript levels in A549 cells where RNF43-HA was transiently expressed. Quantitative

RT-PCR analysis confirmed that RNF43 was unable to cause any significant alteration in p53 transcription (Fig. 4.2.1C).

We next examined p53 ubiquitination status. HEK293T cells were treated with 20uM MG132, proteasomal inhibitor, after co- transfection with plasmids expressing p53-GFP, Ub-myc, RNF43-HA, NP-GFP, MDM2-Flag in different combinations (Fig. 4.2.1D) followed by western blot analysis of harvested cells. We observed a significant increase in ubiquitination status of p53 under RNF43 overexpression. However, NP alleviated RNF43 caused p53 ubiquitination when transfected along with RNF43. MDM2 served as a positive control (Honda et al., 1997).

Next, we examined the ubiquitination status of p53 through Ni-NTA pull-down based ubiquitination assay. HEK293T cells were co-transfected with a combination of plasmids expressing p53-GFP, RNF43-HA, NP-GFP along with Ub-His expressing plasmid. Cells were harvested at 48 h.p.i after treatment with MG132, 5 hours prior to harvesting. Ubiquitinated p53 was pulled with Ni-NTA beads and analyzed through western blotting. As shown in Fig. 4.2.1E, the ladder of poly-ubiquitinated p53 is more abundant in RNF43 transfectants as compared to the control which suggests that RNF43 promotes p53 ubiquitination and degradation.

Moreover, an increase in p53 ubiquitination was observed when RNF43-Flag-HA was co-expressed with NP-GFP as opposed to a diminished level of ubiquitinated p53 in only NP-GFP transfected cells. This convincingly advocates the role of RNF43 in p53 regulation which is targeted by NP to stabilize p53 in IAV infected cells.

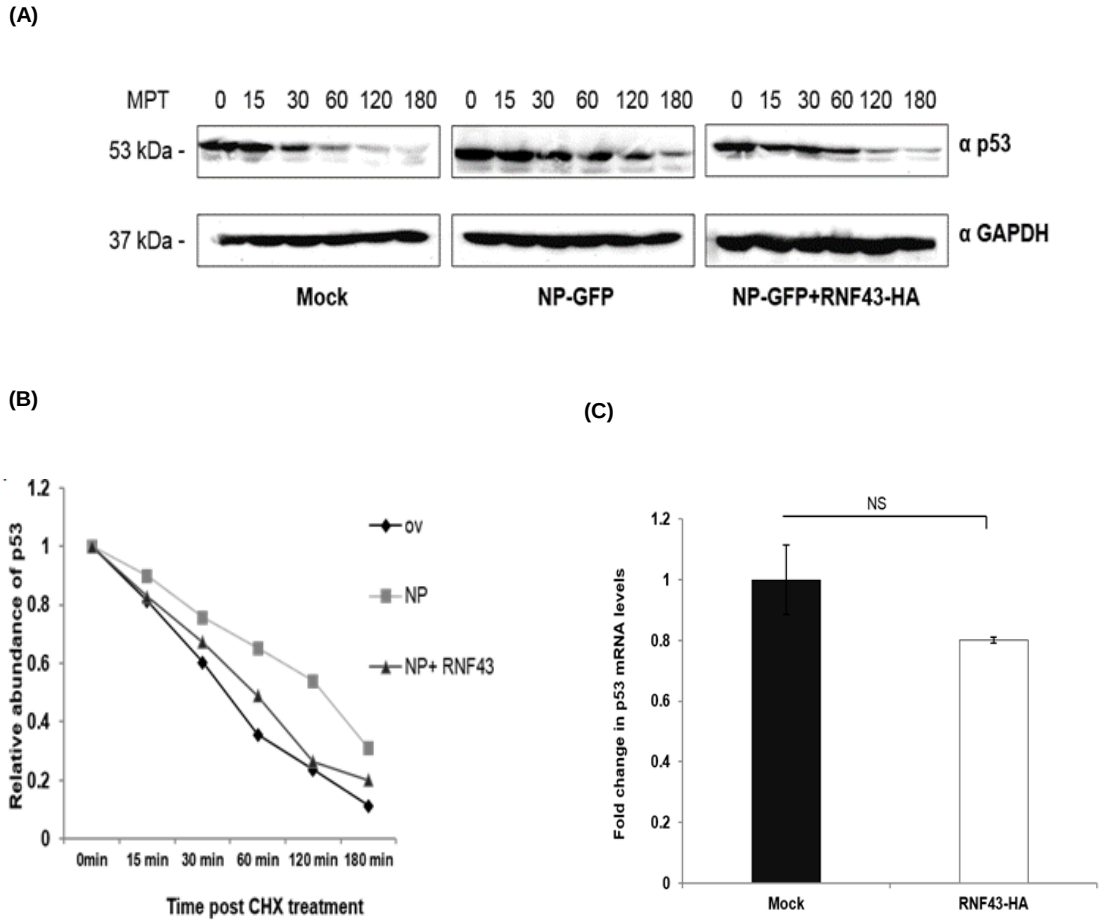

Fig. 4.2.1. NP interacts with RNF43 to stabilize p53 through compromised Ubiquitination of p53 by RNF43. (A& B) A549 cells were transiently transfected with plasmids pEGFPN1 (Mock), pEGFP-NP (NP-GFP) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA) for 48 hrs. The transfectants were treated with CHX at 50µg/ml for the indicated times (minutes) post-treatment (mpt) and subjected to western blot analysis using the indicated antibodies. The quantitative data of immunoblots is shown as line diagram after normalization with GAPDH expression levels. (C) A549 cells were transfected with plasmids pCDNA3.1 (Mock) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA). Cells were harvested after 48h post transfection and processed for mRNA quantification by qRT PCR analysis. The result is shown as mean ± SD of three independent experiments. NS indicates a non-significant statistical difference.

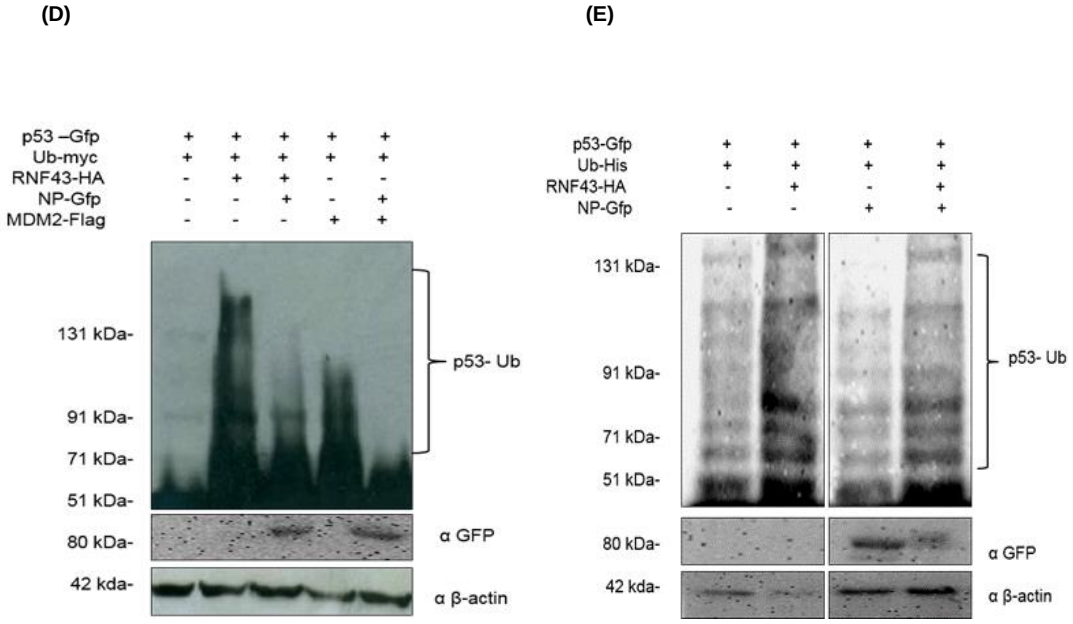

Fig. 4.2.1. NP interacts with RNF43 to stabilize p53 through compromised Ubiquitination of p53 by RNF43. (D) HEK293T cells were transiently transfected with a combination of indicated plasmids and incubated for 48 hours. The transfectants were treated with 20µg/ml MG132, 5 hours before harvest. The cell lysates were subjected to western blot analysis using the indicated antibodies. (E) HEK293T cells were transiently transfected with a combination of indicated plasmids and incubated for 48 hours. The transfectants were treated with 20µg/ml MG132, 5 hours before harvest. Ubiquitinated p53 (Ub-p53) was pulled down using Ni^{2+}-NTA-agarose beads and analyzed by western blotting with indicated antibodies.

4.2.2 RNF43 mitigates NP driven enhancement of p53 transcriptional activity.

Stabilization of p53 by NP prompted us to look into the probable role of NP/RNF43 interaction in an induction of p53 mediated cell functions. Tumor suppressor protein p53 is a transcription factor that regulates the expression of many genes that are crucial in mediating its tumor suppressing activity. Therefore, we checked a dose-dependent effect of NP on p21transcription which is under a direct control of p53 transcriptional activity (el-Diery et al., 1993). A549 cells were transiently co-transfected with p21- luciferase reporter plasmid containing the p53 binding site on its promoter together with indicated concentrations of pEGFP-NP and pcDNA-p53 and a control plasmid, Renilla luciferase pRL-TK. The p21 luciferase activity was measured and normalized to renilla luciferase activity. p21 is a direct target gene of p53 hence a dose-dependent increase in p21 luciferase activity as compared to the mock transfectant suggests an increased p53 transcriptional activity(Fig. 4.2.2A). To probe the significance of NP/RNF43 interaction in NP driven enhanced transcriptional activity of p53, using the similar approach, A549 cells were co- transfected with pEGFPN1or pEGFP-NP expression plasmid alone or together with RNF43-HA expressing plasmid. As speculated, co- expression of RNF43 with NP resulted in an approximate 0.2 fold decrease in p53 transcriptional activity as compared to only NP expressing cells (Fig. 4.2.2B).

We checked the mRNA levels of p21 in RNF43 knocked down HEK293T cells infected with PR8. As compared to NT siRNA treated cells, p21 mRNA levels were almost 10 fold higher in RNF43 depleted cells (Fig. 4.2.2C). p21 protein levels were also observed to be undergoing a notable decrease in the presence of RNF43 when co-expressed along with NP-GFP (Fig. 4.2.2D).

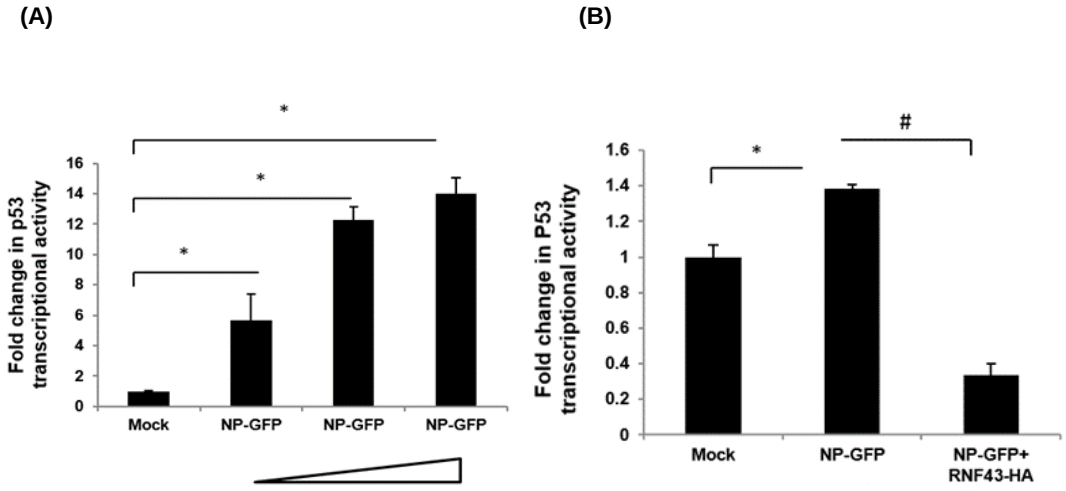

Fig. 4.2.2. RNF43 decreases NP driven increased p53 transcriptional activity and signaling in the cells. (A) A549 cells were transfected with the p21-Luc reporter plasmid, with or without growing amounts of pEGFP-NP (500ng, 750ng, 1µg) in conjunction with pcDNA-p53 and a control plasmid, Renilla luciferase pRL-TK. Luciferase activity of cell lysates of transfectants was analyzed. (B) A549 cells were transfected with the p21-Luc reporter plasmid, pcDNA-p53 and a control plasmid, Renilla luciferase pRL-TK along with plasmids pEGFPN1 (mock), pEGFP-NP (NP-GFP) and pCDNA3.1-RNF43-Flag-HA (RNF43-HA) in the indicated combinations. Luciferase activity was measured. Results in are shown as mean ± SD three independent experiments. * and # indicate statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively.

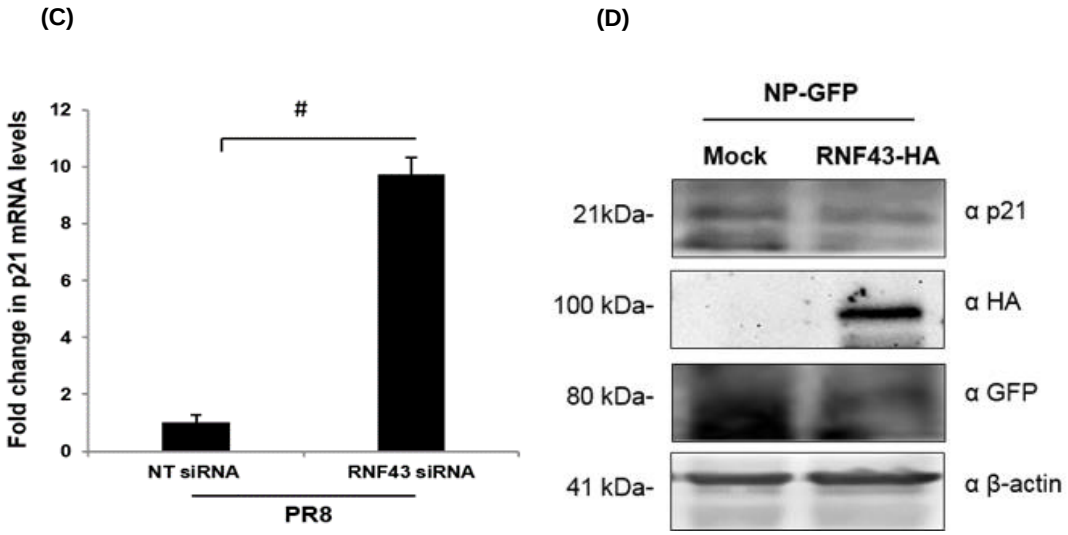

Fig. 4.2.2. RNF43 decreases NP driven increased p53 transcriptional activity and signaling in the cells. (C) HEK293T cells were treated with NT siRNA or RNF43 siRNA for 24h followed by PR8 IAV infection at 1 MOI. Cells were harvested at 24 h post infection and total RNA was extracted followed by p21 mRNA estimation with qRT-PCR. (D) A549 cells were transiently transfected with pEGFP-NP with or without pCDNA3.1-RNF43-Flag-HA and were harvested at 48 hours followed by SDS-PAGE. Western blotting was done using indicated antibodies. Results are shown as mean ± SD three independent experiments. # indicates statistically significant difference at $p < 0.01$.

Acetylation of p53 is an important step to enable p53 mediated transactivation of different factors towards cellular functions (Proietti et al., 2014; Vousden, 2002). We monitored the acetylation of p53 (Lys-382) at different time points post PR8 infection in A549 cells. Acetylation levels of p53 were observed to undergo a time-dependent change as shown in Fig. 4.2.2E. We further assessed the effect of RNF43 on p53 acetylation in IAV infected cells. To achieve the same, we transfected A549 cells with pCDNA3.1 (mock) and pCDNA3.1-RNF43-Flag-HA (RNF43-HA) plasmids followed by PR8 infection after24 hours. Cells were harvested after 24 hours of infection followed by western blot analysis.

A decrease in p53 acetylation in the cells ectopically expressing RNF43 (Fig. 4.2.2F) is likely to be the result of the concomitant increase in RNF43 mediated p53 ubiquitination.

Furthermore, A549 cells were infected with PR8 virus after being transfected with or without RNF43-Flag-HA expressing plasmid construct. 24 h.p.i intracellular protein levels of p53 and its regulated molecules, Bax, and Puma were inspected through western blot analysis. In RNF43 expressing cells, there was an evident decrease in the protein levels of p53 and its downstream effectors, Bax and Puma (Fig. 4.2.2G). The same outline was observed when pEGFP-NP was transfected in conjunction with pCDNA3.1 or pCDNA3.1-RNF43-Flag-HA plasmids (Fig. 4.2.2H).

p21, Bax and Puma are p53 regulated mediators of the latter's cellular functions (Benchimol, 2001; Vogelstein et al., 2000). These findings confirm the role of NP/RNF43 interaction in the activation of p53 signaling pathways.

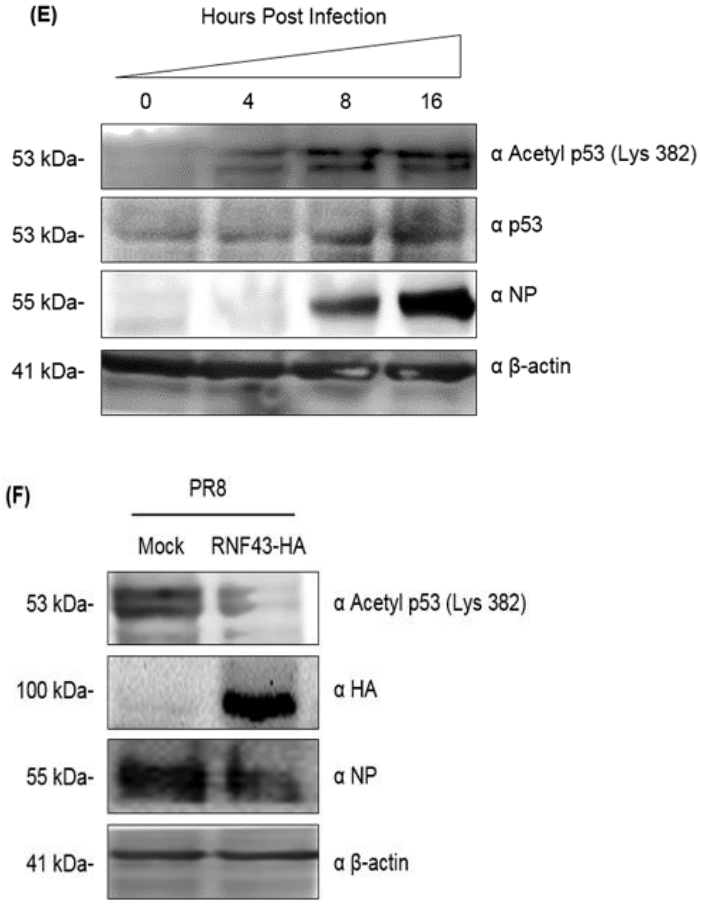

Fig. 4.2.2. RNF43 decreases NP driven increased p53 transcriptional activity and signaling in the cells. (E) A549 cells were seeded in a 6 well plate and were infected with PR8 virus at 1MOI. Cells were harvested at 0, 4, 8, and16 hours post infection, and subjected to western blotting with indicated antibodies. (F) A549 cells were transfected with pCDNA3.1 (mock) and pCDNA3.1-RNF43-Flag-HA (RNF43-HA) plasmids followed by PR8 infection, 24 hours post transfection. The cells were harvested after 24 hours followed by western blot analysis with anti-acetyl p53, HA, NP and β actin antibodies.

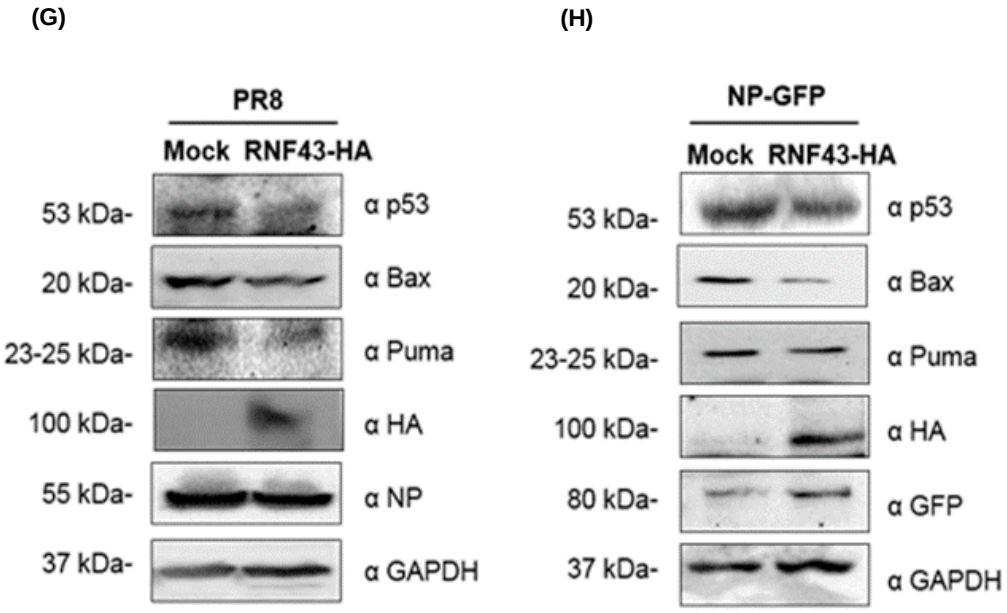

Fig. 4.2.2. RNF43 decreases NP driven increased p53 transcriptional activity and signaling in the cells. (G) A549 cells were transiently transfected with pCDNA3.1 (mock) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA) plasmid constructs and after 24 hrs of incubation, cells were infected with PR8 virus at an MOI of 1 for 24 hrs. (H) Similarly, A549 cells were transiently transfected with pEGFP-NP with or without pCDNA3.1-RNF43-Flag-HA and were incubated for 48 hours. (G-H) Cells were harvested and processed for western blot analysis with indicated antibodies.

4.2.3 RNF43 attenuates IAV NP induced cell death.

To probe the effect of RNF43 on the induction of apoptosis in IAV infected cells, flow cytometry based Annexin V FITC labeling was performed. A549 cells were treated with NT siRNA or RNF43 siRNA and after 24 hours cells were infected with IAV and subjected to annexin V staining. A remarkable 13% of total cell population showed annexin V staining in RNF43 siRNA treated IAV infected cells as against the 2.5% annexin V positive population in NT siRNA treated IAV infected cells. Thus, confirming the anti-apoptotic role of RNF43 in IAV infected cells (Fig. 4.2.3A).

The role of NP/RNF43 interaction in cell death induced by NP was further elucidated in NP microenvironment. 14% of pEGFP-NP transfectants displayed annexin V staining whereas RNF43 overexpression along with NP lowered the annexin V population to 10% (Fig. 4.2.3B).

To investigate the exclusive role of p53 in NP/RNF43 interaction governed apoptosis, p53 null human colon cancer cells, (HCT116 $p53^{-/-}$) were transfected with pCDNA3.1 or pCDNA3.1-RNF43-Flag-HA with or without pEGFPN1 or pEGFP-NP. Annexin V staining of pEGFP-NP transfectants showed no significant difference as compared to the vector control. Similarly, RNF43 co-expression did not cause any change in the annexin V staining profile of NP-GFP expressing cells (Fig. 4.2.3C). This highlights the critical importance of p53 for carrying out NP-induced apoptosis. Most importantly, it draws attention to the critical role of RNF43 in virus-induced p53 mediated apoptosis.

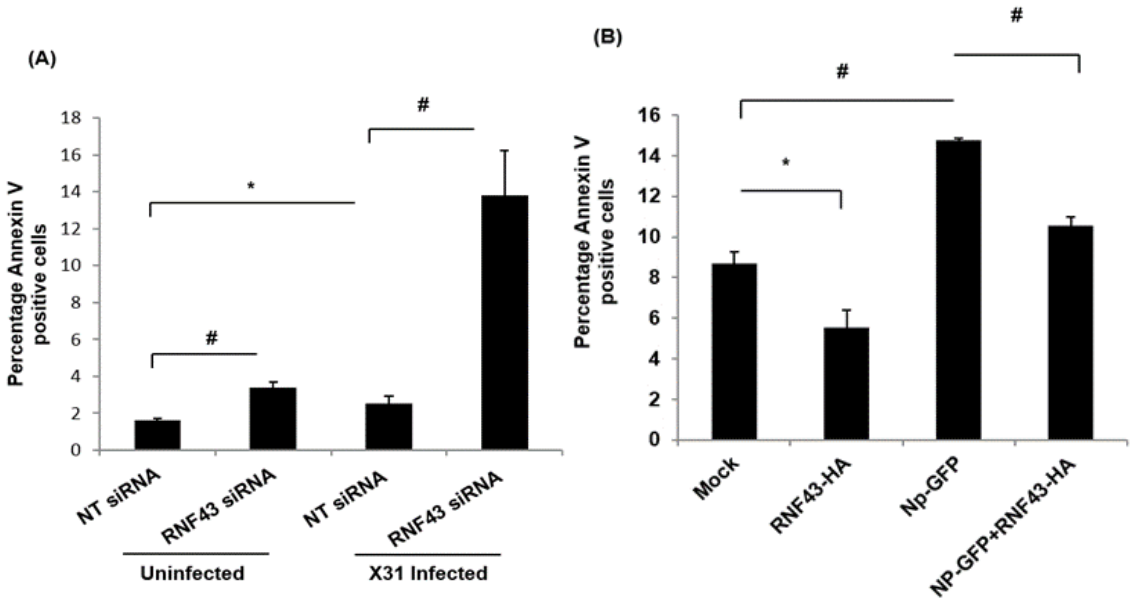

Fig. 4.2.3. RNF43 attenuates IAV NP-induced cell death. (A) A549 cells were treated with NT siRNA or RNF43 siRNA for 24h followed by X-31 IAV infection at 1 MOI. Cells were harvested at 24 h.p.i, stained with Annexin V FITC and subjected to flow cytometry. The percentage of Annexin V positive population is plotted on the graph. (B) A549 cells were transiently transfected with the indicated combinations of plasmids and 48 hours post-transfection cells were harvested and processed for flow cytometric analysis of Annexin V FITC stained population which is plotted on the graph. All graphs represent mean ± SD of three independent experiments. * and # indicate statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively.

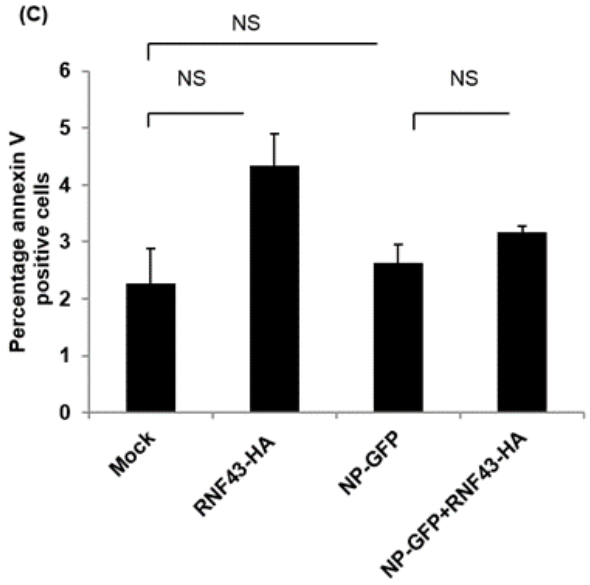

Fig. 4.2.3. RNF43 attenuates IAV NP-induced cell death. (C) $p53^{-/-}$ HCT116 cells were transiently transfected with the indicated combinations of plasmids and 48 hours post-transfection cells were harvested and processed for flow cytometric analysis of Annexin V FITC stained population which is plotted on the graph. Graph represent mean ± SD of three independent experiments. NS refers to non-significant difference.

4.3 DISCUSSION

Owing to their limited size viruses depend on host cellular machinery for their propagation. Similarly, IAV requires an intricate regulatory network of viral and cellular proteins. Ubiquitination is a key regulatory mechanism in the regulation of various cellular activities including signal transduction, transcription, membrane protein trafficking, apoptosis, autophagy and immune responses. Hence, it has become increasingly evident that the multifaceted ubiquitinylation process remains a prime target of a broad range of pathogens including viruses. Viruses may encode E3 ligases or deubiquitinases or may redirect the cellular E3s and deubiquitinases to modulate the ubiquitination status of substrates of choice (Randow and Lehner, 2009). We undertook this study to identify the cellular ubiquitinating or deubiquitinating enzymes that interact with NP of IAV. For this purpose, Yeast- two-hybrid (Y2H) screening was employed which remains one of the traditional yet efficiently reliable methods to identify interacting partners of specific proteins. Although, many high throughput screening methods including, genome-wide RNAi screens, mass spectrometry have recently been developed and utilized but there remain very few host factors that are common among these screens. The downsides of RNAi-based screening are the varying efficiency of knock down and miss of "hits" due to siRNA cytotoxicity. Besides, the Y2H library screening is still the most practical tool in the high-throughput identification of protein-protein interaction. Moreover, several interactions have been validated in the virus micro environment and their functional relevance deciphered (Sharma et al., 2011; Tripathi et al., 2013; Sharma et al., 2014).

Using this system we identified RNF43 as an interacting partner of NP. RNF43 is a member of RING finger family of E3 ligases that mark proteins for proteasomal degradation. Precisely,

E3 ligases, the highly specific mediator of ubiquitination, are extensively exploited by viruses to carry out replication and pathogenesis.

The present study for the first time provides evidence for NP and RNF43 interaction. The conserved nature of the NP–RNF43 interaction between PR8 and X-31 virus strains underlines its significance in viral replication and pathogenesis. We also report that IAV NP and RNF43 predominantly colocalize in the nucleus. IAV infection attenuated RNF43 mRNA as well as protein expression. The sequestration of RNF43 mRNA may well be explained by the known shut off mechanism adopted by many DNA and RNA viruses to inhibit the cellular macromolecular synthesis (Crawford et al., 1981; McGowan et al., 1982). In the case of influenza virus, degradation of preexisting cellular mRNAs leads to degradation of protein synthesis during infection (Inglis, 1982). To establish viral translation system, virus either causes direct protein degradation or target the cellular mRNAs by rendering them more susceptible to different nucleases that can be either cellular or encoded by the virus itself (Beloso et al., 1992). Interaction of viral proteins with the mRNA protecting molecules is another way adopted by the virus to cause cellular mRNA degradation. This shut off is also well established in large DNA viruses including Herpes Simplex Virus (HSV) infection that encodes virion host shut off (Vhs) protein that engages in interaction with cellular translation initiation factor eIF4H to exercise its mRNA degradase activity (Feng et al., 2005).

Overexpression of RNF43 counteracts IAV NP leading to a dose-dependent decrease in NP mRNA and vRNA. There was a drastic increase in viral replication under the influence of RNF43 knockdown. The decline in RNF43 mRNA and protein levels upon IAV infection correlates with the decrease in viral replication by RNF43. It substantiates its role as an

antiviral host protein. E3 ligases, highly specific mediators of ubiquitination, are extensively exploited by viruses to carry out replication and pathogenesis. It is of utmost importance to this study that many other viruses including human oncogenic papillomavirus, HIV and simian virus 40 also either target host ubiquitin ligases or encode ubiquitin ligases to divert their pathway of regulation by altering their specificity (Randow and Lehner, 2009; Arora et al., 2014; Hartmann et al., 2014). Influenza virus exploits the ubiquitination status of many cellular factors to evade host defense mechanisms and achieve maximum replication efficiency (Gack et al., 2009; Su et al., 2013) Thus, it is concluded that RNF43 is targeted by the virus for a strategic attenuation of RNF43 for efficient viral replication and pathogenesis.

Wang et al. illustrated the stabilization of p53 by NP through compromised MDM2-mediated ubiquitination (Wang et al., 2012). In our study, we found that NP-driven stabilization of p53 was impeded by RNF43 via a decreased p53 half-life and a noteworthy augmentation of the ubiquitinated p53 levels in the RNF43 microenvironment. This can be attributed to the posttranslational regulation of p53 by RNF43 through targeting p53 for ubiquitination. These results are in coherence with the fact that RNF43 interacts with p53 (Shinada et al., 2011) and the siRNA caused down regulation of RNF43 led to a significant increase in p53 protein expression in HepG2 and SMMC-7721 cells (Xing et al., 2013). Several E3 ligases along with MDM2 are known to regulate p53 ubiquitination, for example, Pirh2, MdmX, HAUSP, ARF, COP1 and ARF-BP1 (Giaccia et al., 1998; Lee and Gu et al., 2010) Thus, the current study unravels a novel mechanism of p53 regulation via RNF43. Although, a separate study can be undertaken to explore RNF43-mediated p53 regulation but our study provides compelling evidence that RNF43 targets p53 for ubiquitination.

P53 is a transcription factor that controls the expression of many genes including p21, BAX, PUMA which are the known mediators of p53 induced apoptosis in cells (Vogelstein et al., 2000). We speculated that NP must target anti-apoptotic RNF43 protein to induce p53 signaling and apoptosis in IAV infected cells which could prove out to be another mode of apoptosis induction by NP which has recently been identified as one of the anti-apoptotic proteins of IAV. We examined the protein expressions of p53, Bax, and Puma which were shown to be undergoing a profound decrease under the effect of RNF43 over expression in IAV and NP microenvironment. We analyzed the effect of NP on p53 dependent p21 transcription and found a dose-dependent increase in the transcription. It was further found out that RNF43 expression along with NP, attenuated p21 transcription. Furthermore, RNF43 prevented NP induced apoptosis in the cells. However, to deduce the importance of NP/RNF43 interaction in p53 mediated apoptosis we conducted the experiment in p53 null cell lines, HCT116 $p53^{-/-}$. To our astonishment, there was no significant alteration in apoptosis in either NP or RNF43 transfectants. Altogether, our data explains the underlying importance of NP/RNF43 interaction in the induction of p53 mediated cellular apoptosis in IAV infected cells.

Apoptosis induction is one of the hallmarks of IAV infection. IAV being a cytolytic virus induces apoptosis in numerous cell types (Takizawa et al., 1993; Hinshaw et al., 1994; Ludwig et al., 2006). It is considered to be host's defense against the virus but at the same time it is hypothesized that the appropriately timed apoptosis controlled by IAV is important for efficient viral replication (Stray and Air, 2001; Mclean et al., 2009) Accumulation of p53, a critical mediator of apoptosis, in IAV-infected cells is a well-documented phenomenon and in fact is reported to accumulate in a biphasic pattern (Shen et al., 2009).

Our study also demonstrates an accumulation of p53 in IAV-infected cells along with an increasing acetylation levels that corresponds to an increased p53 activity. Turpin et al. (Turpin et al., 2005) demonstrated that p53 is required for IAV-induced cell death but at the same time was important in IFN induction.

However, IFN-dependent antiviral response of p53 is reported to be independent of its pro-apoptotic functions (Munoz-Fontella et al., 2011). In the midst of these conflicting notions regarding the role of p53 in IAV replication and pathogenesis, our study eloquently proves the critical importance of p53 in NP-mediated apoptosis. We previously reported the role of NP in inducing host cell death (Tripathi et al., 2013). Stabilization and accumulation of p53 by NP can be accredited to the pro-apoptotic nature of NP, which, in this case, hijacks RNF43 and withholds its regulatory effects on p53. Together with these findings, the data described here points to a model in which IAV increases p53 downstream signaling and apoptosis by suppressing the RNF43-mediated ubiquitination of p53 (Fig. 4.3.1). Hence, the interaction of NP with RNF43 to modulate p53 ubiquitination is a proof of direct mechanism of apoptosis induction by IAV.

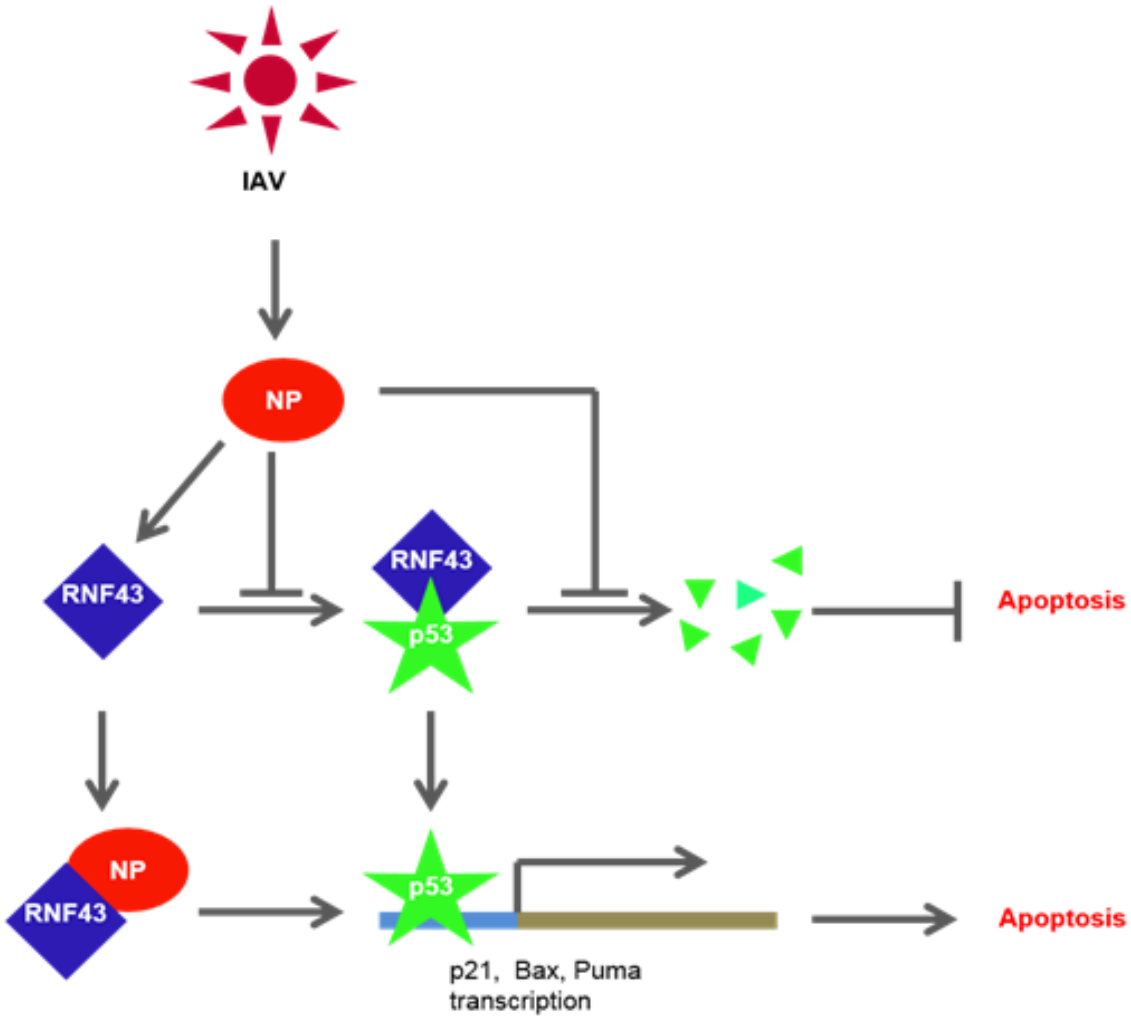

Fig. 4.3.1.The proposed model for the role of NP/RNF43 interaction in the regulation of p53 mediated cell death by IAV NP. The proposed model for the regulation of p53 mediated cell death by IAV NP. p53 is proposed to be regulated by RNF43 through ubiquitylation resulting in its destabilization. NP interacts with RNF43 thereby preventing ubiquitination of p53 and causing its stabilization and accumulation inside the infected cell resulting into activation of p53 signaling cascade including p21, Bax, Puma and eventually results in cell death in IAV infected cell.

CHAPTER 5
Results and Discussion II

5.1 TO INSPECT THE EFFECT OF RNF43 ON INFLUENZA VIRUS MEDIATED CELL CYCLE ARREST IN HOST.

5.1.1 RNF43 enhances expression of S Phase promoting proteins in IAV infected cells.

A study done by Xing et al on the reversing effect of RNF43 on hepatocellular carcinomas illustrated the down-regulation of various cell cycle markers including pRb, CDK4, CDK2 on siRNA-mediated silencing of RNF43 (Xing et al., 2013). This finding correlated with our finding of RNF43 driven decrease in p21 transcription in IAV infected cells. To further explore the suspected role of RNF43 in IAV induced cell cycle arrest we checked the levels of various cell cycle markers including Rb, pRb, E2F1, and CDK4. To begin with, we transfected huh7 (hepatocellular carcinoma) cells with either pCDNA3.1 or pCDNA3.1-RNF43-Flag-HA followed by PR8 infection. There was a marked difference in the pRb and E2F1 protein levels in RNF43 transfected and PR8 infected cells when compared to only PR8 infected cells (Fig. 5.1.1A). Exponentially growing cell cultures are generally asynchronous. Synchronization is particularly useful for examining a particular cell cycle–regulated event. Thus, we proceeded to study cell cycle arrest in synchronized A549 cells through double thymidine block at G1/S phase. For this, A549 cells were first transfected with a various combination of plasmids and were synchronized through double thymidine block. Cells were harvested at different time points post synchronization and were processed for western blotting. Densitometry analysis of the western blots revealed that co expression of RNF43 with NP stabilized CDK4 levels as compared to the only NP expressing cells (Fig.

5.1.1B). Similarly, pRb was evidently more in cells expressing RNF43 along with NP (Fig. 5.1.1C).

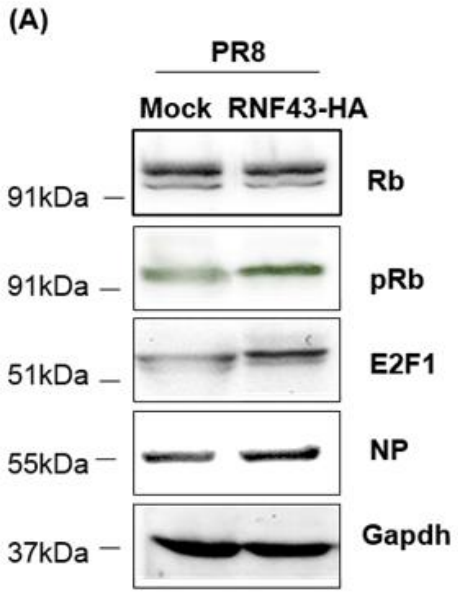

Fig. 5.1.1. RNF43 rescues cell cycle arrest in IAV infected cells. (A) Huh7 cells were transfected with pCDNA3.1 (mock) and pCDNA3.1-RNF43-Flag-HA plasmids. 24 hours post-transfection, the cells were infected with PR8 virus and were harvested for western blot analysis with anti-Rb, anti-pRb, anti E2F1, anti-NP, and anti-GAPDH antibodies, 24 hours post infection.

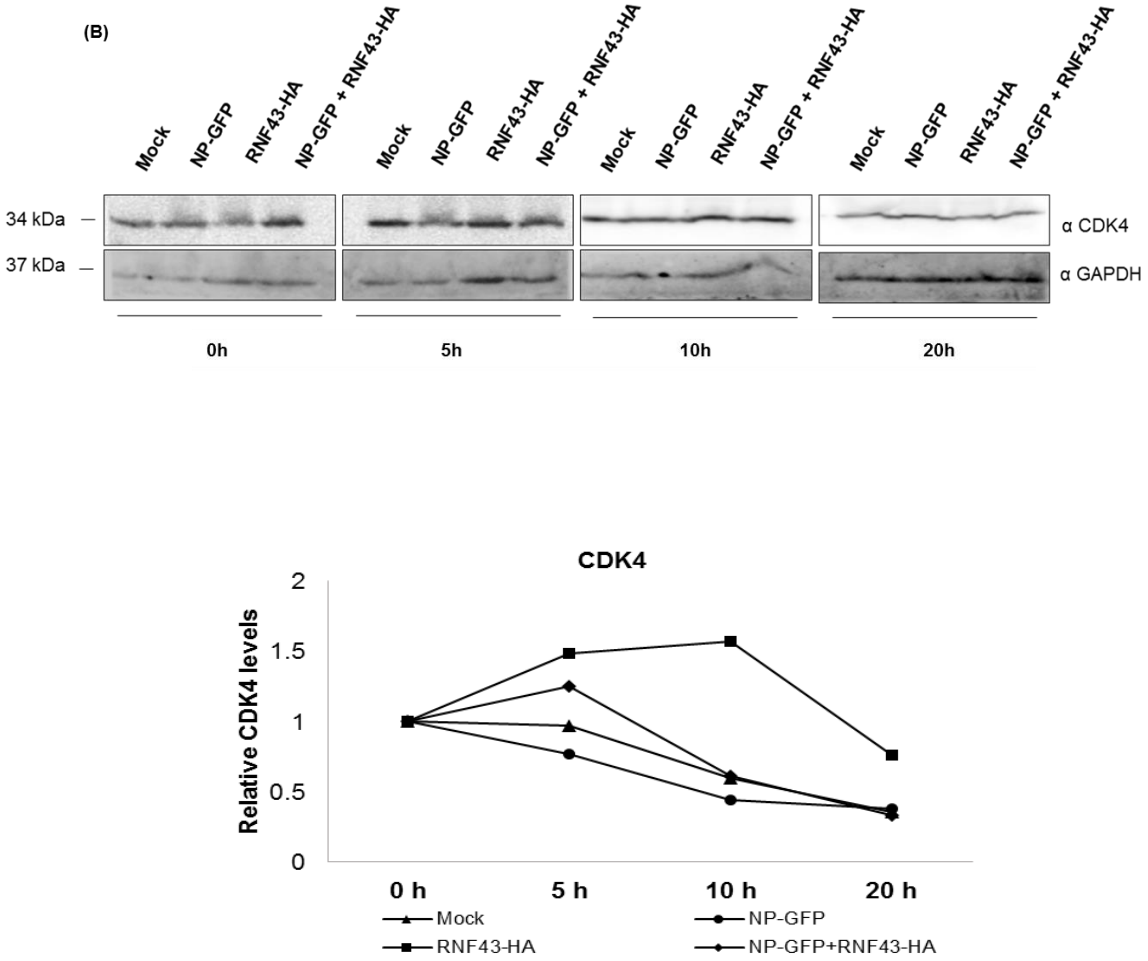

Fig. 5.1.1. RNF43 rescues cell cycle arrest in IAV infected cells. (B) A549 cells were transiently transfected with the indicated combinations of plasmids and 24 hours post transfection cells were subjected to double thymidine block for indicated time periods followed by western blotting with anti-CDK4 and anti-GAPDH. The quantitative data of immunoblots for CDK4 is shown as line diagram after normalization with GAPDH expression levels.

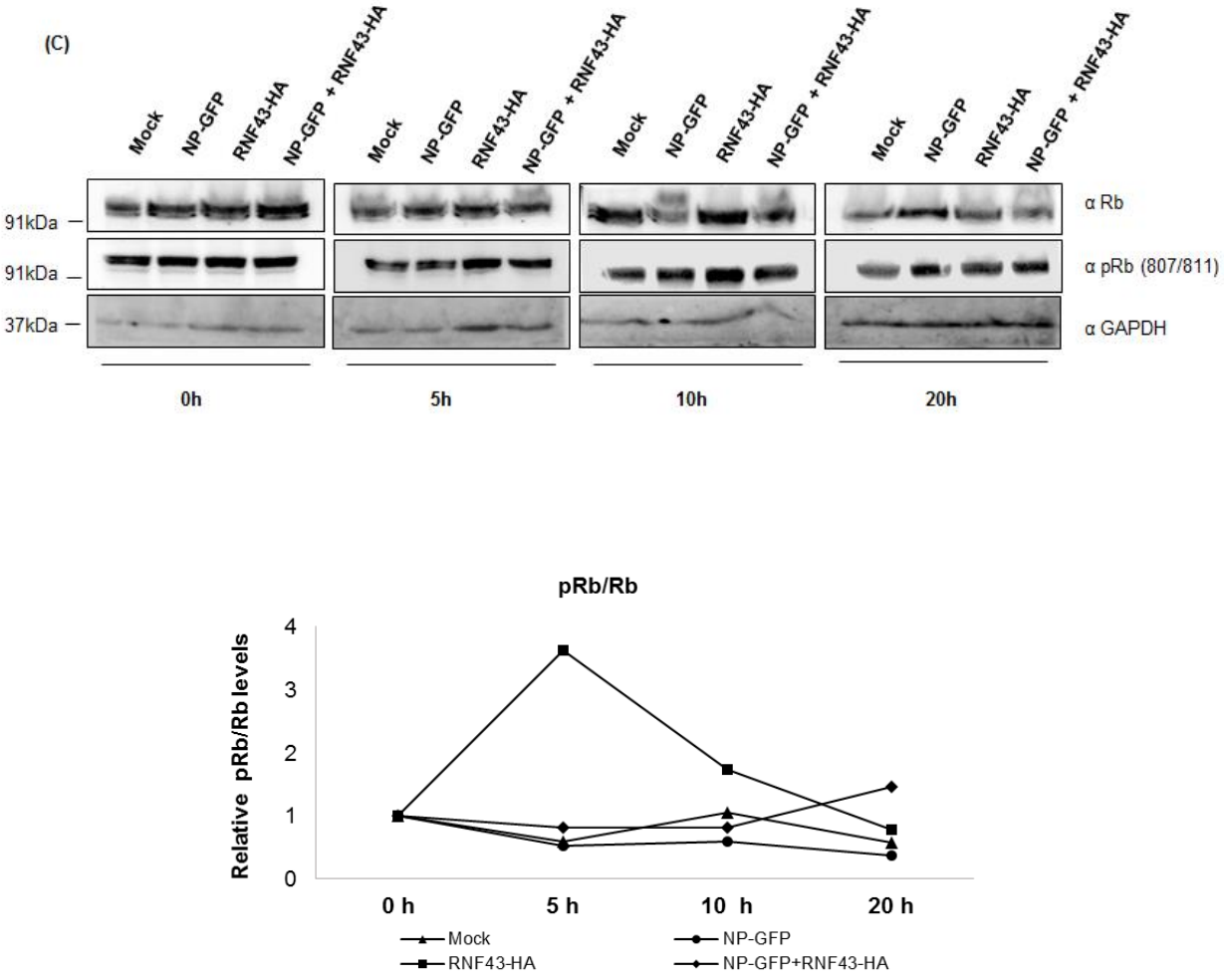

Fig. 5.1.1. RNF43 rescues cell cycle arrest in IAV infected cells. (C) A549 cells were transiently transfected with the indicated combinations of plasmids and 24 hours post-transfection cells were subjected to double thymidine block for indicated time periods followed by western blotting with anti-Rb, anti-pRb, and anti-GAPDH. The quantitative data of immunoblots for pRb/Rb is shown as a line diagram. The data was quantified by first normalizing pRB and Rb levels with GAPDH expression levels followed by pRB normalization with Rb.

5.1.2 RNF43 facilitates entry of cells into S-phase

Further, we decided to perform BrdU incorporation analysis through confocal imaging to conclude the effect of NP/RNF43 interaction on cell cycle progression. BrdU is a synthetic thymidine analog which gets incorporated during DNA replication instead of thymidine during S phase and hence a commonly used method to study cell cycle progression. HEK293 cells were transfected with empty vector, pCDNA3.1 (mock), pCDNA3.1-Myc-NP and pCDNA3.1-RNF43-Flag-HA and incubated for 48 hours. Transfected cells were labeled with 10 μM BrdU, using 5-Bromo-2'-deoxy-uridine labeling and detection Kit I (Sigma Aldrich), for one hour before harvesting. Confocal microscopy of HEK cells showed a significantly lower incorporation of BrdU label in NP expressing cells as compared with the mock (Fig. 5.1.2A). Besides, the number of BrdU-positive cells were also significantly lower in NP expressing cells (~ 4 fold, $p<0.05$) (Fig. 5.1.2B). Nevertheless, the co-expression of RNF43 with NP rescued the cells from the cell cycle arrest caused by NP construed by a remarkable increase in BrdU-positive cells (~ 4 fold, $p<0.01$) (Fig. 5.1.2B). The subdued expression of NP in RNF43 co-expressing cells is in accordance with the previous results wherein ectopic expression of RNF43 led to a dose-dependent decrease in NP transcripts levels.

These observations strongly suggest that NP has a definite role to play in IAV driven cell cycle arrest and NP/RNF43 interaction is partly responsible to mediate this viral strategy.

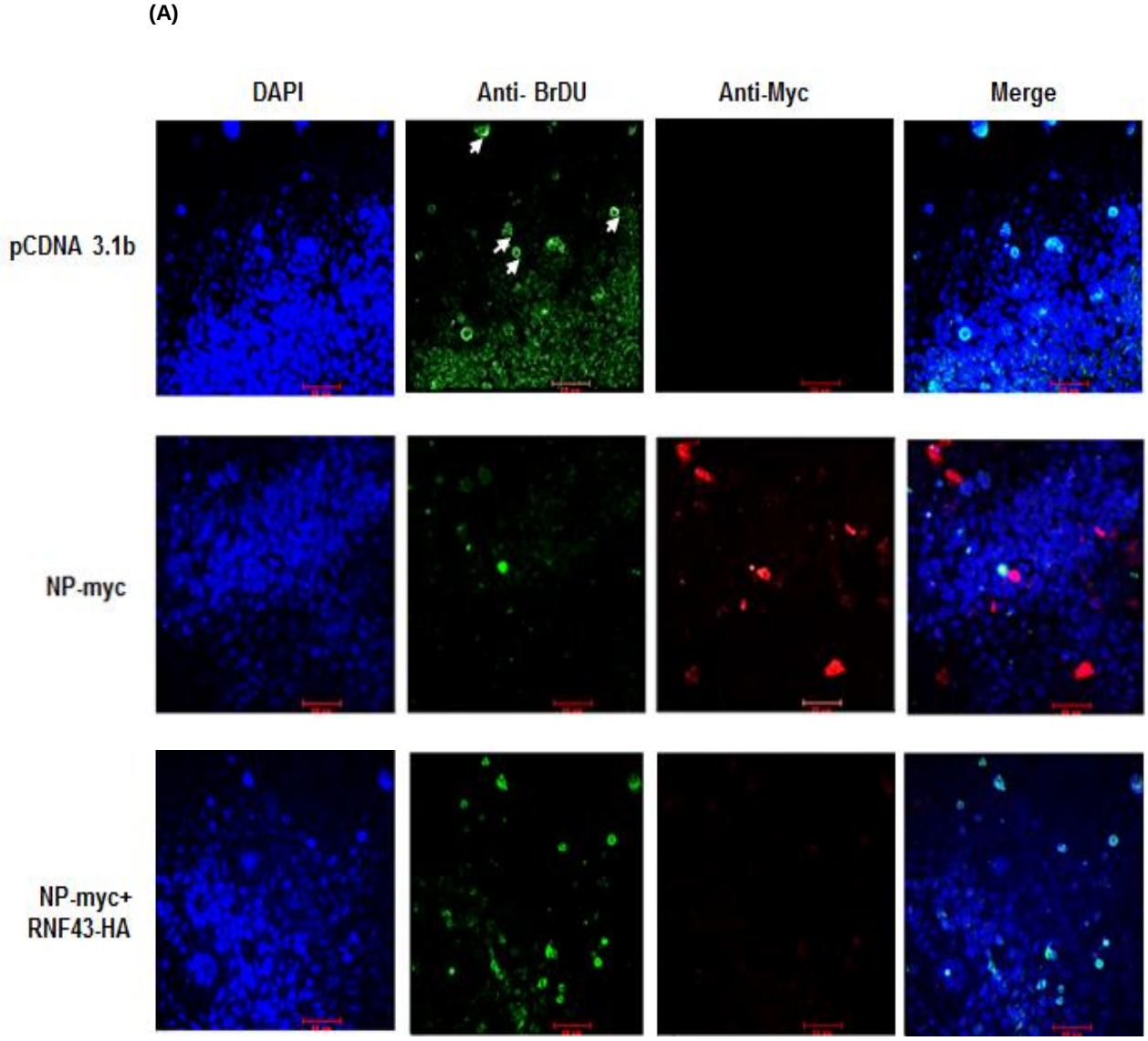

Fig. 5.1.2. RNF43 facilitates S-phase entry of cells. (A) HEK293 cells grown on cover slips were transfected with pCDNA3.1 (mock), pCDNA3.1-Myc-NP and pCDNA3.1-RNF43-Flag-HA. Before fixing at 48 hours post transfection, cells were treated with 10 μM BrdU for one hour. Cells were stained using DAPI for the nucleus, goat anti-mouse secondary antibody conjugated to FITC for BrdU (green) and anti-rabbit secondary antibody conjugated to Alexa-594 for Myc (red) and observed under confocal microscope. Panels are labeled for their respective staining, BrdU (green), NP (red), nucleus (blue).

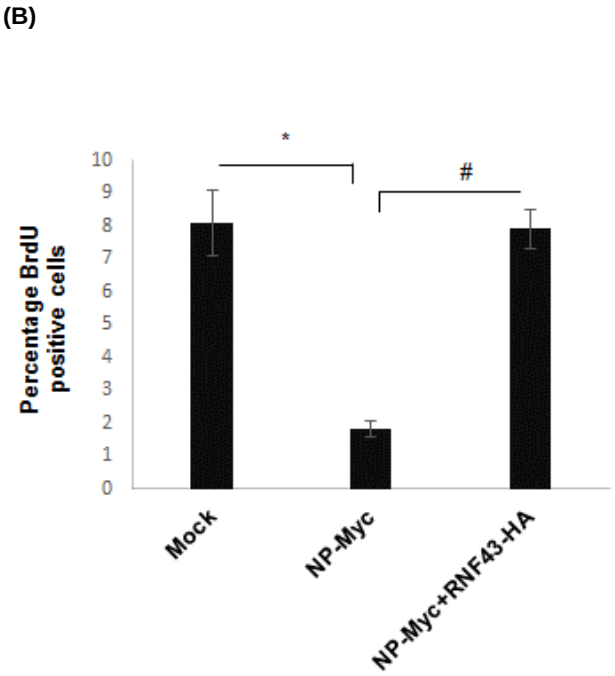

Fig. 5.1.2. RNF43 facilitates S-phase entry of cells. (B) The percentages of BrdU-positive cells are shown as bar diagrams of respective panels. The graph represents mean ± SD of three independent experiments. * and # indicate statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively.

5.2 DISCUSSION

The cell cycle is a series of events taking place in a cell that leads to cell division and growth. The cell cycle is broadly characterized by interphase and M phase. Cells stay in the interphase for the longest duration which is composed of G1, S, and G2 phases, before they can actually enter cell division through mitosis in the M phase. The first gap phase (G1), is characterized by a high translation rate as components required for DNA synthesis are generated. Cells may enter gap 0 (G0) during G1 phase, if mitogenic stimulants are not present where the cell metabolic rate is low or in the case of terminally differentiated cells like neurons. G1 is followed by synthesis (S) phase, where the cellular genome is replicated, and gap 2 (G2) phase. Finally, in mitosis (M) phase, the DNA chromatids and cell contents are split into two daughter cells. The cell cycle is a tightly regulated process marked by various cell cycle checkpoints and regulators including both positive and negative regulators. G1 phase of cell cycle is regulated by cyclin-Cdk complexes and the phosphorylation of the downstream retinoblastoma (Rb) protein. The cyclin D-Cdk4/6 complex is responsible for G1-phase progression, while the cyclin E-Cdk2 complex is required for S-phase entry and DNA replication (Obaya and Sedivy, 2002).

Viruses support their life cycle very often by altering the host cell cycle. Hijacking hosts' cell cycle is a very frequently observed phenomenon seen in the case of various DNA and RNA viruses (Song et al., 2000; Kalejta and Shenk, 2003; Castillo and Kowalik, 2004; Katz et al., 2005; Yuan et al., 2007; Chen et al., 2010). In the case of retrovirus HIV, Vpr protein is known to cause cell death following cell cycle arrest (Stewart et al., 1997). Thus, there is a definite cross-talk between apoptosis and cell cycle arrest. RNF43 being a growth promoting and anti-apoptotic protein has been studied for its role in cell cycle progression

and it was discovered that siRNA-mediated knock down of RNF43 led to a noteworthy decrease in various cell cycle markers including pRb, CDK4, CDK2 (Xing et al., 2013). Although NP has previously not been reported to play a direct role in IAV mediated cell cycle arrest, however, there are confirmed reports of NP caused cellular apoptosis. During our study, we observed an increased p21 expression after ectopic expression of NP which was subdued by transiently co-expressing RNF43. These observations prompted us to further explore the role of NP/RNF43 interaction in carrying out cell cycle arrest. This hypothesis was supported by a noteworthy increase in pRb and E2F1 levels in RNF43 expressing PR8 infected HUH7 cells. We confirmed this hypothesis in thymidine block synchronized A549 cell population. We observed that at 5 hours post release there was a drastic increase in the cellular pRb levels and it corresponded to the high CDK4 levels at the same time point. Interestingly, this sharp rise in the levels of G1/S transition markers was the lowest in NP expressing cells. As expected, the levels of CDK4 and pRb were the highest in RNF43-HA expressing cells. To further establish the cell cycle arrest following NP expression, we proceeded with BrdU incorporation analysis which established that NP causes G0/G1 arrest, where in the NP expressing HEK293 cells showed the least number of BrdU-positive cells. This concluded the NP driven halt in the cell cycle progression at G0/G1 phase.

As reported in the first objective of this study, an increase in p21 expression under NP microenvironment was observed. P21 being under the direct control of p53 suppresses the formation of cyclin E-cdk2 complex that further affects cyclin D1-CDL4/CDK6 complex resulting in the decreased amount of pRb which is important for G1/S transition. The similar hypothesis was proposed by He and coworkers (He et al., 2010). They showed in their study that the G0/G1 phase is favorable for influenza virus replication. Following this study, NS1

was identified as the IAV protein inducing G0/G1 cell cycle arrest through its interaction with Rho A host protein (Jiang et al., 2013). The delay in cell cycle by RNA viruses is also proposed to be a viral strategy to favor assembly since the ER and Golgi apparatus, the organelles essential for translocation of HA, NA and M2 to the cell surface, disassemble into vesicles and cluster during mitosis (Warren, 1993; Lowe et al., 1998). Lastly, one of the first effects of p53 expression is a halt in the cell cycle division by stimulating the expression of p21WAF/CIP1, an inhibitor of cyclin-dependent kinases (CDKs). Apoptosis is another aspect of p53 network mediated by several downstream effectors of p53 including Bax, PUMA etc. (Vogelstein et al., 2000). Thus, our results convincingly prove that along with apoptosis induction, NP/RNF43 interaction is also directed towards causing cell cycle arrest in IAV infected cells.

CHAPTER 6
Conclusion and Future Directions

6.1 Conclusion

Influenza virus remains a major public health threat globally. IAV causes a mild respiratory illness in humans of different age groups. The severity of the disease escalates in the immunocompromised individuals and may result in death in many cases. The biology of influenza genome confers it an evolutionary advantage through constant reassortment and hence the threat of influenza virus pandemics has intensified over the last decade with the emergence of highly resistant virulent strains. The prevalence of NA and M2 inhibitor resistant strains of IAV calls for immediate steps to be taken in order to contain the virus. One of the most promising strategies remain until now is to target the host which is relatively evolutionary static, thus reducing the odds of developing resistance. Due to the minimal genetic material of IAV, the virus remains highly dependent on the host and presents a perfect example of an obligate intracellular parasite. In general, unceasing virus-host interactions throughout virus infection constitute the viral as well as the host strategy, depending upon the outcome of the interaction. A variety of studies are underway to understand these complex molecular interactions and discover novel anti-viral targets based on these interactions.

Ubiquitination and deubiquitination are high-speed processes regulating rapid switch between the active and dormant state of a protein. UPP is paramount in early steps of influenza virus infection. NP is reported to undergo monoubiquitination and deubiquitination of NP results in a decreased viral replication. However, a lot more remains to be explored in terms of UPP and IAV pathogenesis especially due to the fact that around 600 cellular E3 ubiquitin ligases are responsible for maintaining the substrate specificity in the ubiquitination process.

In our study, we discovered the interaction of the structural protein of IAV, NP with the host E3 ubiquitin ligase RNF43. The first part of the study was dedicated to study the interaction of NP/RNF43 and to functionally characterize the interaction. We were able to show the interaction to be conserved between two different viral strains, PR8, and X-31. Confocal imaging of NP and RNF43 at different time points post infection confirmed their co-localization inside the nucleus of the infected host cells. Our results very eloquently prove the anti-viral role of RNF43 wherein RNF43 abetted virus replication construed by the apparently decreased NP mRNA and vRNA levels. The direct proof of the antagonistic effect of RNF43 on IAV replication is provided by the enhanced virus replication after siRNA-mediated silencing of RNF43 in a mini-replicon system. Further characterization of NP/RNF43 interaction revealed that the interaction is directed towards stabilization of p53 in IAV infected host cells. An enhanced ubiquitination of p53 under RNF43 was apparently inundated by NP that was conducive to an enhanced p53 transactivation and p53 signaling concluded by the enhanced levels of P21, Bax, and Puma, the downstream effectors of p53. The physiological outcome of the interaction is the induction of apoptosis in the host cell that was confirmed in X-31 IAV strain as well as in NP microenvironment. Hence, it is concluded that RNF43, a member of the RING finger family of ubiquitin ligases, displays anti-apoptotic and antiviral activities as it counteracts IAV apoptotic effects induced in the virus-infected cells.

In the second part of the study, we have shown the putative role of NP/RNF43 interaction in IAV mediated cell cycle arrest. Although. Until now, there is no direct report stating the role of NP in cell cycle arrest in the infected host cell but through our study, we prove a definitive role of NP in causing G0/G1 phase arrest. The elevated expression levels of the markers of

G1/S transition including pRB, E2F1 and CDK4 were observed in the cells co-expressing RNF43 along with NP. The hypothesis was further strengthened by the BrdU incorporation assay. It provides concrete affirmative results showing that the number of BrdU-positive cells being relatively low in NP expressing Cells. Co-expression of RNF43 with NP led to an increase in S phase entry concluded by the number of BrdU positive cells. Hence, we conclude that the primary effect of NP/RNF43 interaction is the enhanced p53 signaling which is achieved through p53 stabilization by NP through a strategic attenuation of RNF43. Since p53 is a regulator of many downstream pathways mainly apoptosis and cell cycle progression, the resulting cell cycle arrest and apoptotic cell death are argued to be a repercussion of the former's enhanced transactivation.

This study fills the missing link in the virus-induced apoptosis and cell cycle arrest in the host cells. The significance of the study lies in the fact that it reports a viral strategy for its propagation via the interaction with a cellular factor. Most importantly, compelling evidence is provided to prove that RNF43 polyubiquitinates p53 which further leads to its destabilization resulting in a decreased induction of the p53 apoptotic pathway.

This is an overwhelming finding in itself and suggests RNF43 could be a potential pharmacological target namely in developing anti- influenza interventions and also in the cancer field. The feasibility of RNF43 as viable antiviral intervention is supported by the successful phase 1 trial of a combination peptide vaccine for colorectal cancer, containing peptide sequence of RNF43 (Hazama et al., 2014). The risk of an adverse immune response of RNF43 derived peptide vaccine in normal cells is seemingly low and is well tolerated without any adverse effects (Hazama et al., 2014). Moreover, potent cytotoxic T lymphocyte response (CTL) was observed which is known to be antiviral. Although, the effect of NP-

RNF43 interaction on innate and adaptive immune evasion of IAV is yet to be established. Nonetheless, mutations in CTL epitope region of NP of different influenza viruses is well studied. These types of mutations are observed in chronically infecting influenza viruses and constitutes one of the many viral strategies to evade immune recognition by virus-specific T cells (Berkhoff et al., 2005; Horst et al., 2011). Therefore, targeting NP may have an immediate response but does not provide a long term broad spectrum solution to the problem of ever-evolving influenza virus. To tackle this, we propose the targeting of host factors which are less mutable and are more evolutionary static.

6.2 Future Directions

The study undertaken in this thesis paves the way for many future studies. Few of the studies and experiment that can follow up this thesis are:

1. To determine the interacting domains of influenza A virus Nucleoprotein and cellular E3 ligase RNF43.

2. To examine the conserved nature of amino acid sequence in the interacting domains of IAV NP and RNF43 in various IAV strains.

3. To observe the effect of IAV NP siRNA treatment on RNF43 expression and functions during IAV infection.

4. To analyze the effect of RNF34 siRNA on the IAV mediated transcriptomic modulation in the host.

CHAPTER 7
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CHAPTER 8
Appendix

PUBLICATIONS

1. Nailwal H, Sharma S, Mayank AK, Lal SK (2015) The nucleoprotein of influenza A virus induces p53 signaling and apoptosis via attenuation of host ubiquitin ligase RNF43. *Cell Death Dis.* 6, e1768.
2. Nailwal H, Kamra K, Lal SK (2014) H7N9: A killer in the making or a false alarm? *Can J Microbiol* 60, 425–9.

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The nucleoprotein of influenza A virus induces p53 signaling and apoptosis via attenuation of host ubiquitin ligase RNF43

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The interplay between influenza virus and host factors to support the viral life cycle is well documented. Influenza A virus (IAV) proteins interact with an array of cellular proteins and hijack host pathways which are at the helm of cellular responses to facilitate virus invasion. The multifaceted nature of the ubiquitination pathway for protein regulation makes it a vulnerable target of many viruses including IAV. To this end we conducted a yeast two-hybrid screen to search for cellular ubiquitin ligases important for influenza virus replication. We identified host protein, RING finger protein 43 (RNF43), a RING-type E3 ubiquitin ligase, as a novel interactor of nucleoprotein (NP) of IAV and an essential partner to induce NP-driven p53-mediated apoptosis in IAV-infected cells. In this study, we demonstrate that IAV leads to attenuation of RNF43 transcripts and hence its respective protein levels in the cellular milieu whereas in RNF43 depleted cells, viral replication was escalated several folds. Moreover, RNF43 polyubiquitinates p53 which further leads to its destabilization resulting in a decrease in induction of the p53 apoptotic pathway, a hitherto unknown process targeted by NP for p53 stabilization and accumulation. Collectively, these results conclude that NP targets RNF43 to modulate p53 ubiquitination levels and hence causes p53 stabilization which is conducive to an enhanced apoptosis level in the host cells. In conclusion, our study unravels a novel strategy adopted by IAV for utilizing the much conserved ubiquitin proteasomal pathway.
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Influenza A virus (IAV) by far remains the most important of all respiratory viruses, causing severe morbidity and mortality every year.[1] Nucleoprotein (NP) of IAV is a viral RNA genome-encapsulating structural protein that has been implicated in various other indispensable activities for virus replication and pathogenesis-like intracellular trafficking of the viral genome, viral RNA replication, virus assembly[2,3] via its interaction with a plethora of cellular factors like cytoskeleton scaffolding protein α-actinin-4, nuclear import receptor α importin, nuclear export receptor CRM1, DEAD-box helicase BAT1/UAP56 and cytoskeletal element F actin.[4–8]

The evolution of host–microbe interaction is mediated through the orchestration of different viral and host signaling pathways. Similarly, IAV requires an intricate regulatory network of viral and cellular proteins to accomplish successful replication.[9] One of the pathways that have been shown to be maneuvered by the virus is the ubiquitin (Ub) proteasomal pathway (UPP). UPP is a multi-enzyme cascade that involves the sequential action of three different enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase. A completely different class of proteins, known as deubiquitinases, reverses this process by removing the Ub molecules from target proteins.[10] With 400 putative E3 ligases encoded by the mammalian genome, the receptor specificity is maintained by these proteins and hence, are heavily exploited by viruses.[11] IAV protein NS1 is reported to target ubiquitin ligase, TRIM 25 to escape RIG1 recognition[12] and M1 protein reportedly interacts with E3 ligase, Itch.[13] Moreover, recently it has been discovered that NP stabilizes the tumor suppressor protein, p53 through its decreased ubiquitination by ubiquitin ligase, MDM2.[14] In this study, we have identified that NP interacts with E3 ubiquitin ligase, RNF43. RNF43 is a recently identified member of the RING finger family of ubiquitin ligases and has been implicated to be overexpressed in human colorectal and hepatocellular carcinomas with anti-apoptotic and growth-promoting effects that also interacts with HAP95 and NEDL1, an upstream effector of p53.[15–19] A crucial mediator of cellular apoptosis, p53 is present in a latent form in unstressed cells and is regulated through various post-translational modifications like phosphorylation, ubiquitination, sumolyation, neddylation, methylation, acetylation and glycosylation of p53 polypeptide.[20–23] Although accumulation of p53 in IAV-infected cells has been demonstrated,[24–26] it is still not clear whether IAV-induced accumulation of p53 is correlated with its activation and consecutive transactivation of its target genes that could in turn induce apoptosis in infected cells that is considered to be a peculiar feature of IAV pathogenesis.[27–31]

Our study provides evidences that RNF43 prevents cell death by ubiquitinating p53 and thus destabilizing it, *in-vitro*. Importantly, we have shown that IAV attenuates RNF43 on interacting with NP thus stabilizing p53 protein and consequently induces p53 signaling and apoptosis in the host cells.

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Abbreviations: IAV, influenza A virus; NP, nucleoprotein; RNF43, RING Finger 43; Ub, ubiquitin; UPP, ubiquitin proteasomal pathway
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Identification of this new NP–RNF43 interaction could become a promising target for antiviral intervention.

Results

Nucleoprotein of Influenza A virus interacts with human ubiquitin ligase protein RNF43 in a conserved manner. Yeast two-hybrid screening of a human cDNA lung library was conducted to identify potential interacting partners of NP of IAV. We screened a human lung cDNA library using NP from highly pathogenic A/Chicken/Hatay/2004 (H5N1), cloned into plasmid pHybLexA/Zeo, as the bait (Figure 1a). A clone encoding 1071–1622 amino acid region of RNF43 was obtained as the positive interactor which was further confirmed by BLAST analysis.

Full-length RNF43 cDNA was cloned in-frame with the activation domain pYESTrp2 and both NP–pHLZ and RNF43–pYESTrp2 were cotransformed in L-40 strain of *Saccharomyces cerevisiae*. It tested positive for histidine prototrophy and β-galactosidase activity confirming the interaction of full-length RNF43 with NP (Figure 1b).

The NP–RNF43 interaction was further validated by performing co-immunoprecipitation assays. HEK293T cells were transfected with pCDNA3.1-Myc-NP plasmid construct. Myc-tagged NP expression was confirmed using Western blotting as shown in Figure 1c (panel 3). Immunoprecipitation using anti-RNF43 antibody followed by immunoblotting with anti-myc antibody confirmed the interaction of RNF43 with NP (panel 1).

To validate the existence of the said interaction during IAV infection, mammalian lung epithelial A549 cells were infected

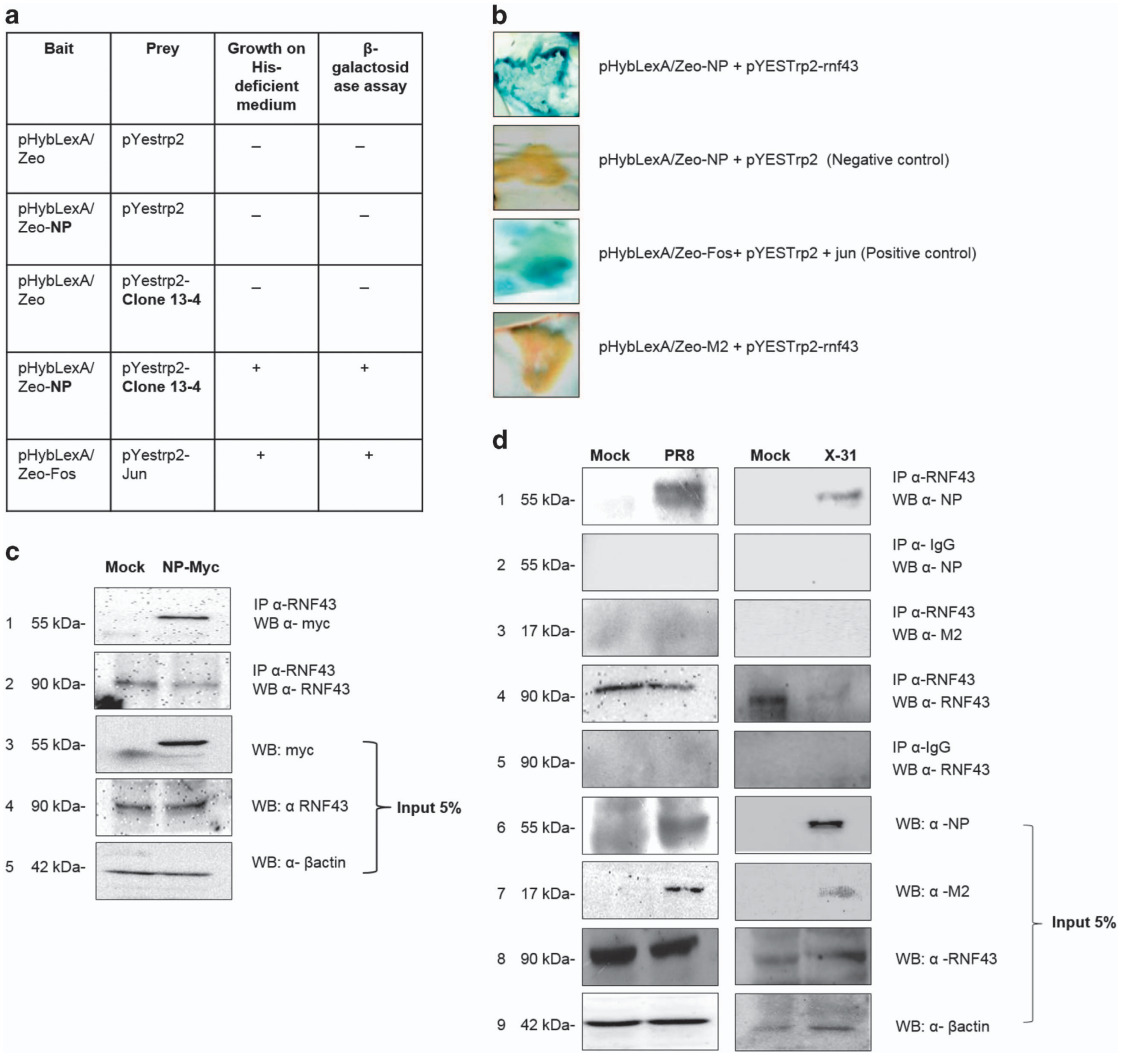


Figure 1 IAV NP interacts with RNF43. (**a**) Tabular representation of the yeast two-hybrid screening of lung cDNA library using NP as the bait protein. The filter β-gal assay for the clone 13-4 is shown as the last column of the table. (**b**) pHybLexA/Zeo-NP and pYESTrp2-RNF43 were cotransformed in L-40 yeast strain and their interaction was verified by β-galactosidase assay. (**c**) Lung epithelial A549 cells were transfected with either empty vector pCDNA3.1 (Mock) or pCDNA3.1-Myc-NP (NP-Myc). Forty-eight hours post transfection, cells were lysed and the lysates were subjected to co-immunoprecipitation with anti-RNF43 antibody followed by Western blotting with anti-Myc antibody. (**d**) A549 cells were infected with PR8 virus and X-31 virus at 1 MOI for 24 h and the whole-cell lysates were used for co-immunoprecipitation assays. Lysates (5%) from the same experiment were subjected to Western blotting with anti-NP (panel 6), anti-M2 (panel 7), anti-RNF43 (panel 8) and anti-βactin (panel 9) antibodies to show the cellular levels of these proteins

with A/Puerto Rico/8/34 virus (H1N1; PR8) and A/Aichi/2/1968 virus (H3N2; X-31) at 1 MOI. The cell lysates were harvested 24 h post infection (24 h.p.i) and subjected to co-immunoprecipitation with anti-RNF43 antibody. NP protein immunoprecipitated with RNF43 antibody thus confirming that NP–RNF43 interaction was conserved between both the virus isolates (Figure 1d, panel 1). Also, co-immunoprecipitation with another IAV protein, M2 (Figure 1d, panel 3), showed that RNF43 antibody was unable to bind the M2 protein in the IAV-infected lysate, proving that RNF43 interacts exclusively with NP of IAV.

RNF43 co-localizes with NP in the nucleus. We sought to ascertain the kinetics and site of interaction of NP and RNF43 after confirming the interaction of these two proteins. A549 cells were transfected with control plasmid pEGFPN1 or pEGFP-NP followed by fixing cells 48 h post transfection and further processing for immunofluorescence analysis. RNF43 was seen to have ubiquitous nucleocytoplasmic localization in mock transfected cells (Figure 2a). However, GFP-tagged NP localized in the nucleus and co-localized with RNF43 majorly inside the nucleus.

Similarly, A549 cells were infected with PR8 at an MOI of 5 and were fixed at 0, 4 and 8 h.p.i (Figure 2b). At 4 h.p.i, NP has been observed to localize within the nucleus. At later stages of infection it is reported to move into the cytoplasm (8 h.p.i).[3] RNF43 as previously observed had nucleocytoplasmic localization but was noted to concentrate inside the nucleus on virus infection (4 h.p.i; Figure 2b). At 8 h.p.i, the two proteins were seen to co-localize inside the nucleus. Thus, it is inferred that the primary site of interaction for the IAV NP and RNF43 is the nucleus of the host cell.

Influenza infection decreases RNF43 mRNA and protein expression. To determine the physiological relevance of this interaction in the influenza virus life cycle, Western blot analysis of the PR8-infected A549 cells harvested at different time points post infection was done (Figure 3a). It showed a gradual decline in RNF43 protein expression with progression of infection reaching to its lowest levels at 48 h. p.i in the infected cells. Using the same technique, protein expression of RNF43 at different MOI of virus was evaluated (Figure 3b). A significant decrease in RNF43 protein levels with increasing virus MOI was in agreement with our previous observation. Protein levels were quantified using densitometric analysis on Image J software (1.46r, NIH, Bethesda, MD, USA).

Subsequently, we studied the mRNA levels of RNF43 after IAV infection. Total mRNA of PR8-infected A549 cells was extracted 24 h.p.i and quantified with quantitative real-time reverse transcription PCR (qRT-PCR). A remarkable 0.5-fold decrease in RNF43 mRNA levels was clearly observed as compared with the mock (Figure 3c).

These contemporaneous regulations of RNF43 transcripts and protein expression levels are indicative of an antagonistic interaction between NP and RNF43 where RNF43 is being attenuated with the progression of virus pathogenesis.

RNF43 acts as an antiviral protein to subside viral replication. Viruses manipulate host proteins to their advantage often by increasing their efficiency of replication thus leading to higher virus titer in infected cells.[4] In order to investigate this aspect, we monitored viral replication after transiently transfecting with pCDNA3.1-RNF43-Flag-HA plasmid at indicated increasing concentrations in HEK293T cells (Figures 4a–c). Empty vector pCDNA3.1 was used as a negative control. After 24 h of incubation, cells were infected with PR8 virus and harvested at 24 h. Quantification of mRNA and vRNA levels of NP was performed through real-time PCR after RNA isolation. RNF43 overexpression resulted in a gradient and a considerable decrease in NP mRNA and vRNA levels with 0.3- and 0.06-fold being the maximum recorded, respectively (Figures 4a and b). The RNF43 overexpression was confirmed with RT-PCR analysis of RNF43 mRNA levels (Figure 4c).

Next, we observed a ~8-fold increase in NP mRNA levels with ~98% silencing of RNF43 (Figure 4d). This drastic alteration in NP mRNA as well as vRNA levels exhibits a convincing antiviral role for RNF43.

In the view of above results we decided to check the effect of RNF43 on influenza virus replicase activity. A549 cells were treated with either non-targeting siRNA (NT siRNA) or RNF43 siRNA followed by co-transfection with plasmids encoding the PR8 polymerase complex genes PB2, PB1, PA and NP in conjunction with a reporter plasmid containing untranslated region of NS1 segment upstream of the luciferase gene driven by the human RNA pol I promoter. Figure 4e shows that there was a sixfold increase in replicase activity in RNF43 siRNA-treated cells as compared with the NT siRNA-treated cells. These findings strongly suggest that RNF43 is a strong inhibitor of virus replication and has antiviral properties.

NP causes p53 stabilization by decreasing p53 ubiquitination via RNF43 ubiquitin ligase. The anti-apoptotic characteristics of RNF43 protein gave us insights to ascertain the importance of the NP–RNF43 interaction in p53 stabilization and signaling. To assess p53 protein stability, 50 μg/ml cycloheximide, an inhibitor of protein synthesis in eukaryotic cells, was administered to A549 cells which were transiently transfected with different expression plasmids, pEGFPN1, pEGFP-NP with or without pCDNA3.1-RNF43-Flag-HA. The half-life of p53 protein was deduced by monitoring its decay over a period of 3 h by Western blot analysis. Half-life of p53 in the NP-GFP and RNF43-Flag-HA co-transfected cells was reduced to 60 min as compared with 120 min in the case of only NP-GFP-transfected cells (Figure 5a). This data clearly proves that p53 is getting stabilized in the NP-transfected cells through the latter's interaction with RNF43 suggesting that NP–RNF43 interaction primarily leads to an enhanced p53 stabilization and accumulation.

Post-translational regulation of p53 via UPP mediated by different E3 ligases is a well-established phenomenon.[22,23] Because of RNF43 protein's E3 ligase activity and a RING domain in its structure we were keen to look at its role in post-translational modification of p53 through ubiquitination and explore the possibility of p53 being a substrate of its ligase activity. To this end, we first checked p53 transcript levels in A549 cells where RNF43-HA was transiently expressed. Quantitative RT-PCR analysis confirmed that RNF43 was

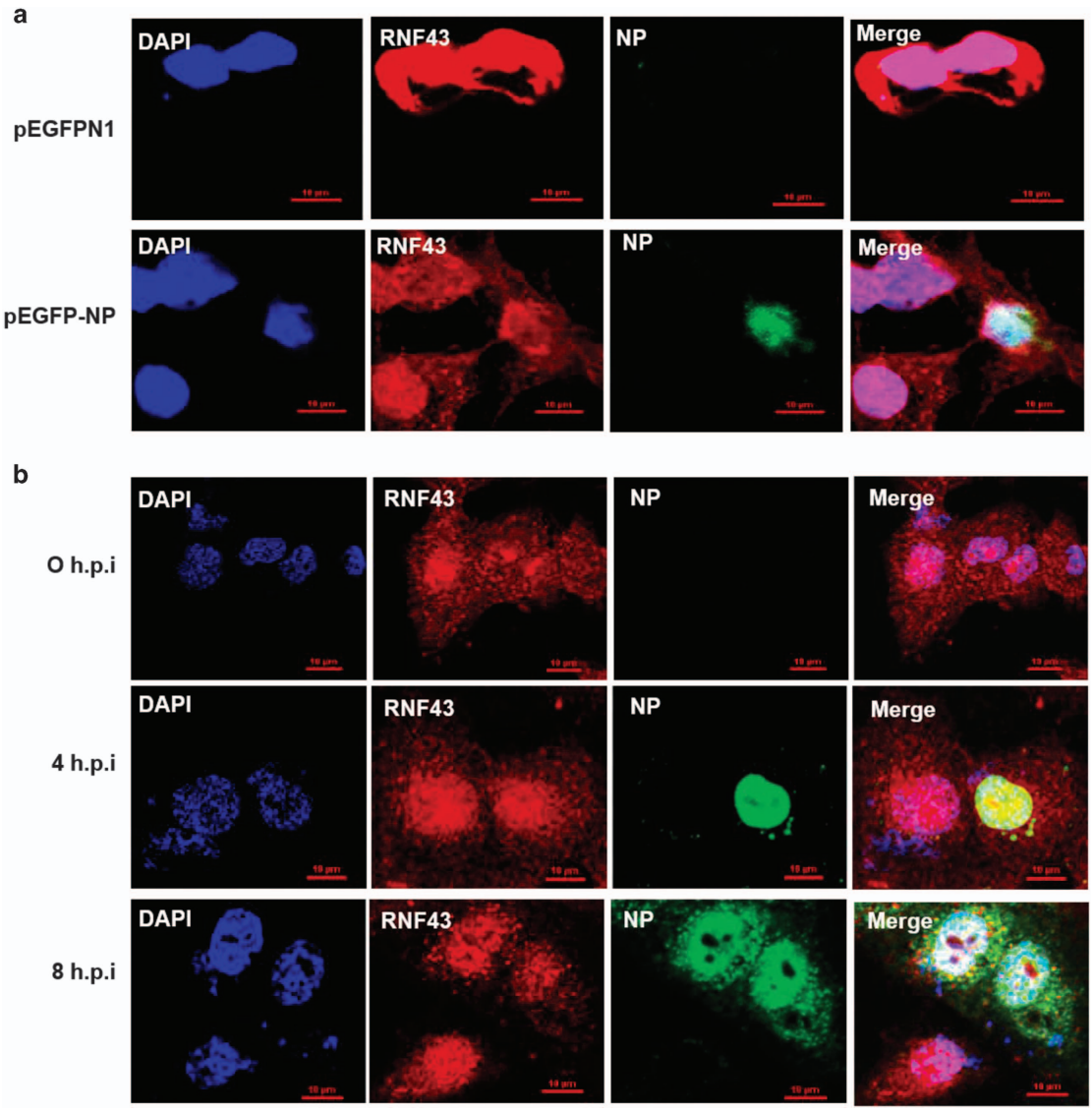


Figure 2 IAV NP and RNF43 co-localize in the nucleus of mammalian cells. (**a**) A549 cells were transfected with pEGFPN1 control plasmid or pEGFP-NP. Cells were fixed after 48 h and stained with DAPI for nucleus and anti-goat secondary antibody conjugated to Alexa-594 for RNF43 (red) and observed under confocal microscope. GFP-tagged NP is shown in green. (**b**) A549 cells were infected with PR8 IAV with 5 MOI and were fixed at the indicated time points. NP was stained using anti-NP monoclonal primary antibody and anti-mouse Alexa488 conjugated secondary antibody (green). RNF43 was stained using specific primary antibody and anti-rabbit Alexa-594 conjugated secondary antibody (red). Panels are labeled for their respective staining, RNF43 (red), NP (green) and nucleus (blue)

unable to cause any significant alteration in p53 transcription (Supplementary Figure S1).

We next examined p53 ubiquitination status. HEK293T cells were treated with 20 μM MG132, proteasomal inhibitor, after co-transfection with plasmids expressing p53-GFP, Ub-myc, RNF43-HA, NP-GFP and MDM2-Flag in different combinations (Figure 5b) followed by Western blot analysis of harvested cells. We observed a significant increase in ubiquitination status of p53 under RNF43 overexpression. However, NP-alleviated RNF43 caused p53 ubiquitination when transfected along with RNF43. MDM2 served as a positive control.[32]

Next, we examined the ubiquitination status of p53 through Ni–NTA pull down-based ubiquitination assay. HEK293T cells were co-transfected with a combination of plasmids expressing p53-GFP, RNF43-HA, NP-GFP along with Ub-His-expressing plasmid. Cells were harvested at 48 h.p.i after treatment with MG132, 5 h before harvesting. Ubiquitinated p53 was pulled with Ni–NTA beads and analyzed through Western blotting. As shown in Figure 5c, ladder of poly-ubiquitinated p53 is more abundant in RNF43 transfectants as compared with the control which suggests that RNF43 promotes p53 ubiquitination and degradation. Moreover, an increase in p53 ubiquitination was observed when RNF43-Flag-HA was co-expressed with NP-GFP as opposed to a diminished level of ubiquitinated p53 in only NP-GFP-transfected cells. This convincingly advocates the role of RNF43 in p53 regulation which is targeted by NP to stabilize p53 in IAV-infected cells.

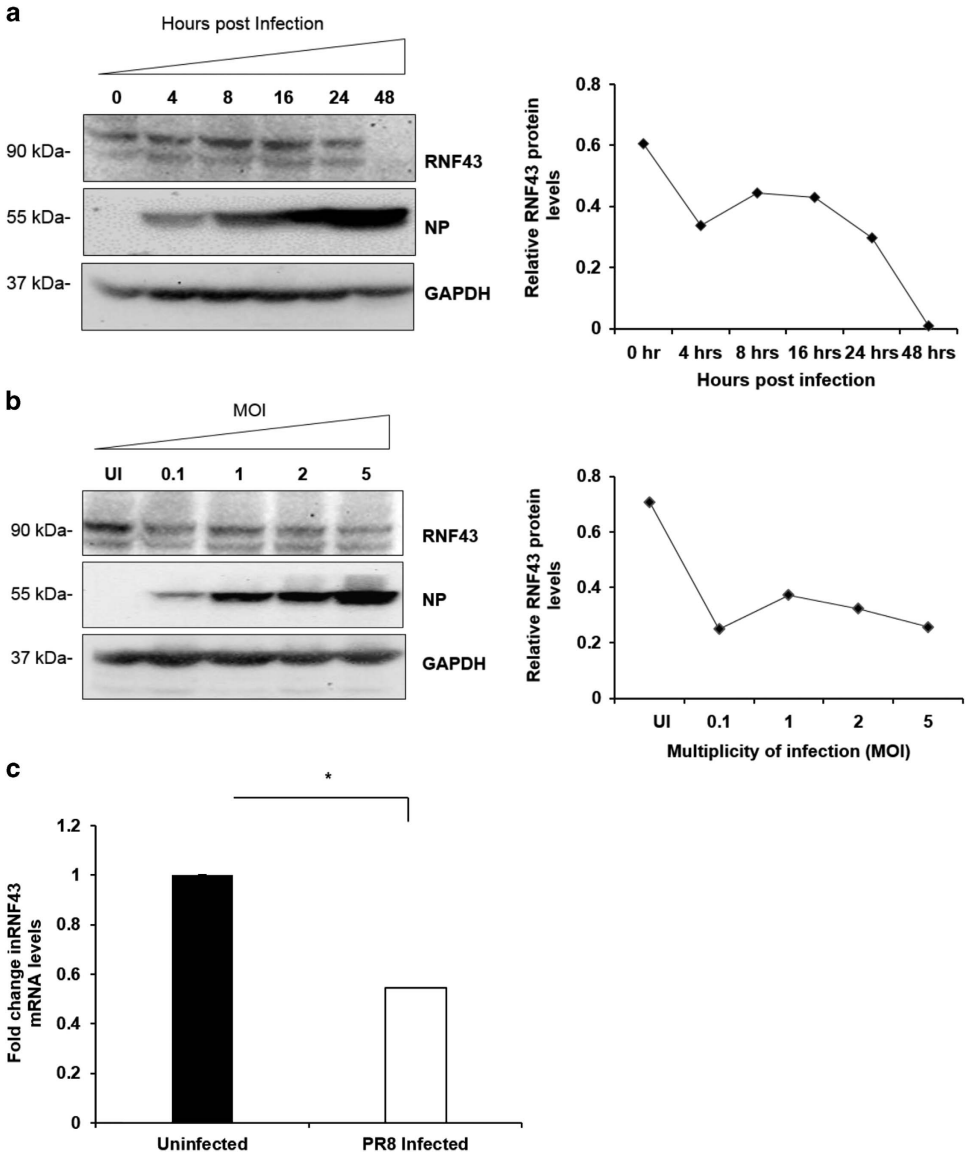


Figure 3 IAV infection decreases abundance of RNF43 at both mRNA and protein levels. (**a**) Lung epithelial A549 cells were infected with PR8 virus at an MOI of 1 and cells were harvested at indicated time intervals post infection and the whole-cell lysate was resolved on SDS-PAGE for Western blot analysis of RNF43, NP and GAPDH. (**b**) A549 cells were infected with PR8 virus at indicated MOIs and harvested at 24-h post infection for Western blot analysis of RNF43, NP and GAPDH. Quantitative representation of the immunoblots of both the experiments is shown as the line diagram after normalization with GAPDH (extreme right). (**c**) A549 cells were mock infected or infected with PR8 virus for 24 h and total RNA was extracted followed by rnf43 mRNA estimation with qRT-PCR. Results are shown as mean of $\pm$S.D. of three independent experiments. * indicates statistically significant difference at $P<0.05$

RNF43 mitigates NP-driven enhancement of p53 transcriptional activity. Stabilization of p53 by NP prompted us to look into the probable role of NP/RNF43 interaction in induction of p53-mediated cell functions. Tumor suppressor protein p53 is a transcription factor that regulates the expression of many genes that are crucial in mediating its tumor suppressing activity.[20,21] Therefore, we checked a dose-dependent effect of NP on p21 transcription which is under a direct control of p53 transcriptional activity.[33] A549 cells were transiently co-transfected with p21 luciferase reporter plasmid containing the p53 binding site on its promoter together with indicated concentrations of pEGFP-NP and pcDNA-p53 and a control plasmid, Renilla luciferase pRL-TK. The p21 luciferase activity was measured and normalized to Renilla luciferase activity. p21 is a direct target gene of p53 hence a dose-dependent increase in p21 luciferase activity as compared with the mock transfectant suggests an increased p53 transcriptional activity (Figure 6a). To probe the significance of NP/RNF43 interaction in NP-driven enhanced transcriptional activity of p53, using the similar approach, A549 cells were co-transfected with pEGFPN1 or pEGFP-NP expression plasmid alone or together with RNF43-HA-expressing plasmid. As speculated, co-expression of RNF43 with NP resulted in an ~0.2-fold decrease in p53 transcriptional activity as compared with only NP-expressing cells (Figure 6b).

We checked the mRNA levels of p21 in RNF43 knocked down HEK293T cells infected with PR8. As compared with NT siRNA-treated cells, p21 mRNA levels were almost 10-fold

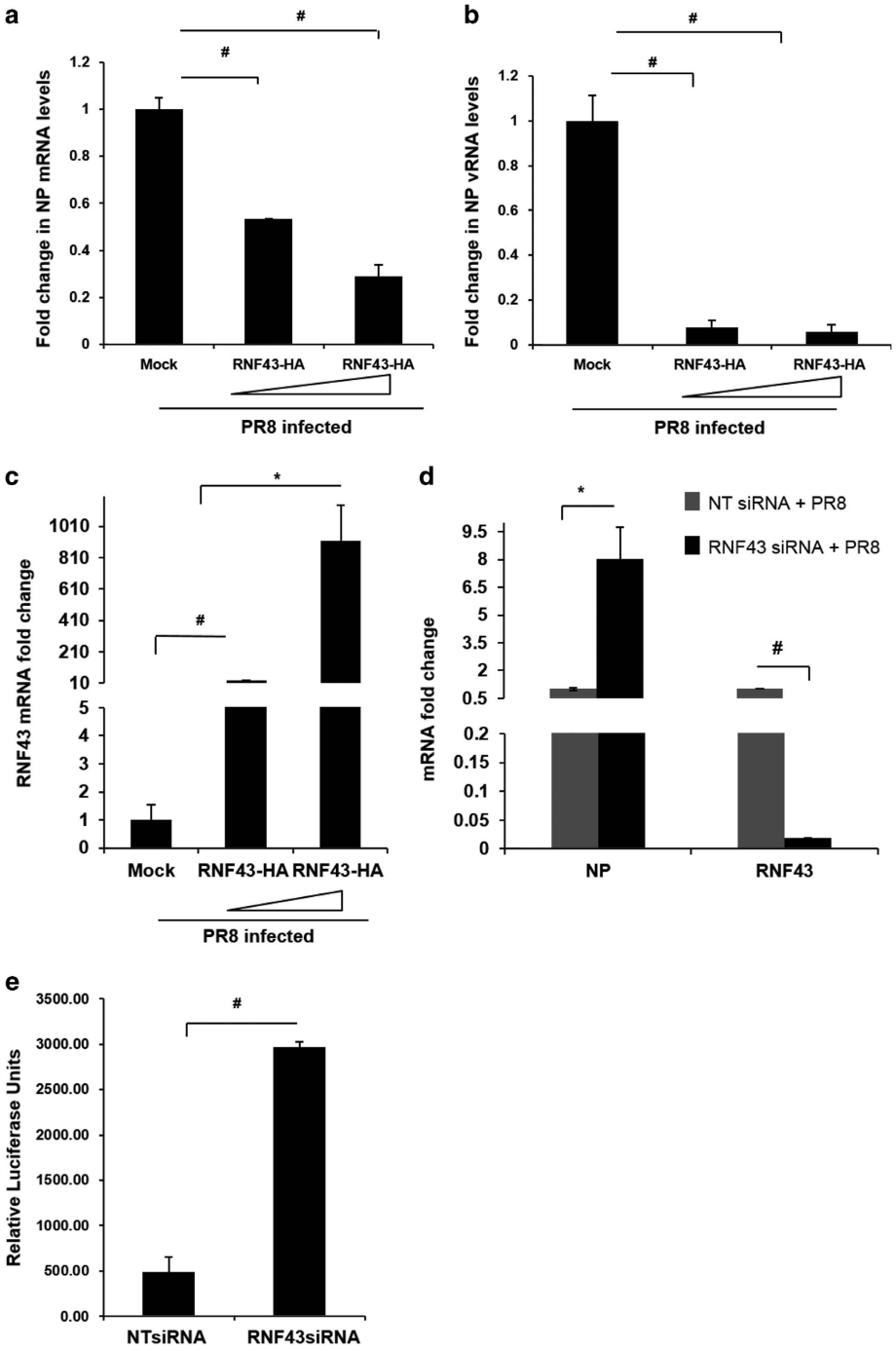


Figure 4 Host factor RNF43 decreases IAV replication. (**a–c**) HEK293T cells were transiently transfected with plasmid pCDNA3.1 (Mock) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA) (1 and 2 μg) followed by PR8 infection and incubated for 24 h. Total RNA was isolated and NP mRNA (**a**), NP vRNA (**b**) and RNF43 mRNA(**c**) levels were estimated through qRT PCR. (**d**) HEK293T cells were treated with NT siRNA or RNF43 siRNA for 24 h followed by PR8 IAV infection for next 24 h. Cells were processed for qRT-PCR analysis of mRNA levels of NP and RNF43. (**e**) A549 cells, pretreated with either NT or RNF43 siRNA were transfected with plasmids-encoding polymerase complex components (PA, PB1, PB2 and NP) derived from PR8 (H1N1 virus) were co-transfected alongside a reporter plasmid containing noncoding sequence from the NS1 segment of influenza A virus and luciferase gene driven by the Pol 1 promoter. The relative luciferase units were calculated after normalization with plasmid pRL-TK (Promega, Madison, WI, USA), which expresses Renilla luciferase that was transfected along. Results are shown as mean ± S.D. of three independent experiments. * and # indicate statistically significant difference at $P<0.05$ and $P<0.01$, respectively

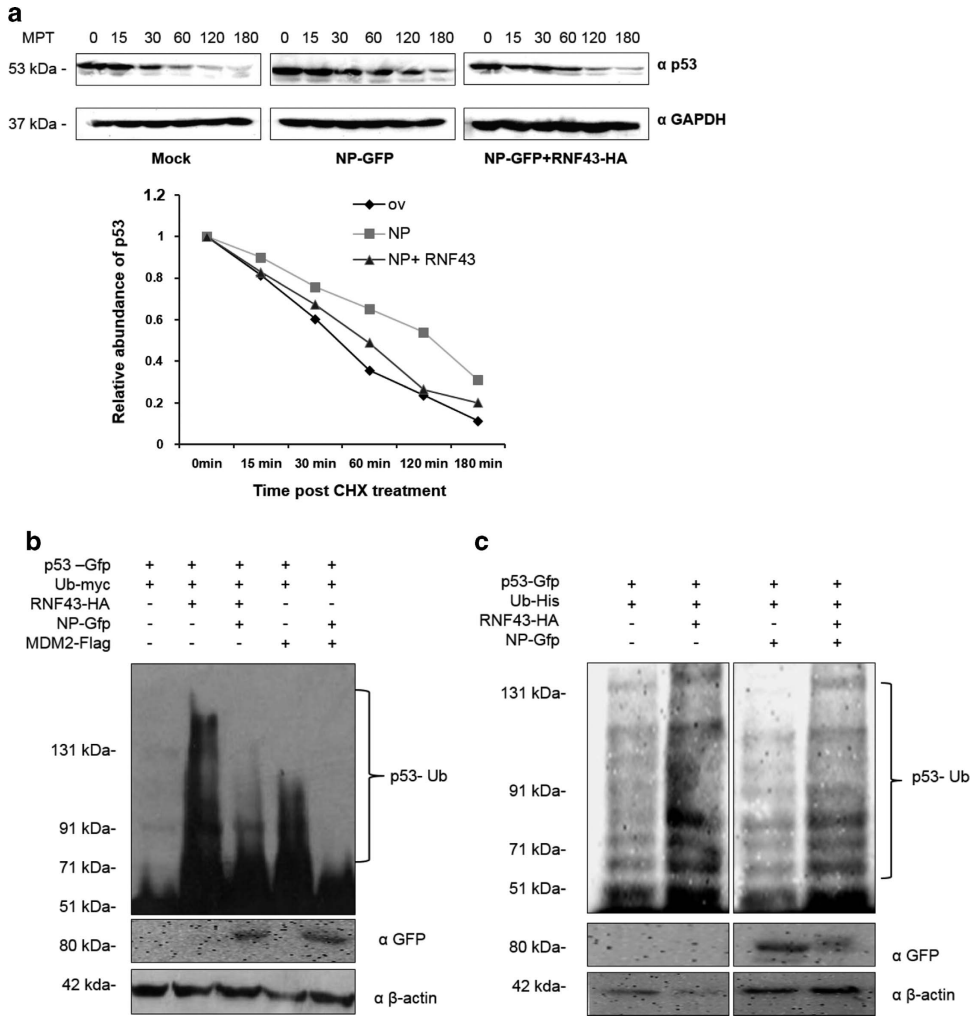


Figure 5 NP interacts with RNF43 to stabilize p53 through compromised ubiquitination of p53 by RNF43. (**a**) A549 cells were transiently transfected with plasmids pEGFPN1 (Mock), pEGFP-NP (NP-GFP) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA) for 48 h. The transfectants were treated with cycloheximide at 50 μg/ml for the indicated times (minutes) post treatment (mpt) and subjected to Western blot analysis using the indicated antibodies. The quantitative data of immunoblots is shown as line diagram after normalization with GAPDH expression levels. (**b**) HEK293T cells were transiently transfected with a combination of indicated plasmids and incubated for 48 h. The transfectants were treated with 20 μg/ml MG132, 5 h before harvest. The cell lysates were subjected to Western blot analysis using the indicated antibodies. (**c**) HEK293T cells were transiently transfected with a combination of indicated plasmids and incubated for 48 h. The transfectants were treated with 20 μg/ml MG132, 5 h before harvest. Ubiquitinated p53 (Ub-p53) was pulled down using Ni^{2+}–NTA–agarose beads and analyzed by Western blotting with indicated antibodies

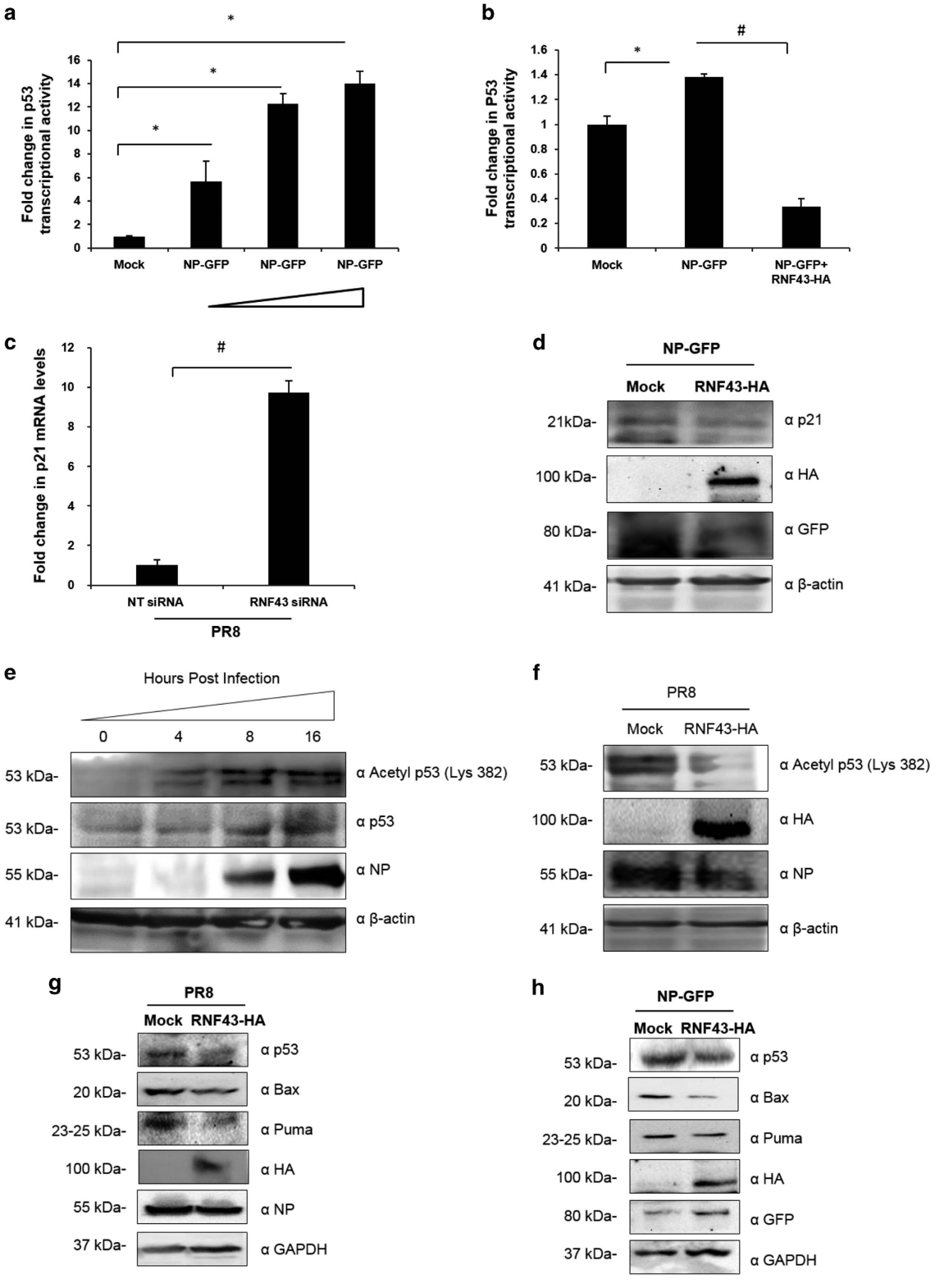
higher in RNF43 depleted cells (Figure 6c). p21 protein levels were also observed to be undergoing a notable decrease in the presence of RNF43 when co-expressed along with NP-GFP (Figure 6d).

Acetylation of p53 is an important step to enable p53-mediated transactivation of different factors towards cellular functions.[34,35] We monitored the acetylation of p53 (Lys-382) at different time points post PR8 infection in A549 cells. Acetylation levels of p53 were observed to undergo a time-dependent change as shown in Figure 6e. We further assessed the effect of RNF43 on p53 acetylation in IAV-infected cells. To achieve the same, we transfected A549 cells with pCDNA3.1 (mock) and pCDNA3.1-RNF43-Flag-HA (RNF43-HA) plasmids followed by PR8 infection after 24 h. Cells were harvested after 24 h of infection followed by Western blot analysis. A decrease in p53 acetylation in the cells ectopically expressing RNF43 (Figure 6f) is likely to be the result of the concomitant increase in RNF43-mediated p53 ubiquitination.

Furthermore, A549 cells were infected with PR8 virus after being transfected with or without RNF43-Flag-HA-expressing plasmid construct. 24 h.p.i intracellular protein levels of p53 and its regulated molecules, Bax and Puma were inspected through Western blot analysis. In RNF43-expressing cells, there was an evident decrease in the protein levels of p53 and its downstream effectors Bax and Puma (Figure 6g). The same outline was observed when pEGFP-NP was transfected in conjunction with pCDNA3.1 or pCDNA3.1-RNF43-Flag-HA plasmids (Figure 6h).

p21, Bax and Puma are p53-regulated mediators of the latter's cellular functions.[20,21] These findings confirm the role of NP/RNF43 interaction in activation of p53 signaling pathways.

RNF43 attenuates IAV NP-induced cell death. To probe the effect of RNF43 on the induction of apoptosis in IAV-infected cells, flow cytometry-based Annexin V FITC labeling was performed. A549 cells were treated with NT siRNA or RNF43 siRNA and after 24 h cells were infected with IAV and subjected to annexin V staining. A remarkable 13% of total cell population showed annexin V staining in RNF43 siRNA-treated IAV-infected cells as against the 2.5% annexin V positive population in NT siRNA-treated IAV-infected cells. Thus, confirming the anti-apoptotic role of RNF43 in IAV-infected cells (Figure 7a).



The role of NP/RNF43 interaction in cell death induced by NP was further elucidated in NP microenvironment. Fourteen percent of pEGFP-NP transfectants displayed annexin V staining whereas RNF43 overexpression along with NP lowered the annexin V population to 10% (Figure 7b).

To investigate the exclusive role of p53 in NP/RNF43 interaction governed apoptosis, p53 null human colon cancer cells, HCT116 ($p53^{-/-}$ HCT116) were transfected with pCDNA3.1 or pCDNA3.1-RNF43-Flag-HA with or without pEGFPN1 or pEGFP-NP. Annexin V staining of pEGFP-NP

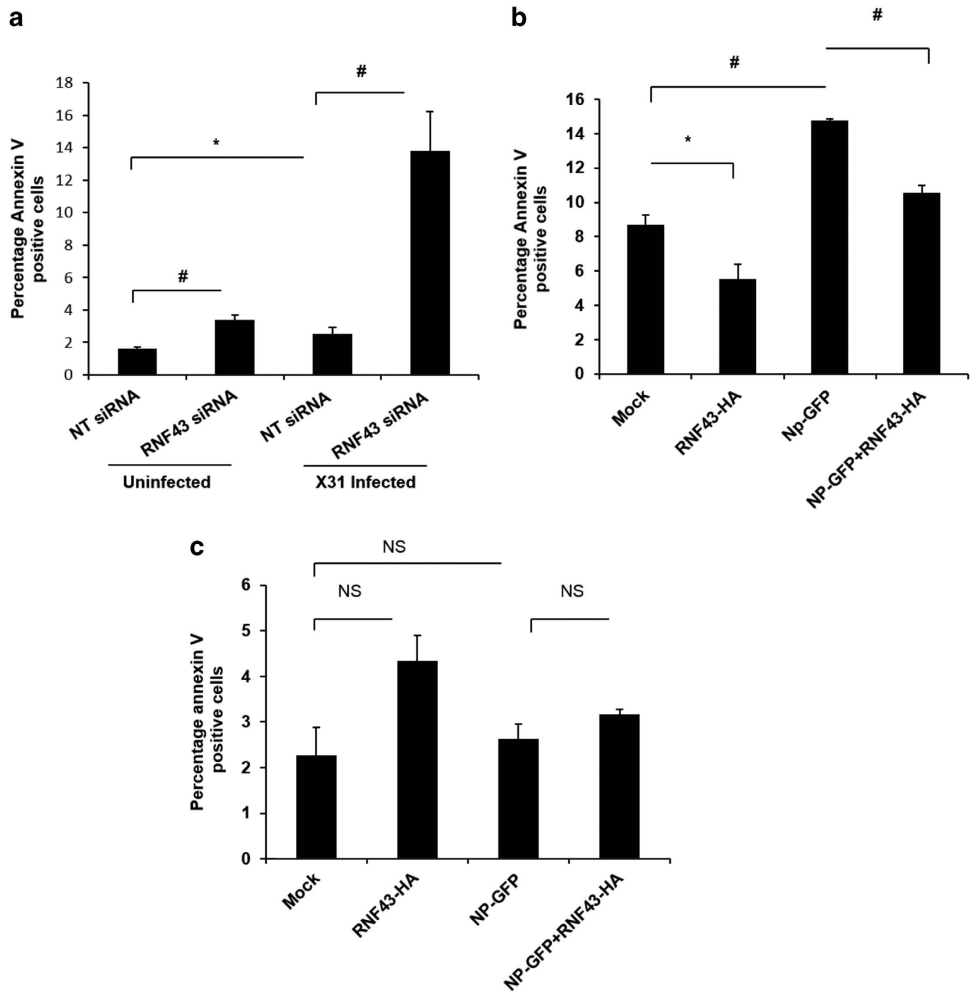


Figure 7 RNF43 attenuates IAV NP-induced cell death. (**a**) A549 cells were treated with NT siRNA or RNF43 siRNA for 24 h followed by X-31 IAV infection at 1 MOI. Cells were harvested at 24 h.p.i, stained with Annexin V FITC and subjected to flow cytometry. The percentage of Annexin V positive population is plotted on the graph. (**b**) A549 cells were transiently transfected with the indicated combinations of plasmids and 48 h post-transfection cells were harvested and processed for flow cytometric analysis of Annexin V FITC stained population which is plotted on the graph. (**c**) Same experiment was conducted in $p53^{-/-}$ HCT116 cells. All graphs represent mean $\pm$ S.D. of three independent experiments. * and # indicate statistically significant difference at $P<0.05$ and $P<0.01$, respectively, whereas NS refers to non-significant difference

Figure 6 RNF43 decreases NP driven increased p53 transcriptional activity and signaling in the cells. (**a**) A549 cells were transfected with p21-Luc reporter plasmid, with or without growing amounts of pEGFP-NP (500 and 750 ng, and 1 μg) in conjunction with pcDNA-p53 and a control plasmid, Renilla luciferase pRL-TK. Luciferase activity of cell lysates of transfectants was analyzed. (**b**) A549 cells were transfected with p21-Luc reporter plasmid, pcDNA-p53 and a control plasmid, Renilla luciferase pRL-TK along with plasmids pEGFPN1 (mock), pEGFP-NP (NP-GFP) and pCDNA3.1-RNF43-Flag-HA (RNF43-HA) in the indicated combinations. Luciferase activity was measured. (**c**) HEK293T cells were treated with NT siRNA or RNF43 siRNA for 24 h followed by PR8 IAV infection at 1 MOI. Cells were harvested at 24-h post infection and total RNA was extracted followed by p21 mRNA estimation with qRT-PCR. (**d**) A549 cells were transiently transfected with pEGFP-NP with or without pCDNA3.1-RNF43-Flag-HA and were harvested at 48 h followed by SDS-PAGE. Western blotting was done using indicated antibodies. (**e**) A549 cells were seeded in a 6-well plate and were infected with PR8 virus at 1 MOI. Cells were harvested at 0, 4, 8, 16 and 24-h post infection, and subjected to Western blotting with indicated antibodies. (**f**) A549 cells were transfected with pCDNA3.1 (mock) and pCDNA3.1-RNF43-Flag-HA (RNF43-HA) plasmids followed by PR8 infection, 24-h post transfection. The cells were harvested after 24 h followed by Western blot analysis with anti-acetyl p53, HA, NP and β-actin antibodies. (**g**) A549 cells were transiently transfected with pCDNA3.1 (mock) or pCDNA3.1-RNF43-Flag-HA (RNF43-HA) plasmid constructs and after 24 h of incubation, cells were infected with PR8 virus at an MOI of 1 for 24 h. (**h**) Similarly, A549 cells were transiently transfected with pEGFP-NP with or without pCDNA3.1-RNF43-Flag-HA and were incubated for 48 h. (**g** and **h**) Cells were harvested and processed for Western blot analysis with indicated antibodies. Results in **a**–**c** are shown as mean $\pm$ S.D. three independent experiments. * and # indicate statistically significant difference at $P<0.05$ and $P<0.01$, respectively

transfectants showed no significant difference as compared with the vector control. Similarly, RNF43 co-expression did not cause any change in the annexin V staining profile of NP-GFP-expressing cells (Figure 7c). This highlights the critical importance of p53 for carrying out NP-induced apoptosis. Most importantly, it draws attention to the critical role of RNF43 in virus-induced p53-mediated apoptosis.

Discussion

Ubiquitination is a key regulatory mechanism in the orchestration of various cellular activities including signal transduction, transcription, membrane protein trafficking, apoptosis, autophagy and immune responses.[36] Hence, it has become increasingly evident that the comprehensive ubiquitinylation process remains a prime target of a broad range of pathogens including viruses. Viruses may encode E3 ligases or deubiquitinases or may redirect the cellular E3s and deubiquitinases to modulate the ubiquitination status of substrates of choice.[11]

The present study for the first time provides evidence that NP interacts with RNF43, a member of RING finger family of cellular E3 ligases that mark proteins for ubiquitination. This interaction was validated in transfected mammalian cells and was also found to be conserved between PR8 and X-31 IAV strains. The conserved nature of the NP–RNF43 interaction underlines its significance in viral replication and pathogenesis.

We also report that IAV NP and RNF43 predominantly co-localize in the nucleus. IAV infection attenuated RNF43 mRNA as well as protein expression and overexpression of RNF43 counteracts IAV NP leading to a dose-dependent decrease in NP mRNA and vRNA. There was a drastic increase in viral replication under the influence of RNF43 knockdown. The decline in RNF43 mRNA and protein levels on IAV infection correlates with the decrease in viral replication by RNF43. It substantiates its role as an antiviral host protein. E3 ligases, highly specific mediators of ubiquitination, are extensively exploited by viruses to carry out replication and pathogenesis. It is of utmost importance to this study that many other viruses including human oncogenic papillomavirus, HIV and simian virus 40 also either target host ubiquitin ligases or encode ubiquitin ligases to divert their pathway of regulation by altering their specificity.[11,37,38] Influenza virus too exploits the host ubiquitination status of many cellular factors to evade host defense mechanisms and achieve maximum replication efficiency.[12,13] Thus, it is concluded that RNF43 is targeted by the virus for a strategic attenuation of RNF43 for efficient viral replication and pathogenesis.

Wang *et al.* illustrated the stabilization of p53 by NP through compromised MDM2-mediated ubiquitination.[14] In our study, we found that NP-driven stabilization of p53 was impeded by RNF43 via a decreased p53 half-life and a noteworthy augmentation of the ubiquitinated p53 levels in the RNF43 microenvironment. This can be attributed to the post-translational regulation of p53 by RNF43 through targeting p53 for ubiquitination. These results are in coherence with the fact that RNF43 interacts with p53[19] and the siRNA caused downregulation of RNF43 led to a significant increase in p53 protein expression in HepG2 and SMMC-7721 cells.[16] Several E3 ligases along with MDM2 are known to regulate p53 ubiquitination, for example, Pirh2, MdmX, HAUSP, ARF, COP1 and ARF-BP1.[22,23] Thus, the current study unravels a novel mechanism of p53 regulation via RNF43. Although, a separate study can be undertaken to explore RNF43-mediated p53 regulation but our study provides compelling evidences that RNF43 targets p53 for ubiquitination.

Apoptosis induction is one of the hallmarks of IAV infection. It is considered to be host's defense against the virus but at the same time it is hypothesized that the appropriately timed apoptosis controlled by IAV is important for efficient viral replication.[39] Accumulation of p53, a critical mediator of apoptosis, in IAV-infected cells is a well-documented phenomenon and in fact is reported to accumulate in a biphasic pattern.[40] Our study also demonstrates an accumulation of p53 in IAV-infected cells along with an increasing acetylation levels that corresponds to an increased p53 activity. Turpin *et al.*[41] demonstrated that p53 is required for IAV-induced cell death but at the same time was important in IFN induction. However, IFN-dependent antiviral response of p53 is reported to be independent of its pro-apoptotic functions.[26] In the midst of these conflicting notions regarding the role of p53 in IAV replication and pathogenesis, our study eloquently proves the critical importance of p53 in NP-mediated apoptosis. We previously reported the role of NP in inducing host cell death.[42] Stabilization and accumulation of p53 by NP can be accredited to the pro-apoptotic nature of NP, which, in this case hijacks RNF43 and withholds its regulatory effects on p53. Together with these findings, the data described here points to a model in which IAV increases p53 downstream signaling and apoptosis by suppressing the RNF43-mediated ubiquitination of p53 (Figure 8). Hence, interaction of NP with RNF43 to modulate p53 ubiquitination is a proof of direct mechanism of apoptosis induction by IAV.

Furthermore, the enhanced p21 transcription under the effect of NP suggests that NP could be involved in carrying out cell cycle arrest following IAV infection[43] thus, paving the way for exploring the role of NP in causing IAV-driven cell cycle arrest.[44,45] Lastly, owing to its antiviral properties and the conserved nature of IAV NP–RNF43 interaction, RNF43 could indeed prove to be an attractive antiviral developing target.

Materials and Methods

Cell culture and viruses. All the cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal calf serum (Hyclone), penicillin–streptomycin solution (100 units per ml; Invitrogen, Grand Island, NY, USA) in 5% CO_2-containing environment. A/*Puerto Rico*/8/34 (PR8) and A/*Aichi*/2/1968 (X-31) strains of IAV were used for viral infection at an MOI of 1 unless specified otherwise.

Transfection and IAV infection. DNA transfections were done with Lipofectamine 2000 (Invitrogen) in NANS medium and after 6 h of incubation; NANS was replaced with 10% fetal calf serum containing DMEM. For virus infections, DMEM supplemented with 0.3% bovine serum albumin (BSA; GIBCO, Grand Island, NY, USA) was used. Cells were washed with phosphate-buffered saline (PBS) followed by virus infection and incubated for virus adsorption at 37 °C. One hour later the virus was removed and cells were incubated with DMEM-containing 0.3% BSA. In mock-infected cells, virus was replaced with PBS.

Plasmids, siRNA and antibodies. NP gene from A/Chicken/Hatay/2004 (H5N1) influenza virus was cloned into pEGFPN1 vector and also in pCDNA3.1-myc vector. Full-length RNF43, cloned into pCDNA3.1-Flag-HA was provided by Clevers

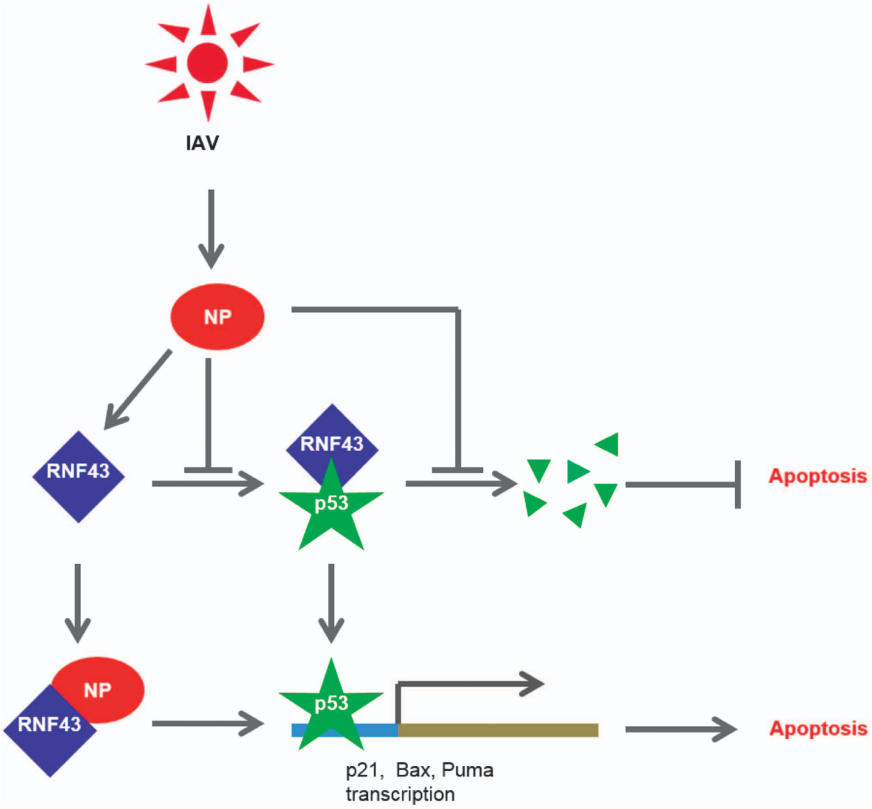


Figure 8 The proposed model for the role of NP/RNF43 interaction in regulation of p53-mediated cell death by IAV NP. The proposed model for the regulation of p53-mediated cell death by IAV NP. p53 is proposed to be regulated by RNF43 through ubiquitylation resulting in its destabilization. NP interacts with RNF43 thereby preventing ubiquitination of p53 and causing its stabilization and accumulation inside the infected cell resulting into activation of p53 signaling cascade including p21, Bax, Puma and eventually results in cell death in IAV-infected cell

and coworkers.[17] RNF43 was also cloned into pYESTrp2 for yeast two-hybrid assay. p53-GFP, MDM2-Flag and Ub-His were kind gifts from Ma and coworkers.[14] p21 luciferase reporter plasmid was provided by Vogelstein and coworkers.[33] Pool of gene specific siRNA against RNF43 was purchased from Santa Cruz, CA, USA. Anti-RNF43 (sc-165398), anti-M2, anti-p53, anti-p21, anti-HA, anti-GAPDH and anti-GFP antibodies were purchased from Santa Cruz Biotechnology, CA, USA. Anti-RNF43 (SAB2102033) and anti-β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Bax and anti-Puma antibodies were purchased from Cell Signaling (Boston, MA, USA). Anti-acetyl p53 (Lys-382) was purchased from Biolegend, San Diego, CA, USA.

Yeast two-hybrid screening. The bait plasmid was constructed by cloning IAV NP coding sequence in-frame with the LexA DNA-binding domain in pHybLexA/Zeo. The prey plasmid was prepared by cloning RNF43 into pYESTrp2. Yeast two-hybrid screening was conducted as described previously.[4]

Western blot analysis. Cells were harvested in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.1% NP-40) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Pleasonton, CA, USA). The lysates obtained were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Co-immunoprecipitation. Cells harvested in the above mentioned lysis buffer were incubated with the primary antibody overnight at 4 °C which was followed by an incubation of 90 min with protein A or protein G sepharose beads (GE Healthcare, Uppsala, Sweden). Beads were then washed three times with chilled PBS. Immunoprecipitates were eluted through boiling the beads in SDS-PAGE sample buffer for 10 min, subjected to SDS-PAGE followed by western blot analysis.

Immunofluorescence microscopy. Cells infected with IAV at 5 MOI or transfected with expression plasmid pEGFP-NP, were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.4% Triton X-100 in PBS for 20 min at room temperature. Fixed cells were then washed twice with PBST (0.2% Tween 20), blocked with 2% BSA for 1 h at room temperature and left at 4 °C for overnight immunostaining with specific primary antibody in 0.5% BSA. Unbound antibodies were washed away with PBST and incubated with secondary Alexa Fluor antibodies (594 488; Invitrogen) solution in 0.5% BSA for 1 h and washed with PBST. Slides were prepared by mounting the cover slips with Prolong Gold anti-fade medium with nuclei staining dye, DAPI (Invitrogen) and sealing the sides of the cover slips. Cell images were taken under ×60 objective lens of Leica DM6000B confocal microscope. Images were processed using NIS Elements AR 3.0 software (Nikon, Melville, NY, USA).

Quantitative real-time PCR analysis. Total RNA was isolated from cells using TRIzol reagent as per the supplier's instructions (Invitrogen). Reverse transcription of 5 μg of mRNA was performed with M-MuLV reverse transcriptase (Fermentas, Waltham, MA, USA) in a 50 μl volume reaction, according to the manufacturer's guidelines. cDNA obtained was diluted 1 : 10 and 2 μl was used in Syber green based quantitative real-time PCR reaction in a volume of 20 μl using the instrument StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used for real-time RT-PCR are RNF43 primers forward GCAGGAGCTACGGGTCATTTC and reverse GATGCTGATGTAACCAGGGGT; NP vRNA primer, CTC GTC GCT TAT GAC AAA GAA G, and NP gene primers (for mRNA) forward CTC GTC GCT TAT GAC AAA GAA G and reverse AGA TCA TCA TGT GAG TCA GAC; p21 primers, forward GTCAGTTCCTTG TGGAGCCG and reverse CTCCAGTGGTGTCTCGGTG; p53 primers, forward GTTTCCGTCTGGGCTTCTTGC and reverse ACGCAAATTTCCTTCCACTCGG; housekeeping gene, GAPDH primers, forward TCA CTG CCA CCC AGA AGA CTG and reverse GGA TGA CCT TGC CCA CAG C. All qRT-PCRs were performed in triplicates. Fold change in mRNA levels were calculated using the delta–delta threshold cycle ($\Delta\Delta$CT) method after normalizing with GAPDH.

Luciferase reporter assay. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions (Promega). The firefly luciferase activity of each sample was normalized to constitutively expressed Renilla luciferase plasmid, pRL-TK. Similarly, for *Renilla* luciferase activity, firefly luciferase plasmid pGL3 was used as an internal control.

Cycloheximide assay. Cycloheximide (Sigma-Aldrich), a protein translation inhibitor, was added to the media at a concentration of 50 μg/ml, 44-h post transfection in A549 cells. Cells were incubated for indicated time periods at 37 °C and harvested in SDS-PAGE sample buffer and subjected to SDS-PAGE followed by western blot analysis.

Ubiquitination assay and purification of His-tagged ubiquitin conjugates. Proteasome inhibitor, Mg132 was used to determine the ubiquitination status of p53 protein. HEK293T cells grown in 60 mm cell culture dishes were transiently co-transfected with the indicated plasmids and treated with 20 μM MG132 (Calbiochem, EMD Biosciences, Billerica, MA, USA) for 5 h before harvesting. Cells were lysed in Triton X-100 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100) and mixed with SDS-PAGE sample buffer followed by western blot analysis with anti-p53 antibody. For purification of His-tagged conjugates, HEK293T cells were treated similarly and were processed as described previously.[46] Concisely, the cells were lysed in a lysis buffer (6 M guanidinium–HCl, 0.1 M Na_2HPO_4–NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0, 5 mM imidazole, 10 mM β-mercaptoethanol) followed by an incubation of 12 h with Ni^{2+}–NTA beads (Qiagen, Hilden, Germany) at 4 °C on a rotating wheel. The beads were then washed as explained previously and incubated with an elution buffer (200 mM imidazole, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol and 5% SDS) for 20 min at room temperature. The eluates were mixed with SDS-PAGE sample buffer and analyzed with western blotting with required antibodies.

Flow cytometry. Annexin V staining of cells was done with Annexin V FITC Assay kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's instructions. Samples were acquired on BD FACS Calibur Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using Flowjo version 9.3.3 software (Tree Star Inc., Ashland, OR, USA).

Statistical analysis. Data are expressed as mean $\pm$ S.E. Means were compared by one-factor analysis of variance followed by Fisher protected least significant difference to assess specific group differences. Differences were considered significant at $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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REVIEW

H7N9: A killer in the making or a false alarm?

Himani Nailwal, Komal Kamra, and Sunil K. Lal

Abstract: Influenza virus remains one of the most important disease-causing viruses owing to its high adaptability and even higher contagious nature. Thus, it poses a constant threat of pandemic, engulfing a large population within the smallest possible time interval. A similar threat was anticipated with the identification of the novel H7N9 virus in China on 30 March 2013. Detection of transmission of the virus between humans has caused a stir with the identification of family clusters along with sporadic infections all across China. In this review we analyze the potential of the novel H7N9 virus as a probable cause of a pandemic and the possible consequences thereof.

Key words: influenza, H7N9, infectious, contagious, pandemic.

Résumé : Le virus de l'influenza demeure l'un des plus importants virus pathogènes en raison de sa grande capacité d'adaptation alliée à sa forte nature contagieuse. Il fait planer le risque constant de pandémie, menaçant de se propager dans une population plus étendue sur une période plus courte qu'à l'habitude. Une menace semblable s'est profilée des suites de l'identification du nouveau virus H7N9 en Chine, le 30 mars 2013. La détection d'une transmission du virus entre des humains a suscité de l'émoi lorsque l'on a repéré des groupes familiaux touchés par des infections en Chine. La présente revue analysera le potentiel du nouveau virus H7N9 comme cause probable d'une pandémie et en étalera les conséquences possibles. [Traduit par la Rédaction]

Mots-clés : influenza, H7N9, infectieux, contagieux, pandémie.

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Introduction

Viruses are notorious for their inherent capability to manipulate themselves and their host to the fullest. They represent the best evolutionary models; continuously evolving to become more "biologically powerful" (Palese and Shaw 2006) and challenging the monotony of their hosts' defense.

Influenzavirus A, *Influenzavirus B*, and *Influenzavirus C* constitute 3 of the 5 genera of the family, *Orthomyxoviridae*, characterized by viruses having a single-stranded negative sense and a segmented RNA genome (Kawaoka et al. 2005). Unlike influenza B virus, known to infect only humans, influenza A virus (IAV) has a vast range of hosts, including birds and mammals. The 8 negatively stranded RNAs of IAV, encode 12 proteins responsible for virus replication, release, and pathogenesis: hemagglutinin (HA); neuraminidase (NA); matrix proteins M2 and M1; nonstructural (NS) proteins NS1 and NS2; the polymerases — polymerase basic 1 and 2 (PB1 and PB2) and polymerase acidic (PA) proteins; the nucleoprotein (NP) (Webster et al. 1992); and a recently discovered pro-apoptotic PB1-F2 protein transcribed from a second reading frame of PB1 (Chen et al. 2001). Subtyping of influenza virus is done through different antigenic determinants to the HA and NA surface glycoproteins of the virus. HA is divided into 2 lineages or groups: group 1 consists of H1a, H1b, and H9 clades, while group 2 consists of H3 and H7 subtypes. With an infidel RNA polymerase, a continuous accumulation of point mutations in these genes, governed by immune selection pressure, gives way to a directional antigenic change. This equips the virus with a stronger immune system escaping mechanism. The continuous "antigenic drift" is the reason behind ever evolving seasonal influenza strains of a single subtype (McCaughey 2010; Rambaut et al. 2008).

Like any other inventive parasite, IAV has also evolved itself to the extent of being undaunted and calamitous. IAV has invented different ways to maintain its propensity, and one of these ways is by crossing the species barrier. Simultaneous infection of a single cell with 2 different IAVs causes an exchange of segments or reassortment between them. This sudden mingling of viruses, known as antigenic shift, forms a novel IAV subtype and, hence, rings the alarm of a pandemic, for example, the triple reassortant H1N2 that emerged in 2001 in North America (Sharma et al. 2009). The capability of the influenza virus to undergo reassortment has made it mightier than expected.

Though extremely rare in RNA viruses, recombination through template switching enhances the virulence of the virus (Wright et al. 2007).

Thus, virulence of lytic viruses like influenza is governed by 2 parameters, namely, high pathogenicity and high transmissibility.

Epidemiology and past pandemics

Constant antigenic variations govern the epidemiology of influenza viruses. Aquatic birds are the natural reservoirs of IAV, in which, it seems, the virus has attained evolutionary status through penultimate adaptation. The orders Anseriformes (ducks, geese, swan) and Charadriiformes (gulls, terns, surfbirds, sandpipers) are their native hosts, with the highest HA (17) and NA (9) subtypes being reported to circulate in them (Wright et al. 2007). However, transmission of the virus to land-based poultry or mammals has led to their rapid evolution (Ludwig et al. 1995). A limited number of influenza subtypes are known to have established themselves in humans (H1N1, H3N2), pigs (H1N1, H1N2), horses (H3N8 and H7N7), and dogs (H3N8) (Peiris et al. 2007).

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Swine have emerged as one of the major players in pandemic outbreaks owing to their having both avian and human influenza virus receptors, α2,3-sialic acid and α2,6-sialic acid, respectively (Sharma et al. 2009). This receptor affinity makes swine efficient "mixing vessels", giving rise to a new reassortant virus and a possible dawn of another pandemic (Ludwig et al. 1995). Apart from reassortment, a continuous replication of avian viruses in pigs may form variants recognizing human type receptors (Ito et al. 1998). This mode of interspecies transfer makes humans vulnerable to influenza pandemics.

Pandemics are the most calamitous manifestations of influenza virus infecting 20%–40% of the human population all over the world, simultaneously. For centuries, influenza virus pandemics have played havoc with nature and have been responsible for mass fatalities across continents. Influenza pandemics date back to 1918, when the deadly "Spanish flu" (H1N1 strain) killed ~25 million people in 25 weeks (Wright et al. 2007). It was followed by "Asian influenza" (H2N2 strain) in February 1957, "Hong Kong influenza" (H3N2 strain) in 1968, "Russian flu" (H1N1 strain) in 1977, and "swine flu" (H1N1 strain) in 2009. In 2009, the H1N1 influenza virus caused the first and till now the only pandemic of the 21st century and was a reassortant between Eurasian swine and North American triple reassortant swine H1N2 virus (Kasowski et al. 2011; Brockwell-Staats et al. 2009). Compared with its ancestor, it came across as a relatively less virulent strain but still managed to establish its pathogenesis throughout the world, with estimated respiratory and cardiovascular deaths around 201 200 and 83 300, respectively, including 80% of people below the age of 65 years (Dawood et al. 2012).

H7N9 outbreak

Since the identification of a novel avian influenza virus, H7N9, on 30 March 2013, 135 laboratory-confirmed human cases of the virus infection and 45 deaths have been reported by the Chinese authorities, which started from Shanghai, Anhui, Jiangsu, and Zhejiang provinces in China (To et al. 2013). The only case of H7N9 outside China was reported from Taiwan but with its source in China (Chang et al. 2013).

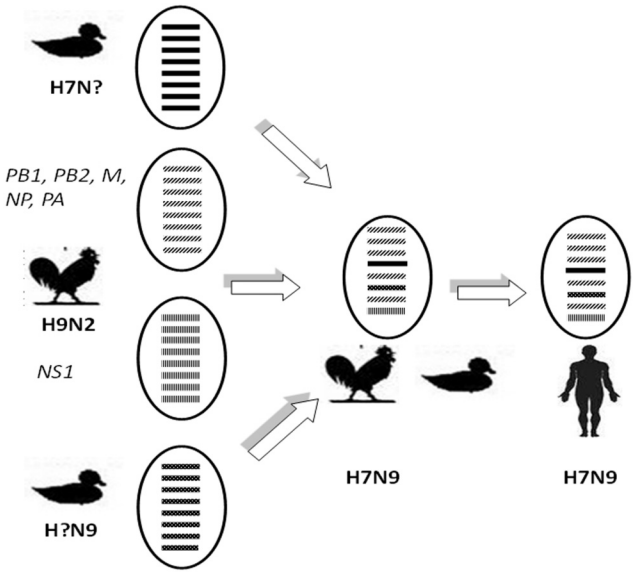
These recent reports are the first cases of human infections with H7N9 avian influenza virus (Parry 2013; Nicoll and Danielsson 2013). Earlier, nonfatal human infections with different subgroups of H7 influenza viruses have occurred all across Europe and America, including H7N2, H7N3, H7N5 (Centers for Disease Control and Prevention 2004; Hirst et al. 2004) with the exception of a highly pathogenic avian influenza virus (HPAI), H7N7, outbreak in the Netherlands in 2003 that caused infection in 89 people with 1 fatality (Koopmans et al. 2004; Du Ry van Beest Holle et al. 2005). Surprisingly, unlike most of these cases that were associated with influenza outbreak in poultry, no poultry outbreak was observed before the onset of human infections in the H7N9 case. It calls for a strict and timely surveillance program in poultry, especially land-based poultry (Jonges et al. 2013).

These cases do not seem to be epidemiologically linked, but since 2 family clusters have been identified, it has raised the alarm of probable human–human transmission (Li et al. 2014). Because of delays in the development of serological responses in persons with H7N9 virus, serological surveying may become challenging (Jonges et al. 2013). The fact that the low pathogenic avian influenza H7N9 virus does not cause any detectable disease in the birds, unlike the HPAI H5N1, makes it highly difficult to trace the source and mode of transmission and, hence, increases the risk of a pandemic.

H7N9 genesis and diversification

H7N9 virus has been reported to circulate in wild birds and ducks, but this is the first time the virus has infected humans.

Fig. 1. Schematic representation of the possible origin of the novel reassortant H7N9 virus and transmission to humans. H7N9 virus contains hemagglutinin and neuraminidase segment of H7 and N9 viruses of duck origin and the remaining segments from chicken origin H9N2 virus. PB1 and PB2, polymerase basic protein 1 and 2; M, Matrix protein; NP, Nucleoprotein; PA, polymerase acidic protein; NS1, nonstructural protein 1.



Phylogenetic studies have indicated that the novel virus is a multiple reassortant carrying gene segments from different H7N9 lineages known to have circulated in the past (Chen et al. 2013; Liu et al. 2013*a*) (Fig. 1). The H7 phylogenetic tree roots back to 3 independent lineages, American, Oceanian, and Eurasian, with H7N9 falling in the Eurasian lineage, with HA having 95% identity with that of H7N3 isolated from ducks in Zhejiang Province in 2011 (Kageyama et al. 2013).

NA, too, has a Eurasian lineage, with the closest related sequences being that of H7N9 viruses isolated from wild ducks in South Korea in February and April of 2011 and low pathogenic avian influenza A (H11N9) viruses isolated in 2010 in the Czech Republic (Liu et al. 2013*a*; Kim et al. 2012).

H9N2 virus that recently circulated in poultry in Shanghai, Zhejiang, Jiangsu, and neighboring provinces of Shanghai, seems to be the closest to the remaining viral genes with >97% identity with their gene sequences (Kageyama et al. 2013; Gao et al. 2013; Liu et al. 2013*a*). This is suggestive of a continuous circulation and genetic exchange between the wild birds, ducks, and chickens, which resulted in a novel low pathogenic avian influenza H7N9 virus capable of infecting humans without any prior devastating effects on the poultry.

Transmission

The H7N9 virus is considerably more easily transmitted to humans from poultry than H5N1 virus, as 77% of the patients infected with H7N9 virus had a history of a prior exposure to poultry (Xu et al. 2013). Until the revelation of 2 family clusters, H7N9 was thought to be milder and inefficiently transmitted between humans. Clinical and virus genome-sequencing studies done in the case of one of the family clusters, a father–daughter duo, acknowledged a direct transmission, which does challenge the earlier assumptions, but the viruses isolated from both the patients showed no other significant mutations conferring higher human-to-human transmission ability (Qi et al. 2013). Moreover, the transmission seems to be more direct and unsustainable.

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Infection and transmission experiments done on ferrets confirmed that the human isolates were able to replicate in the nasal turbinates of these birds (Watanabe et al. 2013) and that there is indeed both direct and airborne infection transmission of the human isolate of H7N9 virus in the form of respiratory droplets (Zhang et al. 2013), but in pigs, the primary mammalian host of IAV, no virus shedding was detected (Zhu et al. 2013).

However, a separate contact transmission study of a human isolate of H7N9 virus, A/Anhui/1/2013, carried out in the guinea pig model, unravels a more profound transmission among cocaged animals and establishment of infection in the lungs at a significantly lower dose of 10^6 plaque-forming units of the virus (Gabbard et al. 2014).

Here, we analyze the biological characteristics of the novel H7N9 virus that could be contributing factors in its transmissibility, pathogenicity, and pandemic potential.

Receptor binding specificity

Host selection is an integral part of influenza virus pathogenesis and is largely determined by HA through its binding to receptors containing terminal α-2,6-linked or α-2,3-linked sialic acid (α-2,6-SA or α-2,3-SA) moieties in birds and mammals, respectively (Das et al. 2010; Rossman and Lamb 2011).

Structural investigations of HA furnish insights into the host adaptability of influenza viruses and clues about the probable pandemic potential of the virus. Cleavage of a newly synthesized 70 kDa HA protein into disulfide-linked HA1 and HA2 is essential for the virus to be infective (Das et al. 2010). The presence of a series of basic amino acids at the cleavage site is a prerequisite for the said event (Peiris et al. 2007). Highly pathogenic viruses like H5 and H7 boast of multiple basic amino acids at their cleavage site, unlike the novel H7N9 virus that possesses only 1 basic amino acid (EIPKGR*GL; * indicates the cleavage site), thus making it a low pathogenic virus (Chen et al. 2013; Kageyama et al. 2013; Liu et al. 2013*a*).

Furthermore, receptor binding studies through biolayer interferometry convincingly prove the dual affinity of the human H7 virus A/Anhui/1/2013, i.e., an affinity for both avian and mammalian receptors but with a preference for avian receptors (Xiong et al. 2013), which may be the possible reason for inefficient binding of H7N9 to epithelial cells of human trachea (Dortmans et al. 2013). However, the avian H7 virus A/Shanghai/1/2013 binds to avian receptors only, similar to another group 2 avian H3 virus. The presence of 8 new mutations in human isolate A/Anhui/1/2013 as compared with avian isolate A/Shanghai/1/2013 could explain this phenomenon. Of these 8 mutations, 4 (S138A, G186V, T221P, and Q226L) are located in the receptor binding site, with the last (i.e., Q226L) being atypical of HPAI H5N1 and seasonal H3N2 viruses (Shi et al. 2013). Substitution of Gln with Leu in human H7 HA is a highly documented phenomenon that generates a hydrophobic patch stabilizing the cis conformation of the receptor analog; ultimately this increases the binding to 2,6-linked galactose as compared with hydrophilic amino acid Gln (Gamblin and Skehel 2010), as present in A/Shanghai/1/2013. However, structural comparative analysis of the interactions of A/Anhui/1/2013 mutant (L226Q) HAs with either avian or human receptor analogs through surface Plasmon resonance also shows dual binding of the mutant (Shi et al. 2013). Therefore, Q226L mutation solely cannot be credited for the receptor switching. Similarly, the presence of Val-186 instead of Asn also favors human receptor binding by increasing hydrophobicity and blocking the occupancy of 2 water molecules that are otherwise a part of the hydrogen bonding network involving 3′ OH of Gal-2 (Xiong et al. 2013). Along with these adaptations, another T160A mutation in all influenza A H7N9 viruses increases binding to human receptors through N-glycosylation site removal at position 158 (H3 numbering; position 149 in H7 numbering) (Baum and Paulson 1990; Wang et al. 2010).

These substitutions are clearly indicative of an evolution of a typical avian H7N9 virus to a more mammalian-adapted one. Interestingly, simultaneous insertion of 2 residues after residue 156 in the receptor binding site of HA results in the protrusion of the otherwise conserved 150-loop to one side that is known to sterically hinder the binding to human receptors (Xiong et al. 2013). Hence, based on this evidence, it would not be wrong to infer that there is a newly attained binding ability to human receptors, but at the same time, the sustained affinity for avian receptors can lead to trapping of the virus in the human upper airways containing α-2,3-linked galactose (Couceiro et al. 1993), thus requiring high doses of virus to cause sustained human-to-human transmission.

It is, therefore, proposed that additional mutations will be needed for the virus to achieve heightened specificity to human receptors (Xu et al. 2013), and the pandemic potential of the virus is still questionable.

Virulence

The other surface glycoprotein, NA, also known as the sialic acid receptor-destroying enzyme, cleaves the α-ketosidic linkage with the adjacent sugar residue in mucus (Baum and Paulson 1990).

In BALB/c mice, the short-NA-stalk viruses were observed to be more virulent than the long-stalk-NA counterparts. However, virulence shot up 10 000 times along with the absence of HA glycosylation. The balance between NA-stalk length and HA glycosylation is maintained for a robust virulence (Wagner et al. 2002; Matsuoka et al. 2009). H7N9 virus also carries such deletions from 69–73 amino acids in the stalk region of NA, which adds to the virulence of the virus (Chen et al. 2013; Kageyama et al. 2013). Moreover, this deletion in the stalk region coincides with the adaptation to terrestrial birds (Zhou et al. 2013), confirming the long-term circulation in these birds prior to infecting humans.

The NS protein of IAV is a multifunctional protein that has the primary function of modulating the host's innate immune response by segregating dsRNA away from 2′-5′-oligoadenylate synthetase and thus inhibiting the interferon α and (or) interferon β induced 2′-5′-oligoadenylate synthetase/RNase L pathway (Garcia-Sastre 2001). At the carboxy terminus, the PDZ (i.e., the postsynaptic density-95 protein, the discs large tumor suppressor protein, and the zonula occludens-1 protein) domain binding motif binds to the cellular PDZ-containing proteins, as observed in NS1 of HPAI H5N1 (1997 and 2003) and H1N1 (1918), thereby affecting the chief cellular pathways involved in protein trafficking, and cell polarity maintenance and organization (Peiris et al. 2007; Jackson et al. 2008). However, all the H7N9 viruses lack this PDZ domain binding motif, which might be another reason for its low pathogenesis and may lead to its attenuation in mammalian cells (Kageyama et al. 2013).

Replication competence

The polymerase complex of influenza viruses plays a pivotal role not just in virus replication but also in determining adaptation of replication in humans (Salomon et al. 2006). Hence, mutational changes in the polymerase complex drive the pathogenic potential of any virus. Out of several predicted substitutions, the prominent substitutions in the PA protein that are associated with H7N9 virus genesis in a host-species-specific manner include K356R, S409N, V100A, and A375S (Liu et al. 2013*b*). Interestingly, back mutation of these human-specific substitutions to avian type showed a radical increase in replication in both mammalian cell lines and the mouse model, giving way to an inference that assembling of H7N9 virus is based on changes that increase as well as decrease replication in humans (Yamayoshi et al. 2014).

Lysine at position 627 instead of glutamic acid is regarded as a significant mutation for avian-to-human transmission, as observed in H5N1 and in the only fatal case of H7N7 outbreak in the

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Netherlands (Hatta et al. 2001; Munster et al. 2007). Following in the lines of these viruses is H7N9 virus, with all the human isolates encoding PB2-627K while the avian and environmental isolates encode PB2-627E (Chen et al. 2013; Kageyama et al. 2013).

Interestingly, up to now, H7N9 has been reported to be less virulent than H5N1 and H7N7; hence, moreso than virulence, PB2-627K imparts the adaptation of avian viruses to humans.

A 90 amino acid protein PB1-F2, encoded by the PB1 gene, induces higher pathogenesis and death of the immune cells (Chen et al. 2001). The possibilities of PB1-F2 being required by the virus for crossing the species barrier and generation of a novel reassortant have been contemplated, and it was observed that a particular mutation at position 66, N66S, is responsible for increased virulence of some of the highly pathogenic strains, including the 1918 H1N1 and H5N1 viruses (Conenello et al. 2007). A recombinant virus carrying the N66S mutation resulted in higher pathogenesis through weight loss and death in mice, as compared with the chimera without it (Tumpey et al. 2004). Interestingly, all the H7N9 strains sequenced till now lack this mutation, thus, it is branded as a low pathogenic IAV (Kageyama et al. 2013).

Resistance towards viral inhibitors

With the sudden onset of any influenza virus, antivirals are the first defensive measures adopted against the virus. Thus, the presence of any kind of mutation in the virus genome that disables the activity of these compounds calls for an emergency.

The M2 ion channel present under the membrane, though with a very low abundance (16 to 20 molecules/virion), plays an important role in the release of viral ribonucleoprotein complexes inside the cytoplasm after virus binding through HA to the sialic acid receptors on the host cell (Nayak et al. 2009). The Adamantane drugs, Amantadine and Rimantadine, are 2 of the antivirals that target the proton flow through the M2 ion channel through binding near serine 31 clusters, hence, blocking the release of viral ribonucleoprotein complexes in the cell (Das et al. 2010). S31N mutation is the most prevalent mutation conferring adamantane resistance, which is found in all 7 novel influenza A (H7N9) viruses sequenced (Chen et al. 2013; Kageyama et al. 2013) thus far.

Moreover, in A/Shanghai/1/2013, at residue 294, substitution of arginine with lysine (R294K) increases the susceptibility of the host to virus pathogenesis, as this mutation bestows the virus with resistance to sialic acid mimicking NA inhibitors oseltamivir and zanamivir (Nayak et al. 2009; Gao et al. 2013).

However, this mutation has never been observed previously in nature, and so it seems to be a result of selective pressure of oseltamivir treatment, which is why this substitution resulted in reduced NA activity and compromised virus growth. Thus, there is still a chance of oseltamivir being a good inhibitor against H7N9 (Wu et al. 2013).

Conclusion

With each passing day, the severity of IAVs is increasing, moreso because of increasing globalization, the ease to reach every nook and corner of the world, and the roaring exchange of diseases at all levels. The widespread circulation and diversification of H7N9 is a proof of the same.

Nevertheless, the novel H7N9 IAV, owing to its inefficient human–human transmission, seems less dangerous and may not pose much threat to humans; this, therefore, puts a question mark on its pandemic potential. Moreover, even the presence of some of the most critical mutations in the current reassortant does not facilitate the dawn of a pandemic, and it is concluded that more mutations are required for the virus to gain a smooth host switching and effective and sustained pathogenesis.

China has a flourishing poultry business that also accounts for the sporadic cases of human infection. Timely surveillance in terrestrial birds and poultry seems to be the ultimate precautionary measure, apart from vaccine development. So, even though speculations of a pandemic are low, they cannot be ignored considering the silent spread of the virus in poultry without any clear clinical symptoms.

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