Influenza A Virus PB1-F2 Protein: its Role in Pathogenesis

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ABSTRACT

Influenza A virus (IAV) causes annual seasonal epidemics and occasional pandemics resulting in significant levels of mortality and socio-economic costs worldwide. PB1-F2 is a small non-structural protein encoded by an alternate +1 open reading frame in the PB1 gene through leaky ribosomal scanning mechanism. PB1-F2 is considered to play important roles in primary influenza virus infection and post-influenza secondary bacterial pneumonia in mice. It is a multifunctional and enigmatic protein with diverse functions attributed to it and the precise contribution of PB1-F2 to the IAV life cycle in avian and mammalian hosts remains largely unknown. In the triple-reassortant H3N2 (TR H3N2) swine influenza virus (SIV) background, we found that PB1-F2 expression did not affect nasal shedding, lung viral load, immunophenotypes, and lung pathology in pigs. On the other hand, in turkeys, deletion of PB1-F2 resulted in early induction of clinical disease and effective transmission among the turkey poults. Interestingly, the virulence associated 66S mutation in PB1-F2 abolished the ability of the IAV to successfully infect turkeys and transmit to in-contacts. These results highlight the strain- and species-specific role of PB1-F2 protein. We also demonstrated that specific amino acid residues in the C-terminal of PB1-F2 determine the pathogenicity of

2009 swine-origin pandemic H1N1 virus in a mouse model. The C-terminal residues 73K, 75R, and 79R together with 66S increased virus replication, decreased type I interferon response, increased infiltration of neutrophils and myeloperoxidase production in lungs resulting in acute respiratory distress syndrome (ARDS) in mice with characteristic clinical and pathological features of acute lung injury (ALI). Further, we found that PB1-F2 induces mitochondrial superoxide production and mitochondrial damage in a sequence dependent manner in IAV-infected lung epithelial cells. PB1-F2-mediated mitochondrial damage promotes Parkin-mediated mitophagy but suppresses the autophagic degradation of damaged mitochondria in the infected lung epithelial cells. Accumulated dysfunctional mitochondria likely to aggravate host cell death and inflammatory responses. Taken together, the present findings enhance our understanding of PB1-F2 protein as a virulence determinant in IAV infection in a species- and strain-specific manner and provide new insights into the impact of genetic changes in PB1-F2 on the host pathogenesis of virulent IAV strains.

Dedicated to my parents Deventhiran and Niranjana,

sister Hamsavardhini, and friends Backiyalakshmi and Giri for

their love and support

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ATTRIBUTION

This dissertation is composed of five chapters: Literature review, general conclusion and three chapters. These chapters are written in a journal format and will be submitted to journals for publication as indicated.

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Adenine nucleotide translocator 3 (ANT3)

Autophagy (Atg)

Avian Influenza viruses (AIV)

Bovine viral diarrhea virus (BVDV)

Chaperone-Mediated Autophagy (CMA)

Chromosomal maintenance 1 (crm1)

Complementary RNA (cRNA)

Cyclic-GMP-AMP synthase (cGAS)

Dendritic cells (DC)

Double membrane vesicles (DMVs)

Dynamin-related protein (Drp 1)

Electron microscopy (EM)

Epstein-Barr virus (EBV)

FADD- like interleukin 1 beta converting enzyme (FLICE)

Heat shock cognate 70 (Hsc 70)

Hemagglutinin (HA)

Hepatitis C virus (HCV)

Herpes simplex virus-1 (HSV-1)

High pathogenic avian influenza virus (HPAI)

Human cytomegalovirus (HCMV)

Human Immunodeficiency virus- (HIV-1)

IFN regulatory factor 3 (IRF 3)

Influenza A Virus (IAV)

Inner mitochondrial membrane (IMM)

Interferon –β (IFN-β)

Interferon (IFN)

Interferon Stimulating genes (ISGs)

Interleukin -1 β (IL-1 β)

Kaposi's sarcoma associated herpes virus (KSHV)

LC3 –interacting region (LIR)

Low pathogenic avian influenza virus (LPAI)

Lower Respiratory tract (LRT)

Lysosomal associated membrane protein type 2A (LAMP-2A)

Major Histocompatibility complex (MHC)

Mammalian target of rapamycin (mTOR)

Matrix protein (M)

Messenger RNA (mRNA)

Microtubule associated light chain 3 (LC3)

Mitochondria antiviral signaling (MAVS)

Mitochondrial DNA (mt DNA)

Mitochondrial membrane potential (MMP)

Mitochondrial targeting sequence (MTS)

Mitofusin (Mfn)

Negative factor (Nef)

Neighbor of BRCA gene 1 (NBR1)

NOD-like receptor family, pyrin domain containing 3 (NLRP3)

Noncoding region (NCR)

Nuclear Export protein/Non-structural protein 2 (NEP/NS2)

Nuclear Localization Signal (NLS)

Nucleoprotein (NP)

Open reading frame (ORF)

Optic atrophy protein 1 (OPA 1)

Outer mitochondrial membrane (OMM)

Pathogen associated Molecular patterns (PAMPs)

Permeability- transition pore complex (PTPC)

Phosphatidylethanolamine (PE)

Phosphatidylinositol 3-kinase (PI3K)

Phosphatidylinositol 3-phosphate (PIP3)

Plasmacytoid dendritic cells (pDCs)

Poly (ADP-ribose) polymerase 1 (PARP1)

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Polymerse Basic 2(PB2)

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Ribonucleic acid (RNA)

Ribonucleoprotein (RNP)

RIG-I like receptors (RLR)

RNA Polymerase I (Pol I)

RNA-dependent RNA polymerase (RDRP)

Sindbis virus (SV)

Single stranded RNA (ssRNA)

Swine Influenza virus (SIV)

Toll-like receptors (TLRS)

Transporter outer membrane (TOM)

Triple reassortant internal gene cassette (TRIG)

Upper Respiratory tract (URT)

Vesicular Stomatitis Virus (VSV)

Viral RNA (vRNA)

Voltage-dependent anion channel 1 (VDAC-1)

GENERAL INTRODUCTION

Influenza A virus (IAV) has the ability to infect a wide range of host species including humans, horses, pigs, dogs, bats, sea mammals, and birds. It is responsible for annual seasonal epidemics in humans, which cause significant morbidity and socio-economic costs worldwide. Occasionally, it leads to pandemics as in the case of 1918 H1N1, 1957 H2N2 and 1968 H3N2 IAV outbreaks causing millions of death worldwide (Wright et al, 2007). The recent pandemic spread in 2009 with a swine origin H1N1 virus spread to more than 200 countries in a short span and is now established as a seasonal strain. Determinants of IAV virulence and transmission are multifactorial. PB1-F2 is a non-structural accessory protein encoded by an alternate +1 open reading frame in the PB1 gene (Chen et al, 2001). The PB1-F2 protein varies in size (Pasricha et al, 2013) and its precise function in the IAV life cycle remains unclear. PB1-F2 is considered to play important roles in primary influenza virus infection and post-influenza secondary bacterial pneumonia in mice (Zamarin et al, 2006; McAuley et al, 2007). It is a multifunctional and enigmatic protein with diverse functions attributed to it; such as immune cell apoptosis, inflammation, enhancing immunopathology, modulating innate immune response, influencing secondary bacterial infections, and increasing viral polymerase activity (Kosik et al, 2013). The functional roles of PB1-F2 have been reported to be strain-specific and species-specific phenomenon (Kosik et al, 2013; Chakrabarti et al, 2013). All three 20th century pandemic IAVs and highly pathogenic avian H5N1 IAVs produce a full length PB1-F2 protein, which is considered an important virulence factor contributing to the pathogenesis of the IAV (McAuley et al,

2010). Unlike previous pandemic IAVs, 2009 swine origin pdm H1N1 (pdm09 H1N1) virus was mild in pathogenicity and does not express a full-length functional PB1-F2 (Hai et al, 2010). Intriguingly, restoration of PB1-F2 in pdm09 H1N1 expression had minimal effects on virulence in pigs, ferrets and mice (Pena et al, 2012). Hence, the precise contribution of PB1-F2 to the pathogenicity of influenza virus in mammalian hosts remains largely unknown.

PB1-F2 predominantly localizes to mitochondria, interacts with mitochondrial proteins such as such as adenine nucleotide translocator 3 (ANT3), voltage dependent anion channel 1 (VDAC1) and causes depolarization of mitochondrial transmembrane potential in a sequence-specific manner (Chen et al, 2001; Chanturiya et al, 2004; Zamarin et al, 2005; Danishuddin et al, 2010). Mitochondria play a central role in a number of cellular processes such as energy production, apoptosis and innate immune signaling (Kubli et al, 2012). The sequestration and degradation of damaged mitochondria through a selective autophagy, known as mitophagy, is critical for maintaining cell viability (Bhatia-Kissova et al, 2012). During IAV infection, oxidative stress results in significantly increased levels of reactive oxygen species (ROS) contributing to enhanced cell death and disease pathogenicity (Akaike et al, 1996; Bove et al, 2006). However, the precise molecular mechanism of ROS-mediated pathogenicity during IAV infection is not yet fully understood. Understanding the molecular mechanisms of PB1-F2 in IAV virulence is key to identify targets for developing vaccine and therapeutic strategies against IAV.

Hypothesis:

We hypothesize that the multifunctional PB1-F2 protein of Influenza A virus is a virulence determinant in a species- and strain-specific manner and specific C-terminal amino acid residues of the protein modulate its functions.

Objectives:

The main objective of the proposal was to better understand the molecular mechanisms underpinning the virulence of the IAV PB1-F2 in hosts. In order to achieve this objective, we set up three aims:

Aim 1. To define the role of PB1-F2 in the pathogenicity and transmission of swine H3N2 IAV in pigs and turkeys

Aim 2. To elucidate the role of C-terminal amino acid residues of PB1-F2 in the pathogenicity of IAV

Aim 3. To characterize the role (s) of C-terminal amino acid residues of PB1-F2 in mitochondrial damage and mitophagy

1. Literature Review

1.1 Influenza A Virus:

1.1.1 Influenza and its Significance:

Influenza, commonly known as the 'flu', is a highly contagious acute viral disease that affects the respiratory tract of birds and mammals with potentially fatal outcomes (Wright et al, 2007). It is an ancient disease, first described by Hippocrates in 412 BC and since been with us (Kuiken et al, 2010). Influenza A virus (IAV) causes significant morbidity and mortality each year in humans. Influenza usually occurs between autumn and spring and causes local epidemics in human especially among infants, elderly, and immunocompromised, clinically manifesting as fever, headache, cough, sore throat, nasal congestion, sneezing, and body aches. According to World Health Organization (WHO), annual seasonal influenza epidemics affect 5-15% of the world's population resulting in 3-5 million cases of severe illness, and 300,000-500,000 deaths (Shapshak et al, 2011).

Occasionally, new influenza virus strains may arise by reassortment or antigenic drift in nature resulting in worldwide pandemics with significant levels of mortality and economic losses (Taubenberger et al, 2010). Although the economic burden of influenza is most prominent during pandemics, the combined annual costs of seasonal epidemics due to sick days, emergency room visits, and medications are significant. The total economic burden of annual influenza epidemics has been estimated to be around $87.1 billion in United States (Molinari et al, 2007). In addition to heavy disease burden in human population, IAV also infect many animal species, sometimes with catastrophic consequences. Influenza remains a major public health threat compounded by global travel, complex human-animal interface and conflicts and thus presenting an ever-increasing challenge to the prevention and control (Kenah et al, 2011).

1.1.2 Classification and nomenclature of Influenza virus:

Influenza viruses belong to the family *Orthomyxoviridae* (Cheung et al, 2007). The family *Orthomyxoviridae* consists of six genera: Influenza virus A, Influenza virus B, Influenza virus C, Thogotovirus, Isavirus, and Quaranjavirus. (Pringle, 1996; Leahy et al, 1997; Krossoy et al, 1999; Fauquet et al, 2005; Presti et al, 2009). The viruses within the *Orthomyxoviridae* family have a negative-sense, single-stranded ribonucleic acid (RNA) genome composed of six to eight segments (Table 1) and their replication takes place in the nucleus of infected cells (Cheung et al, 2007).

Genus	**Species**	**Segments**
Influenza virus A	Influenza A virus	8
Influenza virus B	Influenza B virus	8
Influenza virus C	Influenza C virus	7
Thogoto virus	Thogoto virus Dhori virus	6 7
Isa virus	Infectious Salmon Anemia virus	8
Quaranjavirus	Quaranfil virus Johnston Atoll virus	6

Table 1.1: Summary of characterized genera of the family *Orthomyxoviridae*, their species, and number of genome segments

Influenza viruses are divided into three types (A, B, and C) on the basis of antigenic differences between their nucleoproteins (NP) and matrix proteins (M) (Wright et al, 2007). IAV are further divided into different subtypes based on the antigenic

characteristics of their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). So far, 18 HA (H1-H18) subtypes and 10 NA (N1-N10) subtypes have been identified (Laver, 1984; Fouchier et al, 2005; Tong et al, 2013). IAVs naturally infect a wide range of host species such as humans, pigs, domestic and wild birds, horses, dogs, cats, sea mammals and bats. Wild aquatic birds are the main reservoir of IAVs and serves as source for most of the IAV subtypes found in other animal species (Webster et al, 1992; Horimoto et al, 2001). Recently two IAV subtypes (H17N10 and H18N11) were isolated from fruit bats in South America. This recent discovery shows that in addition to aquatic birds, other mammalian species such as New World bats may serve as an important source of influenza virus genetic diversity (Tong et al, 2012; Tong et al, 2013). Influenza B viruses are mostly found in humans and seals, whereas Influenza C virus have been isolated from humans, pigs, dogs, and cattle (Yuanji et al, 1984; Webster et al, 1992; Manuguerra et al, 1992; Osterhaus et al, 2000; Hause et al, 2014).

Nomenclature system for IAV strains includes their genus, the host of origin (except for humans), location of isolate, the strain number, the year of isolation, followed by the HA and NA subtypes in parenthesis. For example, A/swine/Minnesota/1145/2007 (H3N2) represents an IAV isolated from swine in Minnesota in 2007 with a strain number of 1145, and according to the HA and NA characteristics this virus is assigned as H3N2 subtype. However, no antigenic subtypes have been identified for influenza B and C viruses (Smorodintsev et al, 1982).

1.1.3 Structure of Influenza A viruses:

Influenza viruses are pleomorphic and could be visualized as spherical or filamentous forms by transmission electron microscopy (EM). Spherical viral particles

have a diameter of about 100 nm and are typically characteristic of egg or tissue culture grown viruses. On the other hand, filamentous viral particles with elongated viral structures (~ 300 nm diameter) are frequently observed in fresh clinical isolates (Dawson et al, 1949; Palese et al, 2007). EM of negatively stained IAV particle are characterized by distinctive rod-shaped spikes of HA and mushroom-shaped spikes of NA. These spikes are usually 10-14 long, with approximate ratio of 4 HA: 1NA (Palese et al, 2007). Recent reports suggest that individual virus particles each preferentially package the eight essential RNA segments into the virion (Hutchinson et al, 2010). However, virions with extra genetic segments (9-11 RNA segments per virion) do occur and at most 10% or more virus particles are infectious (Nayak et al, 2013).

Virions of IAV have a complex structure and possess a host-derived lipid bilayer. Two viral glycoproteins, HA and NA and matrix 2 (M2) proteins are embedded in the viral lipid membrane and projects from the surface of the virus (Zebedee et al, 1985; Lamb et al, 1985). The matrix 1 (M1) protein lies beneath the lipid envelope in a layer extending the circumference of the virion (Nayak et al, 2004). Ribonucleoprotein (RNP) complex forms the core of the virus particle and represents the minimum infectious unit. It is comprised of NP and three polymerase subunits [polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acid (PA)] (Caliguiri et al, 1974; Huang et al, 1990; Honda et al, 2002). The nuclear export protein/non-structural protein 2 (NEP/NS2) is also associated with the virus and is found in purified viral preparations (Richardson et al, 1991). In general, IAV particle is composed of about 1% RNA, 5 to 8% carbohydrate, 20% lipid, and approximately 70% protein (Frommhagen et al, 1959).

1.1.4 Genome Organization of IAV:

Each of the eight IAV RNA segments contains a coding sequence flanked by non-coding regions (NCR) at 3' and 5' terminus. The 5' ends of viral RNA (vRNA) segment have 13 conserved nucleotides and the 3' ends have 12 conserved nucleotides among all IAV RNA segments (Robertson, 1979; Desselberger et al, 1980). These conserved nucleotides at the ends of each RNA segment are considered as the core vRNA promoter (Flick et al, 1996). vRNA promoter at 3' and 5' terminus exhibits partial inverted complementarity to each other and is proposed to form a panhandle or a "corkscrew" structure (Hsu et al, 1987; Pritlove et al, 1995; Flick et al, 1996). These proposed secondary structures have been shown to be critical for influenza vRNA promoter activity, endonuclease activity of PB1 and polyadenylation (Pritlove et al, 1999; Dobbyn et al, 2001; Poon et al, 2001).

1.1.5 Proteins of IAV:

The proteome of IAV is highly complex. Eight viral RNA segments of IAV encode at least 17 viral proteins. Segments 1, 4, 5, and 6 are monocistronic, whereas segments 2, 3, 7, and 8 encode multiple proteins (Vasin et al, 2014). In addition to 17 proteins, a hypothetical NEG8 open reading frame (ORF) in the segment 8 genomic strand translating a non-structural protein (nsp) has been predicted (Zhirnov et al, 2007; Clifford et al, 2009).

1.1.5.1 Viral polymerase proteins:

IAV encodes three major polymerase subunits (PB2, PB1 and PA) forming the viral RNA-dependent RNA polymerase (RdRp) complex. These proteins play major roles in virus replication and pathogenicity.

1.1.5.1.1 Polymerase basic 2 (PB2):

The PB2 subunit of the RdRp complex is encoded by RNA segment 1. PB2 plays an important role in the initiation of viral messenger RNA (mRNA) transcription through the process known as 'cap-snatching' (Plotch et al, 1981). During viral infection, PB2 recognizes and binds to the short 5' methylated cap structures of cellular pre-mRNAs inside host cell (Blaas et al, 1982) which are used as primers for synthesis of viral mRNA (Braam et al, 1983; Ulmanen et al, 1983; Shi et al, 1995). The PB2 also plays an important role in influenza virus replication. Mutations in the N-terminal region of PB2 specifically affected virus replication but not transcription (Gastaminza et al, 2003).

1.1.5.1.2 Polymerase basic 1 (PB1):

RNA segment 2 encodes PB1 protein, which is one of the core subunit of the viral RdRp complex. It contains binding sites for PB2 and PA resulting in the formation of an active heterotrimer (Digard et al, 1989). PB1 possess RNA endonuclease activity and it is responsible for the generation of 5' capped primer required for viral mRNA transcription (Rao et al, 2001). In addition to viral transcription, PB1 is also responsible for elongation of the primed nascent viral mRNA and also complementary RNA (cRNA) (Braam et al, 1983; Gonzalez et al, 1999). RNA segment 2 has multiple AUG initiation sites and it encodes two other proteins namely PB1-F2 and N40 through a mechanism called "leaky ribosomal scanning" (Wise et al, 2011). PB1-F2 is a multifunctional 87-101 amino acid protein translated from alternate +1 open reading frame (Chen et al, 2001), whereas N40 is an N-terminally truncated form of the PB1 protein (Wise et al, 2009). N40 interacts with other polymerase subunits and regulate virus replication in a specific genetic

background (Wise et al, 2009). The characteristics of PB1-F2 will be discussed in section 2 of this chapter in detail.

1.1.5.1.3 Polymerase acidic (PA):

PA is the acidic polymerase subunit encoded by the RNA segment 3. Mutational studies revealed that PA plays an essential role in both transcription and replication of IAV (Fodor et al, 2003). In addition, PA possesses proteolytic activity (Sanz-Ezquerro et al, 1995) but the level of proteolysis does not correlate with transcription/replication activity of the protein (Naffakh et al, 2001). PA-X is an newly identified protein encoded by alternate +1 reading frame of segment 3. It is a fusion protein that incorporates 191 amino acids from N-terminal region of PA along with a short 61 amino acids C-terminal sequence that result from +1 ribosomal frame shift. PA-X modulates the host response by repressing expression of a number of host proteins (Jagger et al, 2012). Recently, two additional N-terminally truncated forms of PA proteins namely PA-N155 and PA-N182 were identified. These proteins are translated from the 11^{th} and 13^{th} in-frame AUG initiation site in the RNA segment 3 by leaky ribosomal scanning mechanism (Muramoto et al, 2013). PA-N155 and PA-N182 does not possess polymerase activity when co-expressed with PB1 and PB2. These proteins were shown to play important functions in the replication and pathogenicity of IAV (Muramoto et al, 2013).

1.1.5.2 Envelope proteins:

1.1.5.2.1 Hemagglutinin (HA):

HA is an integral membrane glycoprotein encoded by RNA segment 4. HA forms a spike shaped homotrimer that extends outward from the lipid bilayer and is one of the most abundant viral surface proteins (Wiley et al, 1977). HA binds to sialic acid-

containing receptors on the cell surface and facilitates the attachment of virus to the host cell (Palese et al, 2007). Specificity and affinity of the HA for sialic acid receptors is one of the critical determinants of host tropism and pathogenicity (Rogers et al, 1983). Avian IAVs preferentially bind to sialic acid receptors with N-acetylneuraminic acid linked to galactose sugar by α 2,3 linkage (SA α 2,3 Gal) present in the intestine of birds (Rogers and Paulson, 1983). In contrast, human IAVs prefer to bind sialic acid with an α 2,6-linkage (SA α 2,6Gal) that is present in the bronchial epithelial cells in the upper respiratory tract (URT) (Shinya et al, 2006). In addition, respiratory epithelial cells of swine contain both SA α 2,6 Gal and SA α 2,3 Gal types of receptors, which makes pigs readily susceptible to both human and avian IAVs (Gambaryan et al, 2005). However, this HA specificity is not absolute and it is reported that human and avian epithelial cells can contain both SA α 2,3 Gal and SA α 2,6 Gal receptors (Shinya et al, 2006). For instance, it has been shown that ciliated cells of the lower respiratory tract (LRT) in humans contains SA α 2,3 Gal receptors (van Riel et al, 2006).

HA is synthesized initially as a single polypeptide in a precursor form (HA0) (Copeland et al. 1986). HA0 is cleaved by host trypsin-like proteases into subunits, HA1 and HA2 connected by disulfide linkages (Steinhauer 1999). This post-translation modification of HA0 is required for virus infectivity (Lazarowitz and Choppin 1975; Garten et al, 1983) and a crucial determinant in pathogenicity and in the spread of infection in avian species (Steinhauer 1999). Cleaved HA liberates the fusion peptide at the amino terminus of HA2 which mediates the membrane fusion between the viral envelope and the endosomal membrane facilitating the uncoating process (Skehel et al, 2000). Each molecule of HA consists of a globular head and a stalk (Copeland et al,

1986). The globular head contains the receptor-binding domain as well as antigenic sites and is made up solely of HA1. The stalk is composed of HA2 and partially of HA1. Amino acids at positions 190 and 225 in H1 subtypes and positions 226 and 228 in H3/H2 forms receptor-binding site and plays important role in receptor specificity and interspecies transmission. (Palese et al, 2007; Cheung et al, 2007). IAV HA is recognized by the host adaptive immune system and mounts a vigorous immune response, which results in the formation of neutralizing antibodies. These neutralizing antibodies drive point mutations in HA1 resulting in antigenic drift (Palese et al, 2007).

1.1.5.2.2 Neuraminidase (NA):

The second viral envelope glycoprotein, NA, is encoded by RNA segment 6. The receptor-destroying enzymatic activity of NA helps to cleave the sialic acid receptors and facilitates the release of virus from the infected cell (Gottschalk, 1957). Thus, NA plays an important role in preventing the aggregation of viral progeny and allows spread of viral infection from cell to cell (Palese et al, 1974; Palese et al, 1976). Further studies have also shown that the conserved cytoplasmic tail of NA might control virion morphology and virulence (Mitnaul et al, 1996; Jin et al, 1997). NA also plays an essential role in both viral attachment (Matrosovich et al, 2004) and enhancing late endosome/lysosome trafficking (Suzuki et al, 2005). NA is the second major surface antigen and contains highly variable regions considered as antigenic sites against which neutralization antibodies are produced (Air et al, 1985; Gulati et al, 2002). Increased NA activity correlates with release of influenza particles into the air and thus influences the transmissibility of IAV (Lakdawala et al, 2011).

1.1.5.3 Nucleoprotein (NP):

RNA segment 5 of IAV encodes NP, which is the most abundant protein of the viral RdRp complex. The amino terminus of NP contains an RNA-binding domain that helps to bind viral RNA in a sequence independent manner and thus acts as a scaffold protein (Kobayashi et al, 1994; Albo et al, 1995). It has been proposed that the availability of new soluble NP controls the switching of viral RNA polymerase from transcription to replication. (Hay et al, 1977; Vreed et al, 2004). NP contains nuclear localization signals and acts as a shuttle protein regulating the nuclear import and export of vRNPs during early and late stage of viral infection (Whittaker et al, 1996; Melen et al, 2003).

1.1.5.4 Matrix (M):

RNA segment 7 of IAV encodes three proteins, M1, M2, and M42. Unspliced transcripts encode M1, whereas M2 and M42 are made from a spliced transcript (Wise et al, 2012). M1 determines the morphology of the virion (Roberts et al, 1998; Bourmakina et al, 2005) and plays an important role in virion assembly by recruiting the viral components to the site of assembly at the plasma membrane. M1 interacts both with the cytoplasmic tails of the HA and NA glycoproteins as well as with viral RNP complexes and thus acts as a bridge between inner core components and the membrane proteins (Schmitt et al, 2005). Structural analysis showed that M1 protein contains nuclear localization sequence motif and is necessary for membrane binding, self-polymerization, and the nuclear export of vRNPs from nucleus to cytoplasm (Murti et al, 1988; Talon et al, 1998). M1 is necessary and sufficient for the virus like particles formation indicating that M1 plays viral role in the budding of the virion from the host cell (Gomez-Puertas et

al, 2000; Latham et al, 2001). M1 also interacts with heat shock protein (Hsp) 70 resulting in the activation of caspase-9 and cytochrome complex (cyt c) mediated apoptosis (Halder et al, 2011).

M2 is the transmembrane ion-channel protein and consists of a short ectodomain, a transmembrane domain and endodomain (Pinto et al, 1992; Holsinger et al, 1994; Palese et al, 2007). On an average, 15-20 tetramers of M2 become incorporated into the influenza virion (Zebedee et al, 1988). During acidification in endosomes, M2 allows the influx of protons to enter the virion and thus weakens the interaction of the M1 protein with viral RNP complexes facilitating the uncoating process during viral replication (Bron et al, 1993; Schnell et al, 2008). In addition to uncoating, M2 plays an important role in assembly and budding of IAV virions from the host cell (Hughey et al, 1995; Schroeder et al, 2005). M2 is reported to inhibit macroautophagy, which is important for cell survival. M2 blocks the fusion of autophagosome with lysosomes resulting in enhanced apoptosis of influenza-infected cells (Gannage et al, 2009). The short ectodomain of M2 (M2e) is fairly well conserved among all IAV subtypes and is considered as an attractive target for a universal influenza vaccine candidate (Descamps et al, 2011). M42 is the M2 isoform with an alternative ectodomain and can functionally replace M2 in M2-null viruses (Wise et al, 2012).

1.1.5.5 Non-structural proteins (NS):

RNA segment 8 encodes at least two non-structural proteins NS1 and NS2/NEP. NS1 is the collinear transcription product, whereas the spliced mRNA of segment 8 encodes NS2 (Lamb et al, 1979). NS1 is a multifunctional protein and acts as major virulence factor of IAV by suppressing innate immune defenses of the host. It is not a

structural component of the virion and expressed at high levels in infected cells (Palese et al, 2007). NS1 interacts with variety of host cell proteins and regulates cellular mRNAs nuclear export, splicing, and cellular mRNA polyadenylation resulting in host cell protein synthesis shut-off (Hale et al, 2008). NS1 also functions as an interferon (IFN) antagonist and thus facilitates virus replication inside the host cell (Garcia-Sastre, 2001). The role of NS1 in apoptosis has not been fully established and it was hypothesized that NS1 contributes to both early suppression of cell death that could promote virus replication and late induction of apoptosis thereby facilitating increased release of progeny virions (Hale et al, 2008). In association with M1, NEP/NS2 protein facilitates the export of new vRNP complexes from the nucleus to the cytoplasm and thus plays an essential role in the IAV life cycle (Richardson et al, 1991; Yasuda et al, 1993). In addition to NS1 and NS2, some IAV strains produce an additional protein, named NS3. NS3 is the NS1 isoform with an internal deletion produced by alternatively spliced mRNA. Selman et al. speculated that the NS3 expression could be possibly associated with host adaptation and crossing the species barrier. However, it is still an arguable hypothesis and yet to be established (Selman et al, 2012).

1.1.6 IAV life cycle:

The first step in IAV replication cycle is the attachment of HA to sialic acid-containing receptors on the cell surface (Palese et al, 2007). After attachment, IAV enters the cell via clathrin-mediated endocytosis (Matlin et al, 1981; Lakadamyali et al, 2004). Low endosomal pH triggers a conformational change in HA and exposes fusion peptide at the amino terminus of HA2 subunit resulting in the fusion of viral envelope with the endosomal membrane of host (Stegmann 2000). Ion-channel activity of M2 protein

permits the influx of protons from the endosome into the virion and plays important role in the uncoating process. This acidification results in the disruption of protein-protein interactions and dissociation of viral RNP complexes from the M1 protein resulting in the release of the vRNPs into the cytoplasm (Shimbo et al, 1996; Lear 2003).

Transcription and replication of IAV genome takes place in the nucleus of host cell (Herz et al, 1981). NP, PA, PB1, and PB2 contain nuclear localization signals (NLS) (Boulo et al, 2007). The released vRNPs migrate into the nucleus of host cell via the chromosomal maintenance 1 (crm1) dependent pathway. It binds to various karyopherins such as importin α and β that are involved in nuclear import (Samji et al, 2009). Once inside the nucleus, viral RdRp complex composed of PA, PB1, and PB2 initiates primary transcription of mRNA from viral genomic RNA. Capped 5' ends of the cellular pre-mRNAs obtained through cap-snatching mechanism initiates viral transcription and chain elongation (Dias et al, 2009; Yuan et al, 2009). Viral mRNAs terminate when the polymerase complex stutters near 5-7 uridine stretches at the 5' end of the vRNA (Robertson et al, 1981). After translation of viral proteins in the cytoplasm, the three viral polymerase subunits (PB2, PB1, and PA) along with NP transported back into the nucleus with the help of Hsp 90 for the replication process (Naito et al, 2007). Increased concentration of NP triggers viral genome to switch from transcription to genome replication. However, the mechanism by which NP controls switching from transcription to replication is not understood (Cheung et al, 2007). vRNA segments serve as templates for the production of cRNA in primer-independent fashion (Mikulasova et al, 2000). Once synthesized, positive sense cRNA serves as the template for the production of negative sense vRNA. Newly synthesized vRNAs are assembled into vRNP complexes

and are transported out of the nucleus with the help of M1, and NEP/NS2 (Talon et al, 1998; Whittaker et al, 1996). In the cytoplasm, they accumulate near the microtubule-organizing center and with the help of Rab11 transported to the apical plasma membrane along the microtubule network (Eisfeld et al, 2011; Amorim et al, 2011). Viral membrane proteins (HA, NA, and M2) are synthesized in membrane-bound ribosomes and translocated into the ER, where they undergo post-translational modifications. Viral membrane proteins are subsequently transported via the Golgi apparatus to the apical plasma membrane where final virus assembly and budding takes place (Doms et al, 1993; Holsinger et al, 1995). Interactions between cytoplasmic domains of HA, NA and M2 with M1 vRNPs via M1 induces the signals for assembly and budding of progeny virion (Nayak et al, 2009; Hutchinson et al, 2010). NA cleaves the sialic acid results in the release of progeny virion from the cell surface (Palese et al, 1974; Palese et al, 1976).

1.1.7 Molecular determinants of IAV pathogenicity and transmission:

HA plays an important role in cell attachment and membrane fusion process of IAV life cycle. Specificity of the HA for sialic acid receptors is an important host-range determinant of IAV. As mentioned above, human IAV preferentially binds to SA α 2,6 Gal receptors, whereas avian IAV prefers SA α 2,3 Gal receptors (Rogers et al, 1983). Specific residues within HA that control the receptor-binding specificity have been determined. Change from SA α 2,6 Gal to SA α 2,3 Gal receptor specificity is dictated by E190 and G225 (in human H1) and Q226 and G228 (in human H3). On the other hand, L226 and S228 change avian H5 from SA α 2,3 Gal to SA α 2,6 Gal receptor specificity (Stevens et al, 2004; Medina et al, 2011). Another intriguing aspect is receptor-binding specificity of AIVs circulating in land-based poultry differs from that of viruses found in

aquatic birds. HPAI H5N1 and H9N2 viruses isolated from land based poultry showed substantially lower affinity for SA α 2,6 Gal receptors than viruses from aquatic birds (Matrosovich et al, 1999; Matrosovich et al, 2001). Humans have avian-type SA α 2,6 Gal receptors in lower respiratory tract, which is considered as one of the possible reason for severe pneumonia and limited human-to-human transmission observed during 1997 H5N1 avian influenza outbreak in humans (Medina et al, 2011). Recent research found that four to five amino acid changes around the receptor-binding pocket in the H5 HA conferred efficient respiratory droplet transmission to recombinant H5N1 virus in ferrets. These amino acid changes shift H5 HA from SA α 2,3 Gal receptor specificity to SA α 2,6 Gal receptor specificity (Imai et al, 2012; Russell et al, 2012). In addition to host receptor binding, proteolytic cleavage of HA is necessary for the infectivity of the virus and thus plays a prime role in viral pathogenicity. Cleavage susceptibility of HA0 correlates with the pathogenicity of avian influenza viruses (AIV) in terrestrial poultry (Steinhauer et al, 1999). In Low pathogenic avian influenza (LPAI) viruses, cleavage of HA is mediated by host trypsin-like enzymes, thus infection is restricted to tissues where these enzymes are present. On the other hand, high pathogenic avian influenza (HPAI) viruses contains multi-basic cleavage site that can be recognized by ubiquitous host enzymes resulting in systemic infection (Kawaoka and Webster, 1988).

In addition to receptor specificity, overall viral fitness is crucial for successful virus replication inside the host. Specific mutations in the polymerase subunit, PB2, enhance polymerase activity and are also involved in the adaptation of avian viruses to mammalian hosts. Amino acid at position 627 of PB2 acts as a determinant of host range (Subbarao et al, 1993) and modulates the virulence of IAV (Hatta et al, 2001a). Change

of amino acid 627 from glutamic acid to lysine (E627K) facilitates IAV to cross species barrier from an avian host to a human host (Fouchier et al, 2004). Avian IAVs replicate in the intestinal tract at a temperature of nearly 41^{o}C whereas, mammalian IAVs replicate in the URT at an approximate temperature of 33 °C (Hatta et al, 2007). IAV with PB2 E627K mutation can replicate efficiently in the URT at 33 °C. PB2 E627K mutation is present in most human H1N1 and H3N2 viruses but not in avian influenza viruses. This molecular signature has been associated with increased virulence of HPAI H5N1 virus in humans (Hatta et al, 2001b). Another important determinant of pathogenicity in PB2 is D701N substitution. The D701N mutation in PB2 increases viral replication and also promotes the nuclear localization of PB2 in human cells (Gabriel et al, 2008). NS1 helps influenza virus to overcome host innate immune defenses and is one of the important determinant of influenza virus pathogenicity (Garcia-Sastre et al, 1998). Through retinoic acid-inducible gene-1 (RIG-I), NS1 blocks the recognition of IAV pathogen associated molecular patterns (PAMPs) and thus helps to prevent activation of transcription factors necessary for interferon-β (IFN-β) induction. Mutations in the RNA-binding domain present in the N-terminal region attenuated the interferon antagonistic activity of NS1 (Hale et al, 2008). NS1 also interferes with host interferon stimulating genes (ISGs) expression and thereby interfere with antiviral activity. H5N1 HPAI virus NS1 causes dysregulation of innate immune responses and induces enhanced levels of proinflammatory cytokines in the lungs of infected host (Cheung et al, 2002; Seo et al, 2002). The PDZ ligand domain in the C-terminal region of NS1 acts as an important virulence determinant in 1918 H1N1 and H5N1 HPAI virus (Jackson et al, 2008).

In addition to the above proteins, other gene products such as NA, PA-X, and PB1-F2 also plays important role in the pathogenicity of IAV. Functional balance between HA sialic acid binding affinity and receptor destroying enzymatic activity of NA is crucial for optimal virus replication and transmission (Wagner et al, 2002). Increase in enzymatic activity of NA is correlated with more efficient replication of H7N7 AIV in humans (Munster et al, 2010). Deletion in NA stalk region is an characteristic related to the adaptation of AIV from aquatic birds to land-based poultry. This deletion in the NA stalk region of H5N1 AIV were associated with increased virulence in land-based poultry and in mammalian hosts (Matsuoka et al, 2009; Gabriel et al, 2011). PB1-F2 has broad range of functions such as immune cell apoptosis, modulating innate immune response, enhancing lung immunopathology, influencing secondary bacterial infections, and increasing polymerase activity in a strain- and host-specific manner (Chakrabarti et al, 2013). It is considered to play an important role in both primary influenza virus infection and post-influenza secondary bacterial pneumonia in mice (Kosik et al, 2013). Interestingly, PA-X exerts opposite effects of PB1-F2 on IAV pathogenesis. PA-X acts as a negative virulence regulator by downregulating the host innate immune and cell death responses (Jagger et al, 2012; Hu et al, 2015). Ablation of PA-X expression increases pathogenicity of 1918 H1N1 and HPAI H5N1 viruses in mice and avian species (Jagger et al, 2012; Hu et al, 2015).

1.1.8 Evolution of IAV:

In response to selection pressures, viral population undergoes constant change over time. Wild aquatic bird populations such as *Anseriformes* (particularly ducks, geese, and swans) and *Charadriiformes* (particularly gulls, terns, and waders) harbor IAVs of 16

HA and 9 NA subtypes (Olsen et al, 2006). Phylogenetic analysis combined with the above finding led to the existing hypothesis that all IAVs in terrestrial poultry and mammals were directly or indirectly derived from avian IAV pool resides in wild aquatic birds (Webster et al, 1992). The evolutionary rates at both the nucleotide and amino acid levels are significantly lower for avian IAVs than mammalian viruses (Gorman et al, 1990). In wild aquatic bird populations, IAVs are fully adapted to their host and do not cause disease suggesting evolutionary stasis and optimal adaptation of these viruses to their hosts (Webster et al, 1992). Although nucleotide changes occur at a similar rate in both avian and mammalian IAVs, they do not result in amino acid substitutions in avian IAVs. On the other hand, it has been documented that all viral gene segments of the mammalian and land-based poultry viruses undergo constant amino acid changes (Wright et al, 2007). Extensive phylogenetic analyses have acknowledged the presence of host-specific virus lineages for several IAV proteins except for the HA and NA (Webster et al, 1992). Seven host-specific lineages have been identified for the NP genes: (I) human viruses, (II) classic swine viruses, (III) old H7N7 equine viruses, (IV) recent equine viruses, (V) H13 gull viruses, (VI) North American avian viruses, (VII) Eurasian avian viruses (Wright et al, 2007). PB1 gene phylogenetic tree differs from other IAV genes. PB1 genes of human H1N1 viruses are clustered with H1N1 classic swine viruses indicating common origin from 1918 H1N1 IAV virus. Furthermore, the PB1 genes of human H2N2 and H3N2 viruses form a different sublineage indicating interspecies transmission of avian PB1 genes into human viruses during 1957 H2N2 and 1968 H3N2 pandemics (Kawaoka et al, 1989). The phylogenetic tree of the NS gene is divided into "A allele", which includes all mammalian NS genes and "B allele" that contains avian

NS genes (Treanor et al, 1989). Phylogenetic analysis of human H1N1 and classic swine IAVs indicates common ancestry (Gorman et al, 1991). On the other hand, two sublineages-an American and a Eurasian – can be defined for all eight segments of avian IAV, which indicates an important role for host species in the evolution of IAV gene pools (Donis et al, 1989).

1.1.9 IAV genetics:

Continuous evolution in each of the eight IAV gene segments results in impressive diversity of IAV. Point mutations or antigenic drift, reassortment or genetic shift, and gene recombination, are the three important mechanisms that contribute to the evolution of IAVs (Webster et al, 1982). Substitutions, insertions, and deletions are the central mechanisms for producing variation in IAV. In general, RNA viruses have high rates of mutation on the order of 1 in 10^4 bases due to the lack of an exonuclease activity among RNA polymerases, which limits their proofreading ability during genome replication (Steinhauer et al, 1992). As a result, each round of IAV replication results in generation of different genotypes commonly called as "quasispecies" and the virus with the best fit in the mixed population can become dominant (Domingo et al, 2005). Thus, low fidelity of RNA polymerase helps the virus to evade host immune response effectively and also assists in rapid adaptation to the new host (Manrubia et al, 2005). Antigenic drift is a mechanism that involves the accumulation of a series of point mutations in the viral genome. Immunological pressure on the glycoproteins, HA and NA is thought to drive antigenic drift (Webster et al, 1992). These point mutations lead to amino acid substitutions over time resulting in antigenic changes in the HA and NA proteins (Nobusawa et al, 1991). As a result, new IAV drift variants with the changes in

the antigenic sites of HA and NA escapes virus neutralizing antibodies induced during previous infection or vaccination (Webster et al, 1992). These new antigenic drift variants cause epidemics and exist normally between 2 and 5 years and eventually get replaced by a different variant (Wright et al, 2007). Antigenic drift is the principal reason behind updating influenza vaccine strains annually for the formulation of seasonal flu vaccine to keep-up with the evolving viruses (Carrat et al, 2007).

Reassortment or antigenic shift is the exchange of viral gene segments in cells infected with two different influenza viruses. It involves major antigenic changes in which a new HA or NA subtype that are distinct from previously circulating strains is introduced into the immunologically naïve population, sometimes leading to high infection rates and pandemics (Schild et al, 1982). 1957 H2N2 and 1968 H3N2 pandemic IAV strains were reassortant viruses that contained HA, PB1 and NA or HA and PB1 segments of avian virus origin in a human genetic background. Emergence of pandemic IAV strains underscores the importance of reassortment in genetics and evolution of influenza viruses (Scholtissek et al, 1978; Kawaoka et al, 1989).

Recombination occurs in IAV when the viral polymerase changes templates during replication or when segments of nucleic acid are broken and rejoined (Wright et al, 2007). Homologous recombination is not a common event for negative-sense RNA viruses; however recombination by template switching can take place. Insertion of 54 nucleotides of 28S ribosomal RNA increased the cleavability of avian IAV HA and increased biological fitness of the virus (Khatchikia et al, 1989). Insertion of 21 nucleotides of the M segment or 30 nucleotides of the NP segment into the HA segment have shown to convert low pathogenic avian IAV into high pathogenic phenotype

(Bowes et al, 2004; Hirst et al, 2004; Pasick et al, 2005; Suarez et al, 2004). In another example, serial passage of A/WSN/22 strain with 24-amino acid deletion in the NA stalk produced variants that replicated efficiently in eggs. Sequence analysis revealed that the variants contained sequences originated from the PB1, PB2, or NP genes (Mitnaul et al, 2000). In general, the event is masked by the low biological fitness of the recombinant viruses, however under selective pressure, recombination may provide a selective advantage to the viral fitness (Wright et al, 2007).

1.1.10 Reverse genetics system:

Generation of negative sense RNA viruses from cloned complementary DNA (cDNA) has revolutionized influenza virus research and contributed immensely to our biological understanding of the pathogen (Wright et al, 2007). Reverse genetics systems with negative sense RNA virus took much longer to develop because the viral RNA is not infectious in either genomic or anti-genomic sense. Segmented nature of the IAV genome posed additional challenge, as one must produce a separate vRNA for each gene segment (Pekosz et al, 1999). As mentioned above, the minimum infectious unit comprises of genomic vRNA encapsidated with NP and three polymerase subunits (Neumann et al, 1999). RNP-transfection method was the first reverse genetics system developed for IAV (Enami et al, 1990). *In vitro* synthesized viral RNA is mixed with purified RdRP and NP to reconstitute viral RNP molecules. Genetically altered viral RNP segments were then transfected into eukaryotic cells that were pre-infected with influenza helper virus to provide the remaining seven vRNPs. In order to distinguish the modified virus from the wild type helper virus, strong selections systems were required (Barclay et al, 1995; Castrucci et al, 1995). To overcome the cumbersome process of *in vitro* reconstitution of

RNP complexes, intracellular production of RNP molecules using RNA polymerase I (Pol I) system was established (Neumann et al, 1994). Pol I is an abundant nuclear enzyme, which transcribes ribosomal RNA that does not contain both 5' cap and 3' poly (A) tail (Zobel et al, 1993). The cDNA encoding viral RNA was inserted between Pol I promoter and terminator sequences. Transfection of resultant construct into eukaryotic cells together with infection of helper virus resulted in the generation of recombinant virus (Flick et al, 1996; Pleschka et al, 1996; Zhou et al, 1998). However, both RNP-transfection method and Pol I systems are dependent on a helper virus and a strong selection process. Further, virus generation efficiency is also very low.

Neumann and colleagues overcame this obstacle and established a highly efficient reverse genetics system to generate recombinant IAV entirely from cloned cDNA (Neumann et al, 1999). To generate recombinant influenza virus, plasmids containing a cDNA for each of the eight viral RNA segments flanked by the Pol I promoter and the Pol I terminator/self cleaving hepatitis delta ribozyme are transfected into cells together with protein expression plasmids (PA, PB1, PB2, NP) (Neumann et al, 1999). Hoffmann and colleagues further modified the system and reduced the number of plasmids to 8 from 12. The cDNA for each of the eight viral RNA segments is inserted between Pol I promoter and the Pol I terminator. This Pol I transcription cassette is flanked by the Pol II promoter and polyadenylation signal. After transfection, both Pol I transcripts for vRNA synthesis and Pol II transcripts for mRNA synthesis are derived from the same cDNA template (Hoffmann et al, 2000). This Pol I-Pol II systems allows researcher to introduce any mutation into the IAV genome and study the role of viral proteins in viral life cycle, pathogenesis and host range restriction (Neumann et al, 2001).

1.1.11 Brief history of influenza pandemics:

Pandemics are global disease outbreaks that spread through large portions of human populations across multiple continents in a relatively short period of time. Influenza pandemics occur when a novel virus distinct from previously circulating strains emerges for which a majority of the population has little or no immunity. The first scientific evidence of influenza pandemics was documented using seroarchealogy. The virus causing the first documented 1889 influenza pandemic has not been identified, but sera collected from people living in that period suggest that it was caused by the H2 or perhaps the H3 subtype of IAV (Masurel et al, 1973). The outbreak appeared to start in Russia in the year 1889 and thereafter spread globally causing most deaths among the very young and elderly population (Dauer et al, 1961). The 1918 pandemic influenza outbreak was caused by H1N1 subtype. The 1918/1919 pandemic outbreak, known as "Spanish influenza", remains unprecedented in its severity killing approximately 675,000 people in United States and up to 50 million people worldwide (Johnson and Meuller, 2002). It resulted in high fatality rates among young children, young adults and pregnant women (Morens et al, 2008). Phylogenetic analysis of the 1918 H1N1 virus obtained from preserved human lung tissues from fatally infected patients suggested avian origin (Reid et al, 2004; Rabadan et al, 2006). Further studies demonstrated that high mortality associated with 1918 pandemic H1N1 virus was the result of a virus of greater virulence made worse by bacterial superinfections at a time when antibiotics was not available (Taubenberger and Kash, 2010).

The 1957 pandemic influenza, also known as "Asian Influenza", was caused by H2N2 subtype. The outbreak started in mainland China and further spread to Hong Kong

and other parts of the world. It caused about 70,000 deaths in the United States and more than 1 million deaths worldwide. Heightened mortality was observed, especially in 5-19 year age group, where case fatality rate reached up to 50% (Wright et al, 2007). Phylogenetic analysis indicated that the 1957 pandemic H2N2 virus originated from a reassortment between human and avian viruses. It acquired three novel gene segments (HA and NA of H2 subtype, and PB1) from avian virus and retained remaining five gene segments from the 1918-derived H1N1 lineage (Scholtissek et al, 1978; Kawaoka et al, 1989). Eleven years later, another pandemic outbreak occurred in Southern Asia before spreading most parts of the world. The pandemic of 1968, also known as "Hong Kong Influenza", was caused by H3N2 subtype. This virus was again a reassortant, with HA of H3 subtype and PB1 genes from an avian source and the remaining six gene segments from a circulating human H2N2 virus (Scholtissek et al, 1978; Kawaoka et al, 1989). When compared to previous pandemic outbreaks, the 1968 pandemic were relatively mild in its severity causing 34,000 deaths in the United States. It is possibly due to presence of pre-existing antibodies to N2 protein in human population (Wright et al, 2007).

1.1.12 The 2009 H1N1 influenza pandemic:

In early April 2009, a new H1N1 IAV virus emerged among humans in Mexico and quickly spread to over 200 countries (Scalera et al, 2009). The 2009 pandemic H1N1 virus emerged from multiple reassortment events between triple reassortant North American swine viruses with Eurasian avian-like H1N1 swine influenza viruses (Garten et al, 2009). Phylogenetic analysis of 2009 pandemic H1N1 virus genome revealed that the novel virus was derived from several viruses that are circulating in pigs for more than 10 years. It is a quadruple reassortant virus with PB2 and PA genes from North American

avian virus origin, PB1 gene from human H3N2 virus origin, HA, NP, and NS genes from classical swine virus origin, and NA and M genes from Eurasian avian-like swine virus origin (Neumann et al, 2009; Smith et al, 2009). The virus disproportionately affected children, young adults, and pregnant woman, as compared to the older populations (Baer et al, 2009), which suggests partial immunity to the virus in the older age groups (Rothberg et al, 2010). The 2009 H1N1 pandemic influenza was mostly a mild, self-limiting URT illness with 2-5% of the confirmed cases required hospitalization in the United States (Jain et al, 2009). According to WHO, this pandemic outbreak caused approximately 1.48 million cases and 25,000 deaths worldwide. But, the actual number of cases could be many folds more as most cases were diagnosed clinically and was not laboratory-confirmed (Morens et al, 2010).

Genomic analysis of the 2009 H1N1 pandemic virus indicated the absence of markers associated with high pathogenicity in avian or mammalian species. However, a D222G substitution was found in the HA gene segment of the 2009 H1N1 pandemic virus isolated from patients with acute respiratory distress worldwide (Girard et al, 2010). The D222G variant shift the receptor specificity of HA from SA α2,6 Gal receptors to dual SA α2,3/α2,6 Gal receptors, thus permitting virus replication in the lower respiratory tract (Liu et al, 2010), which is found to be conserved among avian viruses (Matrosovich et al, 2000).

1.1.13 Swine Influenza Virus (SIV):

Influenza in swine is an acute, contagious, respiratory disease whose severity depends on many factors such as host age, virus strain, and secondary bacterial infections (Easterday, 1980). Swine influenza virus (SIV) was first isolated in the year 1930 in USA

(Shope, 1931). H1N1 lineage in North American pig populations were documented from 1918 coinciding with Spanish influenza pandemic outbreak. Until 1998, classical swine H1N1 (cH1N1) lineage with minimal changes was circulating in pigs (Olsen, 2002). However, by late 1998, a novel triple reassortant (TR) H3N2 SIV emerged and become established in North American swine population. It possessed HA, NA, and PB1 gene segments from human IAVs, M, NS, and NP gene segments from cH1N1 SIVs and PA, and PB2 gene segments from avian IAVs (Zhou et al, 1999; Webby et al, 2000). This specific internal gene combination provided high level of fitness for the TR H3N2 SIVs and they spread rapidly through the North American swine population. Over 20% of the pigs sampled were seropositive for the TR H3N2 SIV as early as the year 2000 (Webby et al, 2000). These viruses showed increased propensity to reassort generating a range of reassortants during the following years. For instance, TR H3N2 SIV had undergone reassortment with cH1N1 SIV producing H1N2, reassortant H1N1 (rH1N1) and H3N1 SIVs (Karasin et al, 2000a; Webby et al, 2004; Ma et al, 2006). Currently, the H3N2, rH1N1 and H1N2 SIVs have become endemic and co-circulate in most North American swine population (Choi et al, 2002; Richt et al, 2003; Webby et al, 2003). Reassortant SIVs that have become endemic in swine population contain triple-reassortant internal gene (TRIG) cassette comprising internal genes representing the PA and PB2 genes of avian origin, NS, NP and M genes of classical swine origin, and the PB1 gene of human origin (Vincent et al, 2008; Ma et al, 2009).

1.1.14 Swine as intermediate hosts:

Swine plays an important role in the ecology of influenza viruses. It can support reassortment of viruses from different host species and can also act as maintenance host

that supports adaptation of avian influenza viruses to mammalian host. Pig tracheal cells possess both SA α2,6 Gal and SA α2,3 Gal receptors and have been postulated to be a "mixing vessel" of IAVs from avian and human sources (Ito et al, 1998). Several independent introductions of avian viruses (H1N1, H3N3, H4N6, and H9N2) to pigs have occurred in Europe and Canada (Karasin et al, 2000b; Karasin et al, 2004). However, there has been limited serologic evidence for AIV infection in the pig population of United States (Olsen et al, 2000). Reassortment between avian, swine and human IAV occur in pigs as exemplified by periodical emergence of strains from pigs such as the 2009 pandemic H1N1 (Dawood et al, 2009; Peiris et al, 2009). Hence, surveillance and monitoring of emerging influenza viruses in swine population have become increasingly important from the public health standpoint.

1.1.15 Cross-species transmission of SIV:

Both viral and host factors play a role in cross-species transmission and all eight gene segments are involved in virus species specificity (Horimoto and Kawaoka, 2001; Neumann and Kawaoka, 2006). Cross-species spillover of SIVs occur frequently (Webster et al, 1992) and have been recorded in number of hosts, including humans, turkeys, and ducks (Suarez et al, 2002; Olsen et al, 2003; Webster et al, 2009). Turkeys appear to be most susceptible to SIV infection and numerous instances of swine-to-turkey interspecies transmission events were recorded (Suarez et al, 2002). TR H1N2 and H3N2 SIVs crossed species barrier and caused multiple outbreaks in turkey farms situated in Minnesota and North Carolina. Serological evidence proved that sustained TR SIV transmission occurred between turkeys (Choi et al, 2004; Tang et al, 2005). Similar to swine, turkeys contains both SA α2,6 Gal and SA α2,3 Gal types of receptors.

Introduction of SIVs to turkeys, which may be co-infected with avian viruses, provide opportunities for the emergence of novel reassortants.

Multiple reports of swine-to-human transmission have been reported and almost all of the major global lineages of SIVs have been detected in humans. Comprehensive report of SIV infections in humans in the year 2007 found that 14% of these infections were lethal (Myers et al, 2007). It has been shown that the people who work closely with pigs were at higher level of risk for zoonotic SIV infection (Myers et al, 2006). Elevated serologic titers have been detected in high-risk groups including farmers, meat processing workers, and veterinarians indicating occupational exposure to the SIV infection (Vincent et al, 2008). Most of the SIV infections in humans were typically self-limiting with the exception of one outbreak occurred in early 1976 among military personnel at Fort Dix, New Jersey. The outbreak resulted in 1 death and severe respiratory illness in 13 soldiers (Webster et al, 2009). The cause of the outbreak was determined to be a cH1N1 virus. Serological studies showed that as many as 230 soldiers were infected indicating substantial human-to-human transmission of cH1N1 virus (Gaydos et al, 1977). When the 2009 pandemic H1N1 virus became enzootic in swine population, the virus continued to reassort with other circulating SIVs. In recent years, infection with novel H3N2 variant virus (H3N2v) containing M gene segment from 2009 pandemic H1N1 virus in the circulating SIV H3N2 virus background was detected in human populations (Nelson et al, 2012). Infections with H3N2v have been mostly associated with prolonged exposure to pigs especially at agricultural fairs and rarely result in sustained person-to-person transmission (Greenbaum et al, 2015).

1.1.16 Novel H7N9 outbreak in China: Implication for influenza evolution and cross-species transmission:

In 2013, a novel H7N9 (nH7N9) AIV emerged in humans in China. nH7N9 is an reassortant avian-origin IAV originating from the H7N3, H7N9, and H9N2 AIV (Lai et al, 2013). The nH7N9 viruses isolated from humans contained features related to human adaptation such as Q226L mutation in the receptor-binding site of HA that enhances virus binding to human sialic acid receptors. It also contained E627K and D701N mutations in the PB2 that augments virus replication in the human URT (Gao et al, 2013; Chen et al, 2013). *In vivo* experiments in ferrets showed that the nH7N9 can replicate both in URT and LRT of infected animals and also could transmit efficiently via direct contact among ferrets (Zhu et al, 2013). Live poultry markets provide an suitable environment for the selection of mutants that facilitate nH7N9 binding to receptors in URT leading to virus replication and spread (Koopmans et al, 2013). It also contained deletions in NA stalk region, which is an characteristic of AIV adaptation from aquatic birds to land-based poultry (Lai et al, 2013).

Aquatic birds such as ducks and geese are natural reservoirs of AIVs. Land-based poultry such as chicken, quail, and turkeys contains both SA α 2,3 Gal to SA α 2,6 Gal receptors in their trachea and intestine (Gambaryan et al, 2002; Wan et al, 2006; Guo et al, 2007; Costa et al, 2012). The mixing of multiple species of poultry along with mammals in live poultry markets provides a conducive environment for reassortment and interspecies transmission of IAV (Bao et al, 2013). Some of the LPAI viruses such as H9N2, H7N2, H7N7, and H7N9 does not possess multiple basic amino acids at the HA cleavage site probably leading to subclinical infections and silent spread among the birds

in live poultry markets (Belser et al, 2008; Jonges et al, 2011; Shi et al, 2013). Researchers believe that the asymptomatic spread of AIV in live poultry markets facilitate the viruses to acquire mutants essential for human receptor specificity and overcoming the species barrier (Nguyen et al, 2001). Hence, the live poultry market is important in the emergence, spread, and maintenance of reassortant IAV and in the selection of mutants capable of cross-species transmission (Fournie et al, 2012).

1.1.17 H5N2 outbreak:

Since 2012, H5N2 outbreaks occurred sporadically in domestic birds around the world (Lee et al, 2014). Analysis of H5N2 isolates collected from Taiwan in the year 2012 showed that these viruses contain multiple basic amino acids at the HA cleavage site indicating high pathogenicity to chickens. Further, phylogenetic analysis showed that the virus was generated by reassortment between 1994 Mexican-like H5N2 virus and a local enzootic H6N1 virus. High similarity of the H5N2 virus to the Mexican vaccine strain indicates that the inadequately inactivated or attenuated vaccines might have caused this outbreak (Lee et al, 2014). In 2015, H5N2 outbreaks were recorded in chicken and turkey farms in the Mid-Western region of the United States. Migratory waterfowl was suspected to be involved in the current H5N2 outbreak (CIDRAP, 2014).

Very recently, the US Department of Agriculture (USDA) reported to the World Organization for Animal Health (OIE) that H5N2 was found in a wild pintail duck, and a H5N8 virus was found in a captive wild gyrfalcon that was fed on hunter-killed birds from Whatcom County, Washington State, which borders the Abbotsford area of British Columbia, the site of recent H5N2 outbreaks in poultry (Roos, 2014). These viruses appear to be similar to viruses found in Canada, Italy, Japan, Germany and other

countries in domestic and wild fowl with a popular view on transmission through migratory aquatic birds. But, this notion is being questioned by many in the U.S (Roos, 2015).

1.2 IAV PB1-F2 Protein:

1.2.1 Discovery of PB1-F2 and its properties:

As described above, gene segment 2 of IAV has multiple AUG initiation sites and it encodes three proteins, namely, PB1, PB1-F2 and N40 (Wise et al, 2009; Wise et al, 2011). PB1-F2 is a small non-structural protein encoded by the alternate +1 open reading frame through leaky ribosomal scanning mechanism. It was first discovered in A/PuertoRico/8/34 (H1N1) (PR8) IAV strain while searching for novel peptides recognized by $CD8^+$ T cells during IAV infection. It has a short half-life of approximately 30 minutes and undergoes rapid proteasome-dependent degradation (Chen et al, 2001). The PB1-F2 protein varies in size from 11 to 101 amino acids long with truncations at the amino (N) or carboxy (C) termini have been reported (Pasricha et al, 2012). Many of the IAV strains encode a full-length PB1-F2 protein of 90 amino acids and a molecular weight of 10.5 kDa (Lamb et al, 2001).

1.2.2 Structure of PB1-F2 Protein:

Initial studies found that the PB1-F2 protein has propensity to form an amphipathic helix comprising amino acid residues Leu (69)-Phe(83) of the protein. (Chen et al, 2001). Further studies showed that PB1-F2 protein consists of two independent structural helical domains connected by a flexible and unstructured hinge region. Hydrophobic C-terminal domain contains one extended helix with major oligomerization domain, whereas disordered “spaghetti strand” N-terminal domain consists of two short

helices (Henklein et al, 2005; Bruns et al, 2007). PB1-F2 molecule has an inherent tendency to undergo oligomerization resulting in the formation of variably sized membrane pores in planar lipid bilayers (Chanturiya et al, 2004; Bruns et al, 2007). In another study, PB1-F2 protein was classified as a member of intrinsically disordered protein, which can switch from a random to alpha (α)-helical or beta (β)-sheet secondary structure depending on the environment. It was shown that PB1-F2 protein self-oligomerize resulting in the formation of amyloid-like fibers in infected cells that leads to permeabilization of cellular membranes in a sequence-specific manner (Chevalier et al, 2010). PB1-F2 has also been characterized as a phosphoprotein and its function is regulated by protein kinase C (PKC)-mediated phosphorylation. Thr (27) and Ser (35) residues of the PB1-F2 protein have been mapped as PKC phosphorylation sites (Mitzner et al., 2009).

1.2.3 Prevalence of PB1-F2 protein:

Comprehensive GenBank search revealed that different subtypes of IAV encode varying sizes of PB1-F2 like 90 aa full-length form (63.71%), 57 aa C-terminally form (21.2%) and 52 aa N-terminally truncated form (11.3%) (Pasricha et al, 2012). Interestingly, the proapoptotic and proinflammatory role of PB1-F2 is mapped to C-terminal end (Alymova et al, 2011; Alymova et al, 2014), whereas the N-terminal end is reported to regulate the PB1 expression and polymerase activity (Kosik et al, 2011). However, the effect of C- and N-terminally truncations and its functional utility in different host still remain unanswered. It would be interesting to explore whether IAV balances its pathogenicity by mutating PB1-F2 in strain-specific manner. The 1918 H1N1 virus that caused Spanish flu encoded a full-length 90 aa PB1-F2 (McAuley et al, 2007).

However, since 1949, most H1N1 isolates from human and swine harbor incomplete PB1-F2 protein with truncation either at the N- or C-terminal ends. PB1-F2 of human H1N1 viruses terminates after 57 aa whereas classic swine H1N1 sequences have in-frame stop codons after 11^{th}, 25^{th} and 34^{th} codons (Zell et al, 2007; Pasricha et al, 2012). Similarly, 42% of swine IAV isolates (H1N1, H1N2, H3N2) from Europe also exhibits stop codons after 11^{th}, 25^{th} and 34^{th} codons (Zell et al, 2007).

Analysis of PB1-F2 sequences of H2N2 and H3N2 subtypes revealed that 98.8% and 82.03% of the isolates express a full-length PB1-F2 protein, respectively (Pasricha et al, 2012). The 1957 H2N2 and 1968 H3N2 pandemic IAV encoded a full-length PB1-F2 protein whereas the recent 2009 pandemic H1N1 virus encodes a 11-amino-acid C-terminally truncated form of PB1-F2 protein (McAuley et al, 2010; Hai et al, 2010). Nearly all avian IAV isolates encode a full-length PB1-F2 including the recent highly pathogenic zoonotic avian IAV strains such as H5N1, H7N7, H7N9, and H9N2 (Alymova et al, 2014). However, recent H7N9 isolates from China contain a N-terminal truncated (52aa) and C-terminal truncated (76aa) PB1-F2 (Wei et al, 2015). The presence of full-length PB1-F2 in most of the avian IAV isolates suggests that this protein has some functional utility in bird's gut infection during viral life cycle. It has been shown that PB1-F2 is important for disease severity and systematic spread of H1N1 virus in mallard ducks (Schmolke et al, 2011).

1.2.4 Cellular and humoral response to the PB1-F2 protein:

PB1-F2 protein was first discovered based on its ability to generate a robust $CD8^{+}$ cytotoxic T cells response during PR8 IAV infection (Chen et al, 2001; La Gruta et al. 2008; Thomas et al, 2013). During IAV infections, antibodies are generated against both

structural proteins and non-structural proteins. But only antibodies generated against HA and NA can effectively neutralize virus infectivity. Human immune system recognizes PB1-F2 protein and therefore has the ability to induce both humoral and cell-mediated immune responses (Krejnusoa et al, 2009). Anti-PB1-F2 antibodies were detected in the convalescent sera of humans recovering from the H5N1 infection during the 2004 outbreak in Vietnam (Khurana et al, 2009). Antibodies against PB1-F2 protein could be detected both in mouse and human convalescent sera via immunoprecipitation or immunofluorescence assays. During IAV infection, antibodies are induced both against the C-terminus and N-terminus part of PB1-F2 protein (Krejnusova et al, 2009; Khurana et al, 2009; Kosik et al, 2013). However, the biological relevance of anti-PB1-F2 specific antibodies seems to be very complex. PB1-F2 specific antibodies directed against N-terminus of PB1-F2 do not significantly affect the course of infection whereas antibodies against C terminus of the protein reasonably protect mice against IAV infection (Kosik et al, 2013).

1.2.5 Subcellular localization and apoptosis:

PB1-F2 protein localizes predominantly to mitochondria, but is also found diffusely distributed in the cytoplasm and in the nucleus of infected cells (Chen et al, 2001). PB1-F2 protein has been shown to permeabilize mitochondrial membranes resulting in loss of mitochondrial membrane potential (MMP) and efflux of cyt c that activates the caspase cascade and initiates apoptosis. However, the exact mechanism by which PB1-F2 induces apoptosis is not clear. Initial studies proposed that the C-terminus of the PB1-F2 protein contains mitochondrial targeting sequence (MTS) and forms a positively charged amphipathic α-helix. Region 69 to 82 aa was mapped as minimal MTS

and 65 to 87 aa was mapped as optimal MTS. Hydrophobic stretch of PB1-F2 protein interacts with the transporter outer membrane (TOM) receptor facilitating the translocation of α-helix through the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM). This process results in the loss of MMP leading to the initiation of apoptosis (Gibbs et al, 2003). However, precise mapping of MTS is surrounded with some discrepancies. Using different fusion FLAG tags, one research group mapped region 46 to 75 aa as the MTS region, of which Lys (73)-Arg (75) was found minimally essential for functional MTS. It was shown that PB1-F2 protein affects the cell cycle and retains the mitochondria in the Synthesis (S) phase. Mitochondria are fragmented in S phase and its morphology altered resulting in the loss of MMP and activation of apoptosis (Yamada et al, 2004). PB1-F2 with optimal MTS primarily localized to mitochondria, whereas PB1-F2 with less optimal MTS are prevalently localized to cytoplasm and nucleus (Chen et al, 2010).

Structural studies with synthetic PB1-F2 protein shown that PB1-F2 α-helix interacts with lipid head group charges and a transmembrane IMM electric field. It results in the incorporation of PB1-F2 α-helix in the membrane leading to the formation of potential dependent membrane pores in planar lipid bilayers. These membrane pores depolarizes MMP resulting in apoptosis (Chanturiya et al, 2004). However, another study claimed that self-oligomerization of PB1-F2 protein leads to the formation of non-selective water-filled protein pores in planar lipid membranes. These non-selective protein pores disrupts IMM depolarizing MMP and initiates cell death (Henkel et al, 2010). PB1-F2 protein also indirectly interacts with mitochondrial membrane proteins such as adenine nucleotide translocator 3 (ANT3) present in IMM and voltage-dependent

anion channel 1 (VDAC1) present in OMM resulting in the formation of the permeability-transition pore complex (PTPC). ANT3 interacts with C-terminus and VDAC-1 interacts with both N- and C-terminus of PB1-F2 protein. PTPC play a important role in the permeabilization of the mitochondria and activates apoptosis pathway (Zamarin et al, 2005; Danishuddin et al, 2010). Phosphorylation of PB1-F2 protein at Thr (27) and Ser (35) aa position by PKC was found to be important for caspase 3 activation and promotion of cell death (Mitzner et al, 2009). Till date, there is no consensus on how PB1-F2 interacts with mitochondria and the consequences of such interaction.

1.2.6 Proapoptotic role of PB1-F2 protein is cell-type- and virus strain-specific:

Proapoptotic effect of PB1-F2 protein was observed in a cell-type dependent manner. It was more conspicuous in immune competent cells than in epithelial cells, thus affecting the ability of the host to mount effective immune response against IAV infection (Chen et al, 2001; Coleman, 2007). Initial studies proposed that proapoptotic function of PB1-F2 of PR8 IAV causes cell death of antigen-presenting cells (such as macrophages and dendritic cells) resulting in delayed virus clearance by host immune system (Zamarin et al, 2006). It has also been shown that PB1-F2 protein of 1918 H1N1 virus increases pathogenicity by aggravating cell death responses in infected mice (Kash et al, 2006; McAuley et al, 2007). It was also found that the ability of PB1-F2 protein to cause apoptosis is virus-strain specific. PB1-F2 peptide studies showed that PR8 PB1-F2 causes cell death in both epithelial cells and immune cells whereas 1918 H1N1 PB1-F2 induced apoptosis only in immune cells. However, in the context of viral infection, only PR8 virus induced apoptosis in both epithelial cells and immune cells and none of the

20th century pandemic strains induced apoptosis (McAuley et al, 2010). On the other hand, PB1-F2 in pandemic IAV strains such as 1957 H2N2, 1968 H3N2, and HPAI H5N1 virus does not causes apoptosis in both epithelial and immune cell lines. Further, it was found that PB1-F2 protein of PR8 IAV promotes mitochondrial pathway of apoptosis through the activation of the pro-apoptotic Bcl-2 family effector proteins BAX and BAK (McAuley et al, 2010). Virus-strain specific nature of its proapoptotic function is further confirmed by another study that showed PB1-F2 protein of H5N1 strain does not localize to mitochondria and also does not promote cell death (Chen et al, 2010). Taken together, the proapoptotic function of PB1-F2 is found to be cell-type- and virus strain-specific and does not affect pathogenesis of IAV in general (McAuley et al, 2010).

1.2.7 Co-localization of PB1-F2 protein with PB1 regulates polymerase activity:

The PB1-F2 protein indirectly regulates viral polymerase activity by interacting with PB1 protein of polymerase complex. PB1-F2 co-localizes with PB1 resulting in the nuclear retention of PB1 protein in the late phase of replication and thus enhances polymerase activity. Lack of PB1-F2 during *in vitro* IAV infection resulted in the cytoplasmic localization of PB1 and thereby decreases viral polymerase activity. Mutant viruses that lack functional PB1-F2 reading frame showed impaired spread in cell monolayers resulting in smaller plaque phenotype (Mazur et al, 2008). The effect of PB1-F2 protein on viral polymerase activity was found to be cell-type- and virus strain-specific (McAuley et al, 2010). Further studies identified that the presence of PB1-F2 protein resulted in the increase of multiple viral proteins such as NP, M1, and NS1 expression in addition to PB1 protein. It was also found that N-terminal region of PB1-F2 protein was responsible for the increased PB1 and other viral protein expression (Kosik et

al, 2011). However, the effects of PB1-F2 on *in vitro* polymerase activity did not translate to differences in lung viral load and IAV pathogenicity in mice (McAuley et al, 2010).

1.2.8 Contribution of the PB1-F2 protein to IAV pathogenicity:

1.2.8.1 Inflammation and pathogenic effects of PB1-F2:

PB1-F2 protein plays an important role in the pathogenicity of primary influenza virus infection in mammalian and avian hosts. Effects of PB1-F2 appear to be largely mediated through interactions with the host immune system. Multiple studies showed that PB1-F2 protein from certain IAV modulates inflammatory responses manifested as enhanced infiltration of neutrophils, monocytes, and macrophages into the lungs and airways, accompanied by increases in proinflammatory cytokines. This results in extensive consolidation and tissue damage in the lungs of infected host (Zamarin et al, 2006; McAuley et al, 2007). In particular, it was shown that C-terminal region of PB1-F2 increases cellularity in bronchoalveolar lavage fluid and enhanced lung inflammatory response resulting in increased lung immunopathology (McAuley 2010). Four amino acid residues 62L, 75R, 79R, and 82L in the C-terminal region of PB1-F2 protein are mapped for the proinflammatory phenotype of IAV (Alymova et al, 2011). All the 20th century pandemic IAVs such as 1918 H1N1, 1957 H2N2, and 1968 H3N2, and the HPAI H5N1 as well contained all four inflammatory residues. In addition, recently emerged zoonotic AIVs such as H7N7, H7N9, and H9N2 strains also contain all four inflammatory markers (Alymova et al, 2014). High throughput screening using yeast two-hybrid system identified macrophage migration inhibitory factor (MIF) as PB1-F2 interacting partner. MIF is an important regulator of innate immunity and acts as

inflammatory cytokine by suppressing anti-inflammatory effects of glucocorticoids. PB1-F2 interaction with MIF could mediate proinflammatory function during IAV infection (Guan et al, 2012).

1.2.8.2 PB1-F2 modulates innate immune response:

IAV infected cells mount a strong antiviral response to restrict virus replication. During IAV infection, viral RNA is recognized by RIG-I and type I interferon (IFN) is produced resulting in antiviral state (Chignard et al, 2005; Chignard et al, 2007; Varga et al, 2011). IFN antagonism strategies used by IAV are more complex and it possesses multiple IFN antagonists (NS1, PB1, PB2, PA, PB1-F2) (Garcia-Sastre et al, 1998; Graef et al, 2010; Iwai et al, 2010). PB1-F2 protein exhibits IFN antagonistic activity by interfering with RIG-I RNA-sensory pathway at the level of mitochondria antiviral signaling (MAVS) protein complex and inhibiting the activation IFN regulatory factor 3 (IRF 3). They also found that IFN antagonism function of PB1-F2 works in conjunction with NS1. (Varga et al, 2011; Dudek et al, 2011). In contrast, another group reported that PB1-F2 protein exacerbates IFN-β expression through the activation of NF-kB pathway during IAV infection of epithelial cells but not during immune cells infection (Delmas et al, 2010). Analysis of global transcriptional response of the mouse respiratory tract during IAV infection showed that PB1-F2 protein strongly influences the early host response by significantly increasing the expression of genes associated with inflammation and identified IFN-γ as a central regulator (Delmas et al, 2011). It is interesting to hypothesize that PB1-F2 of highly pathogenic strains antagonizes early viral response resulting in increased viral growth, which in turn leads to an dysregulated cytokine and inflammatory response in lungs. It was also shown that PB1-F2 activates NOD-like

receptor family, pyrin domain containing 3 (NLRP3) inflammasome, which induces secretion of pyrogenic cytokine interleukin-1β (IL-1β) resulting in increased cellular recruitment to the airways and lung inflammation during IAV infection in mice (McAuley et al 2013).

1.2.8.3 Role of PB1-F2 in exacerbating secondary bacterial infection:

IAV predisposes hosts to secondary bacterial infections during or immediately following infection. Bacterial pneumonia is a significant contributor to morbidity and mortality in patients infected with IAV (Simonsen, 1999). *Streptococcus pneumoniae, Staphylococcus aureus,* and *Streptococcus pyogenes* are the major pathogens responsible for the secondary bacterial pneumonia (Sethi, 2002; Huber et al, 2012). Multiple factors such as damage to respiratory epithelium, depressed mucociliary clearance, altered host immune responses, loss of alveolar macrophages contributes to increased bacterial cell adherence and tissue invasion during IAV infection (McCullers, 2011). Bacterial co-infection significantly modulates the lung immune responses. For instance, *S. pneumoniae* negatively regulates the influenza-specific CD8+ T cell response resulting in increased morbidity and mortality (Blevins et al, 2014). Increased inflammation due to expression of PB1-F2 alters lung environment promoting both the frequency and severity of secondary bacterial pneumonia. It was also shown that PB1-F2 of 1918 H1N1 virus predisposes the lungs of infected mice for secondary bacterial infections more efficiently than PR8 IAV indicating strain-specific function (McAuley et al 2007). Presence of cytotoxic residues (68I, 69L and 70V) in the PB1-F2 of PR8 IAV enhanced lung pathology and predisposed to secondary bacterial infection in mice (Alymova et al, 2014). Further studies found that proinflammatory motif (62L, 75R, 79R, and 82L) in the

PB1-F2 of 1968 pandemic H3N2 virus enhance lung inflammation and primes for secondary bacterial pneumonia in mice. Conversely, presence of noninflammatory motif (P62, H75, Q79, and S82) as found in seasonal human H3N2 isolates mediated antibacterial effect (Alymova et al, 2011). It was proposed that proinflammatory phenotype present in the pandemic IAV that emerged from avian reservoir is detrimental to the virus and hence during evolution and adaptation to the mammalian lungs, mutation from proinflammatory to noninflammatory motif with antibacterial function would be of benefit to virus. At present, it is not known when in the course of IAV infection, secondary bacterial pathogens invade and induce pneumonia in the host. It is also not clear whether there are any differences between different bacteria in establishing post-influenza secondary pneumonia.

1.2.9 N66S polymorphism:

Alignment of amino acid sequences of isolates collected during 1997 H5N1 outbreak in Hong Kong showed that N66S polymorphism was highly conserved among high virulence phenotypes in mice. *In vivo* studies showed that serine at position 66 instead of asparagine in the PB1-F2 of 1997 H5N1 and 1918 H1N1 viruses caused cytokine dysregulation in lungs resulting in increased pathogenicity in mice (Conenello et al, 2007a; Conenello et al, 2007b). Transcriptional profiling of lungs from PB1-F2 66S-infected mice showed lower levels of IFN-β, RIG-I, Mx-1, and STAT-1 until day 3 post infection indicating modulation of innate immune responses by N66S variant. Thus, N66S mutation allows PB1-F2 to delay interferon response, allowing for unchecked viral replication, increased infiltration of monocytes and neutrophils, cytokine dysregulation in lungs and ultimately leading to increased disease severity in mice (Conenello et al, 2011).

H5N1 PB1-F2 with N66S mutation does not localize to mitochondria and distributed diffusely throughout cytoplasm and nucleus. N66S polymorphism efficiently invades the CNS and replicates in the brain of mice indicating neurotropic spread and enhanced pathogenicity in mice. However in ducks, mutation at position 66 played only a minor role in pathogenesis. This indicates that N66S polymorphism probably impacts pathogenicity only in mammals but not in avian species (Schmolke et al, 2011). GenBank search for the presence of N66S polymorphism in IAV strains showed that it is mostly present in avian isolates (77.9%). On the other hand, human and swine isolates carries N66S mutation very minimally (Chakrabarti et al, 2013).

1.2.10 2009 H1N1 PB1-F2 puzzle:

20th century pandemic IAVs such as 1918 H1N1, 1957 H2N2, and 1968 H3N2 have full-length PB1-F2 protein. In contrast, PB1-F2 of 2009 pdmH1N1 contains three in-frame stop codons at 12, 58, and 88 aa position preventing the expression of full-length protein. Restoration of PB1-F2 expression had minimal effects on its virulence in mouse and swine models. Although N66S mutation recreated in PB1-F2 of pdm09 H1N1 modulate early immune response, it failed to exacerbate virulence significantly in mice and swine (Hai et al, 2010; Pena et al, 2012). When the amino acid sequences of PB1-F2 protein of different IAVs were compared, the C-terminus of restored PB1-F2 of 2009 pdmH1N1 virus differs significantly from other 20th century pandemic viruses and highly pathogenic H5N1 avian influenza viruses. It does not contain most of the molecular signatures considered necessary for virulence (Alymova et al, 2014). Thus, it is compelling to argue that the possession of certain unique residues in hitherto pandemic

viruses is what makes them lethal while other strains despite their high transmission potential fail to induce high mortality due to the lack of these unique residues.

1.3 Autophagy and Mitophagy:

1.3.1 Autophagy:

Autophagy is a ubiquitous catabolic process in which cytoplasmic proteins and organelles are sequestered and degraded in lysosomal dependent manner. The term autophagy derived from Greek means "self-eating" (Levine et al, 2004). It is evolutionarily conserved from eukaryotes to metazoans (Dong et al, 2013). Basal level of autophagy is found in most cells. It is required for the normal clearance of misfolded proteins and damaged/old organelles, which are potentially deleterious and can cause cell damage. Deregulated autophagy plays an important role in the pathogenesis of many human diseases such as neurodegenerative diseases, cancer, infectious diseases and inflammatory bowel disorders. (Levine et al, 2008). Based on the type of cargo delivery, there are three types of autophagy – chaperone-mediated autophagy, microautophagy, and macroautophagy (autophagy) (Mizushima et al, 2008; Ravikumar et al, 2010).

1.3.1.1 Microautophagy:

Microautophagy involves direct engulfment of soluble or particulate cellular constituents into lysosomes. It translocates cytoplasmic cargo into the lysosomes for degradation via direct invagination and vesicles then pinch off into the lysosomal lumen. Microautophagy of soluble substrates are induced by nitrogen starvation or rapamycin via complex regulatory signaling pathways (Li et al, 2012). Vacuolar transporter chaperone, VTC complex is important for the process of microautophagy. The main functions of

microautophagy are maintenance of organelle size and membrane composition, and cell survival under nitrogen restriction (Uttenweiler et al, 2007; Mijaljica et al, 2011).

1.3.1.2 Chaperone-mediated autophagy:

Chaperone-Mediated Autophagy (CMA) is a selective autophagic pathway for the degradation of soluble cytosolic proteins in lysosomes (Cuervo, 2009). In contrast to the other forms of autophagy, CMA does not require the formation of intermediate vesicles, membrane fusion, or membrane deformity of any type. Instead, soluble substrate proteins from the cytosol directly cross the lysosomal membrane to reach the lumen by a translocation protein complex (Kaushik et al, 2008; Cuervo et al, 2014). Cytoplasmic protein substrates are recognized by a chaperone complex, consisting of heat shock cognate 70 (Hsc70) and its cochaperones, present in the cytoplasm (Orenstein et al, 2010). The substrate proteins are identified by the presence of a peptapeptide target motif (KFERQ-like motif) in its amino acid sequence and are targeted to the lysosomal membrane (Dice, 1990; Hoffman et al, 2012). At the lysosomal membrane, the targeted protein interacts with lysosomal receptor, lysosomal associated membrane protein type 2A (LAMP-2A), and directly imported across the lysosomal membrane into lumen via lysosome resident chaperone (Cuervo and Dice, 1996). Dysfunction of CMA is associated with several human neurodegenerative diseases and aging (Massey et al, 2006).

1.3.1.3 Macroautophagy (Autophagy):

In macroautophagy (herein autophagy), a *de novo* isolation membrane is generated, which sequesters cytoplasm and organelles destined for lysosomal degradation. This isolation membrane then elongates and seals into a double-membrane

vesicle – the autophagosome. After lysosomal fusion, cytoplasmic and/or microbial cargo present inside the autophagosomes are degraded by lysosomal enzymes (Mizushima et al, 2002; Yang et al, 2010). There are two types of macroautophagy: Non-selective autophagy occurs under stress condition to provide energy and macromolecules through the catabolism of intracellular constituents to maintain essential cellular processes until the return of normal growth conditions. On the other hand, Selective/cargo-specific autophagy occurs under nutrient-rich conditions that plays main role in removal of damaged or unwanted cell organelles and toxic protein aggregates from the cytoplasm. (Youle et al, 2011).

1.3.2 Mechanisms of autophagy:

It involves four sequential stages and over 30 Autophagy (Atg) gene products have been identified that play important roles in the regulation and execution of autophagy (Xie et al, 2007).

1.3.2.1 Autophagy induction and vesicle nucleation: Mammalian target of rapamycin (mTOR) kinase plays a central role in constitutively suppressing induction of autophagy by binding and phosphorylating the ULK1/2-Atg13-FIP200 complex. Under autophagy promoting conditions such as nutrient deprivation or rapamycin (a selective inhibitor of mTOR) treatment, mTOR is repressed leading to hypophosphorylation of the ULK1/2-Atg13-FIP200 complex. This process results in the induction of autophagy and allows the isolation membrane to expand (Hosokawa et al, 2009). Autophagy induction activates phosphatidylinositol 3-kinase (PI3K) Vps 34, which promotes the formation of phosphatidylinositol 3-phosphate (PIP3) residues on the isolation membrane. Vps 34 associates with other protein such as Beclin 1, Vps15, Atg14 and forms the minimal class

III PI3K complex to mediate the formation of the phagophore. Phagophore serve as docking sites for other autophagy-promoting proteins (Itakura et al, 2008). Beclin 1 is the central modulator of autophagy.

1.3.2.2 Autophagosome elongation and completion: Growth and closure of the autophagosome membrane involves two ubiquitin-like conjugation systems, Atg12 and microtubule-associated light chain 3 (LC3) protein, which associate with the phagophore and promote its curvature and expansion. After cleavage by the protease Atg 4, E2-like enzyme Atg3 adds phosphatidylethanolamine (PE) to the C-terminus of the cleaved LC3. This process results in the formation of LC3-II or LC3-PE, which stably associates with both outer and inner membranes of the growing autophagosome. LC3 is the most commonly used marker for studying autophagy (Klionsky et al, 2008).

1.3.2.3 Docking and Fusion: Next step of autophagy involves the fusion of completed autophagosome with lysosomes and maturation into autophagolysosomes. It appears that lysosomal protein LAMP-2 and GTP-binding protein Rab 7 aid in this process. But the process is not very well understood (Kudchodkar et al, 2009).

1.3.2.4 Vesicle breakdown and degradation: Upon autophagolysosome formation, the lysosomal cathepsins B, D, and L as well as other enzymes degrade inner membrane and its luminal constituents. Byproducts such as amino acids and lipids are exported back into the cytoplasm to generate new macromolecules (Xie et al, 2007; Levine et al, 2008).

It is also important to understand the mechanism by which cytoplasmic cargo is selected for autophagic sequestration. In addition to targeting proteins for proteasomal degradation, ubiquitin plays an important in directing protein aggregates and oligomeric proteins for autophagy (Kim et al, 2008; Kirkin et al, 2009b; Korolchuck et al, 2009a).

Sequestosome 1 (SQSTM1/p62) and neighbor of BRCA gene 1 (NBR1) proteins can bind to both ubiquitin and LC3 and serves as adapters to target ubiquitinated proteins to autophagosomes (Pankiv et al, 2007; Kirkin et al, 2009a; Korolchuck et al, 2009b). After the fusion of autophagosomes with lysosomes, p62 gets degraded indicating the completion of autophagy process (Bjorkoy et al, 2005). Disruption in the process of autophagy leads to the accumulation of p62 with ubiquitin-containing aggregates resulting in cellular stress (Rusten et al, 2010).

1.3.3 Interplay between apoptosis, necrosis, and autophagy:

Depending on the cellular context and death trigger, both apoptosis and necrosis modes of cell death often interact in a balanced fashion with autophagy, which is predominantly considered as a cell survival mechanism (Nikoletopoulou et al, 2013). The crosstalk between apoptosis and autophagy is mediated by functional interaction of Beclin-1 and anti-apoptotic proteins (Pattingre et al, 2005). Apoptotic stimuli can lead to caspase-mediated cleavage of Beclin 1 and thereby blocks the autophagy process (Vellai et al, 2005). On the other hand, Lee et al. showed that anti-apoptotic protein such as FADD-like interleukin 1 beta-converting enzyme (FLICE)-like inhibitor proteins also act as a negative regulator of autophagy indicating complex cross talk between the autophagic and apoptotic machinery (Lee et al, 2009). Similar to apoptosis, the interplay between autophagy and necrosis is also a complex phenomenon. Autophagy has been shown to either suppress or promote necrosis (Bell et al, 2008; Bonapace et al, 2010). Inhibition of autophagy via the mTOR-signaling pathway results in the activation of necrosis pathway (Wu et al, 2009). On the other hand, activation of poly (ADP-ribose) polymerase 1 (PARP1), an important player in the activation of necrosis promotes

autophagy either through inhibition of the mTOR signaling pathway (Gwinn et al, 2008) or through activation of ULK1 complex (Egan et al, 2011).

1.3.4 Autophagy and Viral Infections:

Being a cellular housekeeper, autophagy plays an important role in health and disease. As obligate intracellular parasites, survival of viruses is intimately linked to their ability to not only exploit host cellular machinery for their replication but also able to circumvent cellular anti-viral defenses that prevent their replication. In response to viral infections, autophaghy machinery can serve as an intrinsic immune defense and also influence the innate & adaptive immune system responses against them. By co-evolving with hosts, viruses have developed diversified strategies to either avoid or exploit this machinery for their own survival (Kudchodkar et al, 2009).

1.3.4.1 Antiviral roles of autophagy and autophagy-related proteins:

Invading microorganisms like virus and bacteria are captured and trafficked to autophagosomes for lysosomal degradation in a process called Xenophagy (Gutierrez et al, 2004; Knodler et al, 2011; Wild et al, 2011). Xenophagy induced by viral infections was first studied in the context of CNS infection with Sindbis virus (SV). Increased Beclin 1 expression in brains of mice during SV infection resulted in lower viral titers, fewer neuronal apoptotic cells in parallel with protecting mice against fatal SV encephalitis (Liang et al, 1998). Xenophagy have also been demonstrated against viruses such as Herpes Simplex Virus-1 (HSV-1), and Vesicular Stomatitis Virus (VSV) (Orvedahl et al, 2007; Shelly et al, 2009).

In addition to direct elimination of invading bacteria/viruses, autophagy participates in other aspects of immunity (Levine and Deretic, 2007; Schmid and Munz,

2007). Autophagy activates innate immune signaling by deliverying viral nucleic acids to endosomal toll-like receptors (TLRs). TLR7 ligand single stranded RNA (ssRNA) intiates potent autophagy and plays vital role in eliminating intracellular microbes (Delgado et al, 2008). Autophagy and/or autophagy genes has also been shown to play a role in delivering ligands to TLRs for activation and section of antiviral factors like type I IFNs. In plasmacytoid dendritic cells (pDCs), Atg5 is necessary for the delivery of cytosolic replicating viral RNA of SV and VSV to endosomal TLR 7 and subsequent IFN production (Lee et al, 2007).

Autophagy augments adaptive immune responses by promoting viral antigen processesing and presentation of endogenous viral antigens to major histocompatibility complex (MHC) class I and class II molecules (Dong et al, 2013). Autophagosomes constitutively fuse with MHC-II loading compartments in professional antigen presenting cells (like dendritic cells, B cells and macrophages) and facilitates efficient recognition of viral antigens by $CD4^+$ T cells. It was first demonstrated in Epstein-Barr virus (EBV) infection. This contribution of autophagy to $CD4^+$ T cells activation has been demonstrated in Epstein-Barr virus (EBV), IAV and VSV infection (Paludan et al, 2005; Comber et al, 2011; Morris et al, 2011). In addition to MHC II presentation, autophagy also promotes the presentation of endogenous viral antigens on MHC class I molecules and the activation of $CD8^+$ T cells. For example, during late stages of HSV-1 infection, autophagy facilitated the presentation of viral glycoprotein gB on MHC class I molecules (English et al, 2009). However, it is important to note that most of the antiviral effects of autophagy are context-dependent and are yet to be explored in great detail.

1.3.4.2 Subversion of autophagy by viruses:

As herpesvirus establishes latency for the life of the hosts, it evolved numerous mechanisms to subvert host immune defenses. All three subfamilies of herpesvirus (α, β, γ) encode viral proteins that subverts autophagy pathway. α herpesvirus HSV-1 encodes ICP 34.5 protein that blocks the induction of autophagy by targeting Beclin 1 (Orvedahl et al, 2007) and PKR mediated phosphorylation of eIF2α (Talloczy et al, 2006). γ herpesviruses like murine gamma herpesvirus 68 (γHV68) and Kaposi's sarcoma associated herpesvirus (KSHV) encode viral Bcl-2, a Beclin 1 interacting protein that prevents autophagy at early stage (Sinha et al, 2006; Ku et al, 2008). On the other hand, β-herpesvirus human cytomegalovirus (HCMV) inhibits autophagy by activating the mTOR-signaling pathway and renders infected cells resistant to autophagy (Chaumorcel et al, 2008). In addition to herpesviruses, human immunodeficiency virus -1 (HIV-1) is another virus well known for its ability to subvert host immune system. Upon HIV-1 infection, autophagy is suppressed in myeloid, lymphoid and neuronal cells. Monocytes or $CD4^+$ T cells infected with HIV-1 shows greatly reduced number of LC3-PE, Beclin 1 and also autophagosome formation resulting in increased virus replication (Zhou et al, 2009). Negative factor (Nef), accessory protein of HIV-1, inhibits maturation of autophagosomes into autolysosomes by targeting Beclin 1 and thereby protects HIV from degradation (Kyei et al, 2009). HIV-1 was also shown to evade early immune responses by rapidly shutting down autophagy in dendritic cells (DC) through interaction between HIV-1 envelope protein (Env) and DC surface receptors (Blanchet et al, 2010)

1.3.4.3 Proviral roles of autophagy and autophagy-related proteins:

Autophagy pathway or components of the autophagy machinery have been found to be involved in proviral roles for several DNA and RNA viruses. Many of these viruses either induce *de novo* synthesis of autophagy membranes or block autophagy maturation or both to serve as a scaffold for genome replication (Kudchodkar et al, 2009). It was first discovered in poliovirus-infected cells where autophagy promotes both viral replication and nonlytic release of virions (Jackson et al, 2005). Subsequent research have shown that DNA viruses such as Hepatitis B virus, porcine circovirus type 2, procine reproductive and respiratory syndrome virus also utilizes core autophagic machinery to promote viral DNA replication (Tian et al, 2010; Zhu et al, 2012; Sun et al, 2012).

Lipophagy is a selective autophagy process through which lipid droplets can be selectively degraded by lysosomes to release free fatty acids (Singh et al, 2009). Dengue virus modulates host lipid metabolism by inducing lipophagy for viral replication (Heaton et al, 2010). Hepatitis C virus (HCV) exploits components of the autophagy machinery to initiate early viral genome replication and also to conceal viral RNA in autophagosome-like double membrane vesicles (DMVs) to evade cytosolic RNA sensors and host antiviral responses (Kanda et al, 2008; Chen et al, 2008; Dreux et al, 2009; Tanida et al, 2009; Shrivastava et al, 2011; Kuo et al, 2012). It was also shown that autophagy plays an important role in IAV and coronavirus replication (Zhou et al, 2009; Mayer et al, 2012). A recent study suggests that immunity-associated GTPase family M (IRGM) may be a common target of RNA viruses that subverts autophagy for viral replication (Gregoire et al, 2011).

Moreover, viruses can exploit autophagy-related proteins for viral replication in an autophagy-independent manner. Fox example, Coronaviruses recruits nonlipidated LC3-I onto MHV-Induced DMVs necessary for viral replication in autophagy-independent manner (Reggiori et al, 2010). Viral exploitation of LC3 in an autophagy-independent manner in bovine viral diarrhea virus (BVDV) is another interesting example. Cytopathogenic strain of BVDV incorporates an LC3 gene fragment into its genome and utilizes cellular LC3-specific protease Atg4B to facilitate the processing of the viral polyprotein and for BVDV replication (Fricke et al, 2004).

1.3.5 Autophagy and IAV:

IAV infection enhances autophagosome formation and plays a key role in virus replication. Depletion of endogenous LC3 and Beclin 1 by RNA interference greatly impaired virus replication in lung epithelial cells (Zhou et al, 2009). Further studies showed that matrix protein 2 (M2) stimulate autophagy by interacting with Beclin 1 but blocks the fusion of autophagosomes with lysosomes leading to increased accumulation of autophagosomes. This process of autophagy inhibition significantly increases host cell apoptosis in immune cells indicating that there is a delicate balance between IAV-mediated autophagy induction and induction of cell death (Gannage et al, 2009; Rossman et al, 2009). M2 plays important role in IAV assembly and budding (Chen et al, 2008). It was also shown that the cytoplasmic tail of M2 contains LC3-interacting region (LIR), which causes relocalization of LC3 to the plasma membrane in IAV-infected cells at the time of virus budding. Thus IAV M2 protein via its LIR hijacks the autophagy machinery to provide suitable resources for viral budding (Beale et al, 2014). M2 is the only IAV protein studied in the context of autophagy and role of other IAV proteins is yet to be

explored. Given the central importance of autophagy to host defense, involvement of other IAV viral proteins in the modulation of autophagy pathway cannot be ruled out.

1.3.6 Mitophagy:

In addition to classical autophagy, various organelle specific forms of autophagy such as ribophagy (degradation of ribosomes), pexophagy (degradation of peroxisomes) and mitophagy (degradation of mitochondria) also occur in virus-infected cells (Chiramel et al, 2013; Xia et al, 2014). Mitophagy is a well-studied type of cargo-specific autophagy that mediates the selective removal of mitochondria and occurs under normal physiological conditions (Bhatia-Kissova et al, 2012). It has been shown to be essential for steady-state turnover of mitochondria and for the adjustment of mitochondrion numbers to match metabolic demand (Kissova et al, 2004; Tal et al, 2007). Mitophagy also plays an important role in specialized developmental processes such as stem cell maintenance and differentiation (Vazquez-Martin et al, 2012), reticulocyte maturation (Kundu et al, 2008; Schweers et al, 2007), and removal of paternal mitochondria in fertilized oocytes (Sato and Sato, 2011). In addition to normal physiologic process, mitophaghic activity occurs as a quality control process to remove damaged mitochondria. It involves targeted and selective engulfment of damaged or dysfunctional mitochondria and subsequent lysosomal degradation (Taylor et al, 2011; Ding et al, 2012; Jin et al, 2013).

1.3.7 Mitochondrial dynamics and regulation of mitophagy by PINK1/Parkin pathway:

Mammalian mitochondria, which form a highly dynamic reticular network, constantly undergo cycles of fission and fusion that helps to preserve proper

mitochondrial function (Scott et al, 2010). Imbalance in the fission/fusion equilibrium or mitochondrial damage can lead to degradation of mitochondria by mitophagy (Twig et al, 2008). Membrane-anchored dynamin family members, mitofusin (Mfn) 1 & 2 and single dynamin family member, optic atrophy protein 1 (OPA 1) mediates the fusion of healthy mitochondrial fragment. Mfn 1 and Mfn 2 mediate fusion of outer mitochondrial membranes whereas OPA 1 mediates fusion between inner mitochondrial membranes (Twig et al, 2011). Mitochondrial fusion is stimulated by energy demand and stress. It helps to maintain integrity and homogeneity of the mitochondrial network and also protects against loss of mitochondrial DNA (mtDNA) (Anton et al, 2013).

Mitochondria play a crucial role in both apoptosis and mitophagy. Impairment of mitochondrial function results in activation of both mitophagy and mitochondrial apoptosis in the same cell. In order to prevent cell death, damaged mitochondria are sequestered by autophagosomes and degraded before apoptosis pathway is triggered. Damaged mitochondria undergo dynamin-related protein 1 (Drp 1) - mediated fission before mitophagy. Mitochondrial fission helps to generate new organelles and facilitates quality control by isolating damaged mitochondria from the intact network and its selective removal by mitophagy (Westermann et al, 2010; Youle et al, 2012). The phosphatase and tensin homolog-induced putative kinase 1 (PINK1)/Parkin pathway is vital in regulating mitophagy in cells (Kubli et al, 2012). Under basal condition, PINK1 are regularly cleaved by mitochondrial proteases and found at very low level on mitochondria. Similarly, E3 ubiquitin ligase Parkin is present predominantly in the cytoplasm under normal condition. Loss of mitochondrial potential leads to the accumulation of PINK1 on the outer mitochondrial membrane and subsequent

recruitment of the E3 ubiquitin ligase Parkin to mitochondria (Narendra et al, 2008; Suen et al, 2010; Narendra et al, 2010). This process promotes ubiquitination of mitochondrial proteins such as Mfn1, Mfn2, and VDAC-1 (Geisler et al, 2010; Gegg et al, 2010; Poole et al, 2010). Ubiquitination serves as a signal for autophagic degradation of mitochondria (Pankiv et al, 2007; Kirkin et al, 2009b). Autophagy adaptor protein SQSTM1 plays an important role in the process of mitophagy. It binds to LC3 on the phagophore via its LC3-interacting domain and to ubiquitinated proteins via its ubiquitin-associated region (Seibenhener et al 2004; Pankiv et al, 2007). It has been reported that SQSTM1 is recruited to mitochondria in a Parkin-dependent manner and knockdown of the protein substantially inhibits mitophagy (Geisler et al, 2010; Ding et al, 2010). Binding of SQSTM1 to ubiquitinated mitochondrial proteins tethers the damaged mitochondria to the LC3-positive autophagosome for engulfment (Kubli et al, 2012).

1.3.8 Types of mitophagy:

There are 3 types of mitophagy. In type I mitophagy induced by nutrient deprivation, Beclin 1/PI3K activation results in the formation of preautophagic structures (PAS). PAS grow into cup-shaped phagophores that envelop and sequester individual mitochondria into autophagosomes. This process often occurs in coordination with mitochondrial fission. Mitochondria maintain their membrane potential during the process and depolarization occurs after sequestration. Subsequently, the mitophagosome fuses with lysosomes, and hydrolytic digestion of the entrapped mitochondria occurs (Lemasters, 2014). In type II mitophagy, mitochondrial injury causes membrane depolarization resulting in the formation of LC3-labelled membrane vesicles. These membrane vesicles then tethered to the depolarized mitochondria resulting in the

formation of autophagosome and subsequent degradation in lysosomes. This process occurs independent of Beclin 1/PI3K pathway. PI3K inhibitors such as 3-methyladenine can be used to distinguish type I and type II mitophagy (Lemasters et al, 2006). In type III micromitophagy, mitochondria derived vesicles containing oxidized mitochondrial proteins bud off from mitochondria and transit into multivesicular bodies. These multivesicular bodies subsequently fuses with lysosomes resulting in vesicular acidification and degradation (Lemasters, 2014).

1.3.9 Role of mitophagy in inflammation and innate immune signaling:

Mitochondria play a central role in many important cellular functions such as energy production, apoptosis and innate immune signaling. Damaged mitochondria have detrimental consequences for cell viability and function of immune system (Lazarou et al, 2015). Dysfunctional mitochondrion releases excess reactive oxygen species (ROS) and mtDNA within the cell (Kurihara et al, 2011). ROS acts as damage associated molecular patterns that can activate both NLRP3 inflammasome and mitophagy (Zhang et al, 2013). NLRP3 inflammasome is an important player of innate immune response through its signaling to induce pyrogenic cytokine IL-1β production (Abderrazak et al, 2015). In response to various stimuli, ablation of autophagy proteins such as LC3β, Beclin 1, or Atg5 enhanced NLRP3 activation and IL-1β secretion. Selective removal of dysfunctional mitochondria through mitophagy prevents cytosolic accumulation of ROS and mtDNA, and thereby restricts NLRP3 inflammasome activation (Zhou et al 2011; Nakahira et al, 2011). NLRP3 inflammasomes senses dysfunctional mitochondria, which may explain the frequent association of mitochondrial damage with inflammatory diseases (Ding and Yin, 2012). The cytosolic DNA sensor cyclic-GMP-AMP synthase

(cGAS) has been shown to activate the process of autophagy via Beclin 1 (Liang et al, 2014). Recently, West et al. showed that mtDNA released in response to damaged mitochondria is recognized by the cGAS sensor pathway leading to robust induction of type I IFNs and IFN-stimulated genes (West et al, 2015).

As mentioned above, there is a complex crosstalk between autophagy and necrosis. The receptor-interacting protein serine/threonine kinases (RIPK) play an important role in the process of necrosis. Necroptosis, which is a programmed necrotic cell death, is the result of the complex interplay between RIPK, calcium, and mitochondria (Jain et al, 2013). Excessive ROS formation due to mitochondrial dysfunction results in the activation of necroptosis pathway (Denecker et al, 2001). During IAV infection, receptor interacting protein kinase 2 (RIPK2)-mediated mitophagy negatively regulates ROS production, NLRP3 inflammasome and inflammation. $RIPK2^{-/-}$ bone-marrow-derived dendritic cells showed increased accumulation of damaged mitochondria resulting in greater ROS production and activation of NLRP3 inflammasome (Lupfer et al, 2013).

RIG-I like receptors (RLR) recognizes RNA viruses via MAVS protein located on the OMM and induces expression of potent antiviral factors such as type I IFNs and proinflammatory cytokines (Seth et al, 2005). Mitochondria acts as a point of intersect between mitophagy and RLR signaling. Autophagy deficient immune cells showed enhanced stimulation of RLR signaling following VSV infection. Wild-type cells showed reduced levels of MAVS and damaged mitochondria are removed by mitophagy. However in autophagy deficient immune cells, increased accumulation of dysfunctional mitochondria lead to increased levels of MAVS and enhanced RLR signaling through

mitochondrial ROS (Tal et al, 2009). Taken together, these studies show that mitophagy is not only important for basal mitochondrial quality control but also during infection to modulate inflammation and innate immune signaling.

1.3.10 Subversion of mitophagy by viruses:

Antiviral immune responses and apoptosis are the two major limiting factors for viral infections. Emerging evidence shows that viruses subvert mitophagy to enhance viral replication by mitigating innate immune responses and cell death mechanism (Xia et al, 2014). Hepatitis B and Hepatitis C viruses (HBV and HCV) subverts PINK1/Parkin-mediated mitophagy for virus replication. HCV induces mitochondrial translocation of Parkin resulting in the removal of damaged mitochondria. Blocking mitophagy by knocking down PINK1 and Parkin genes impairs HCV replication (Kim et al, 2013b; Kim et al, 2014). PINK1/Parkin- mediated mitophagy assists HBV to circumvent mitochondrial apoptosis by reducing cyt c release (Kim et al, 2013a). Removal of damaged mitochondria attenuates cell death and thus promotes viral persistence in infected hepatocytes (Kim et al, 2014).

1.4 References:

1. Abderrazak A, Syrovets T, Couchie D, El Hadri K, Friguet B, et al. (2015) NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases. Redox Biol 4: 296-307.

2. Air GM, Ritchie LR, Laver WG, Colman PM (1985) Gene and protein sequence of an influenza neuraminidase with hemagglutinin activity. Virology 145: 117-122.

3. Ait-Goughoulte M, Kanda T, Meyer K, Ryerse JS, Ray RB, et al. (2008) Hepatitis C virus genotype 1a growth and induction of autophagy. J Virol 82: 2241-2249.

4. Alymova IV, Green AM, van de Velde N, McAuley JL, Boyd KL, et al. (2011) Immunopathogenic and antibacterial effects of H3N2 influenza A virus PB1-F2 map to amino acid residues 62, 75, 79, and 82. J Virol 85: 12324-12333.

5. Alymova IV, York IA, McCullers JA (2014) Non-avian animal reservoirs present a source of influenza A PB1-F2 proteins with novel virulence-enhancing markers. PLoS One 9: e111603.

6. Amorim MJ, Bruce EA, Read EK, Foeglein A, Mahen R, et al. (2011) A Rab11- and microtubule-dependent mechanism for cytoplasmic transport of influenza A virus viral RNA. J Virol 85: 4143-4156.

7. Bao CJ, Cui LB, Zhou MH, Hong L, Gao GF, et al. (2013) Live-animal markets and influenza A (H7N9) virus infection. N Engl J Med 368: 2337-2339.

8. Barclay WS, Palese P (1995) Influenza B viruses with site-specific mutations introduced into the HA gene. J Virol 69: 1275-1279.

9. Beale R, Wise H, Stuart A, Ravenhill BJ, Digard P, et al. (2014) A LC3-interacting motif in the influenza A virus M2 protein is required to subvert autophagy and maintain virion stability. Cell Host Microbe 15: 239-247.

10. Bell BD, Leverrier S, Weist BM, Newton RH, Arechiga AF, et al. (2008) FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. Proc Natl Acad Sci U S A 105: 16677-16682.

11. Belser JA, Blixt O, Chen LM, Pappas C, Maines TR, et al. (2008) Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. Proc Natl Acad Sci U S A 105: 7558-7563.

12. Bhatia-Kissova I, Camougrand N (2013) Mitophagy: a process that adapts to the cell physiology. Int J Biochem Cell Biol 45: 30-33.

13. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, et al. (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171: 603-614.

14. Blaas D, Patzelt E, Kuechler E (1982) Cap-recognizing protein of influenza virus. Virology 116: 339-348.

15. Blanchet FP, Moris A, Nikolic DS, Lehmann M, Cardinaud S, et al. (2010) Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic cells impairs early innate and adaptive immune responses. Immunity 32: 654-669.

16. Blevins LK, Wren JT, Holbrook BC, Hayward SL, Swords WE, et al. (2014) Coinfection with Streptococcus pneumoniae negatively modulates the size and composition of the ongoing influenza-specific CD8(+) T cell response. J Immunol 193: 5076-5087.

17. Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, et al. (2010) Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J Clin Invest 120: 1310-1323.

18. Boulo S, Akarsu H, Ruigrok RW, Baudin F (2007) Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. Virus Res 124: 12-21.

19. Bourmakina SV, Garcia-Sastre A (2005) The morphology and composition of influenza A virus particles are not affected by low levels of M1 and M2 proteins in infected cells. J Virol 79: 7926-7932.

20. Bowes VA, Ritchie SJ, Byrne S, Sojonky K, Bidulka JJ, et al. (2004) Virus characterization, clinical presentation, and pathology associated with H7N3 avian influenza in British Columbia broiler breeder chickens in 2004. Avian Dis 48: 928-934.

21. Braam J, Ulmanen I, Krug RM (1983) Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell 34: 609-618.

22. Brockwell-Staats C, Webster RG, Webby RJ (2009) Diversity of influenza viruses in swine and the emergence of a novel human pandemic influenza A (H1N1). Influenza Other Respir Viruses 3: 207-213.

23. Bron R, Kendal AP, Klenk HD, Wilschut J (1993) Role of the M2 protein in influenza virus membrane fusion: effects of amantadine and monensin on fusion kinetics. Virology 195: 808-811.

24. Bruns K, Studtrucker N, Sharma A, Fossen T, Mitzner D, et al. (2007) Structural characterization and oligomerization of PB1-F2, a proapoptotic influenza A virus protein. J Biol Chem 282: 353-363.

25. Caliguiri LA, Compans RW (1974) Analysis of the in vitro product of an RNA-dependent RNA polymerase isolated from influenza virus-infected cells. J Virol 14: 191-197.

26. Carrat F, Flahault A (2007) Influenza vaccine: the challenge of antigenic drift. Vaccine 25: 6852-6862.

27. Castrucci MR, Kawaoka Y (1995) Reverse genetics system for generation of an influenza A virus mutant containing a deletion of the carboxyl-terminal residue of M2 protein. J Virol 69: 2725-2728.

28. Chakrabarti AK, Pasricha G (2013) An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. Virology 440: 97-104.

29. Chanturiya AN, Basanez G, Schubert U, Henklein P, Yewdell JW, et al. (2004) PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. J Virol 78: 6304-6312.

30. Chaumorcel M, Souquere S, Pierron G, Codogno P, Esclatine A (2008) Human cytomegalovirus controls a new autophagy-dependent cellular antiviral defense mechanism. Autophagy 4: 46-53.

31. Chen CJ, Chen GW, Wang CH, Huang CH, Wang YC, et al. (2010) Differential localization and function of PB1-F2 derived from different strains of influenza A virus. J Virol 84: 10051-10062.

32. Chen E, Chen Y, Fu L, Chen Z, Gong Z, et al. (2013) Human infection with avian influenza A(H7N9) virus re-emerges in China in winter 2013. Euro Surveill 18.

33. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306-1312.34. Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, et al. (2002) Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 360: 1831-1837.

35. Cheung TK, Poon LL (2007) Biology of influenza a virus. Ann N Y Acad Sci 1102: 1-25.

36. Chevalier C, Al Bazzal A, Vidic J, Fevrier V, Bourdieu C, et al. (2010) PB1-F2 influenza A virus protein adopts a beta-sheet conformation and forms amyloid fibers in membrane environments. J Biol Chem 285: 13233-13243.

37. Chiramel AI, Brady NR, Bartenschlager R (2013) Divergent roles of autophagy in virus infection. Cells 2: 83-104.

38. Choi YK, Goyal SM, Joo HS (2002) Prevalence of swine influenza virus subtypes on swine farms in the United States. Arch Virol 147: 1209-1220.

39. Choi YK, Lee JH, Erickson G, Goyal SM, Joo HS, et al. (2004) H3N2 influenza virus transmission from swine to turkeys, United States. Emerg Infect Dis 10: 2156-2160.

40. Chu CM, Dawson IM, Elford WJ (1949) Filamentous forms associated with newly isolated influenza virus. Lancet 1: 602.

41. Clifford M, Twigg J, Upton C (2009) Evidence for a novel gene associated with human influenza A viruses. Virol J 6: 198.

42. Coleman JR (2007) The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages. Virol J 4: 9.

43. Comber JD, Robinson TM, Siciliano NA, Snook AE, Eisenlohr LC (2011) Functional macroautophagy induction by influenza A virus without a contribution to major histocompatibility complex class II-restricted presentation. J Virol 85: 6453-6463.

44. Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P (2007a) A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog 3: 1414-1421.

45. Conenello GM, Palese P (2007b) Influenza A virus PB1-F2: a small protein with a big punch. Cell Host Microbe 2: 207-209.

46. Conenello GM, Tisoncik JR, Rosenzweig E, Varga ZT, Palese P, et al. (2011) A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response in vivo. J Virol 85: 652-662.

47. Copeland CS, Doms RW, Bolzau EM, Webster RG, Helenius A (1986) Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J Cell Biol 103: 1179-1191.

48. Costa T, Chaves AJ, Valle R, Darji A, van Riel D, et al. (2012) Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. Vet Res 43: 28.

49. Cuervo AM (2010) Chaperone-mediated autophagy: selectivity pays off. Trends Endocrinol Metab 21: 142-150.

50. Cuervo AM, Dice JF (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273: 501-503.

51. Cuervo AM, Wong E (2014) Chaperone-mediated autophagy: roles in disease and aging. Cell Res 24: 92-104.

52. de Wit E, Munster VJ, van Riel D, Beyer WE, Rimmelzwaan GF, et al. (2010) Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host. J Virol 84: 1597-1606.

53. Delgado MA, Elmaoued RA, Davis AS, Kyei G, Deretic V (2008) Toll-like receptors control autophagy. EMBO J 27: 1110-1121.

54. Denecker G, Vercammen D, Steemans M, Vanden Berghe T, Brouckaert G, et al. (2001) Death receptor-induced apoptotic and necrotic cell death: differential role of caspases and mitochondria. Cell Death Differ 8: 829-840.

55. Desselberger U, Racaniello VR, Zazra JJ, Palese P (1980) The 3' and 5'-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene 8: 315-328.

56. Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, et al. (2009) The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458: 914-918.

57. Dice JF (1990) Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem Sci 15: 305-309.

58. Digard P, Blok VC, Inglis SC (1989) Complex formation between influenza virus polymerase proteins expressed in Xenopus oocytes. Virology 171: 162-169.

59. Ding WX, Ni HM, Li M, Liao Y, Chen X, et al. (2010) Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. J Biol Chem 285: 27879-27890.

60. Ding WX, Yin XM (2012) Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem 393: 547-564.

61. Domingo E, Escarmis C, Lazaro E, Manrubia SC (2005) Quasispecies dynamics and RNA virus extinction. Virus Res 107: 129-139.

62. Doms RW, Lamb RA, Rose JK, Helenius A (1993) Folding and assembly of viral membrane proteins. Virology 193: 545-562.

63. Dong X, Levine B (2013) Autophagy and viruses: adversaries or allies? J Innate Immun 5: 480-493.

64. Donis RO, Bean WJ, Kawaoka Y, Webster RG (1989) Distinct lineages of influenza virus H4 hemagglutinin genes in different regions of the world. Virology 169: 408-417.

65. Dreux M, Gastaminza P, Wieland SF, Chisari FV (2009) The autophagy machinery is required to initiate hepatitis C virus replication. Proc Natl Acad Sci U S A 106: 14046-14051.

66. Dudek SE, Wixler L, Nordhoff C, Nordmann A, Anhlan D, et al. (2011) The influenza virus PB1-F2 protein has interferon antagonistic activity. Biol Chem 392: 1135-1144.

67. Easterday BC (1980) The epidemiology and ecology of swine influenza as a zoonotic disease. Comp Immunol Microbiol Infect Dis 3: 105-109.

68. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, et al. (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331: 456-461.

69. Eisfeld AJ, Kawakami E, Watanabe T, Neumann G, Kawaoka Y (2011) RAB11A is essential for transport of the influenza virus genome to the plasma membrane. J Virol 85: 6117-6126.

70. El Bakkouri K, Descamps F, De Filette M, Smet A, Festjens E, et al. (2011) Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J Immunol 186: 1022-1031.

71. Enami M, Luytjes W, Krystal M, Palese P (1990) Introduction of site-specific mutations into the genome of influenza virus. Proc Natl Acad Sci U S A 87: 3802-3805.

72. English L, Chemali M, Duron J, Rondeau C, Laplante A, et al. (2009) Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. Nat Immunol 10: 480-487.

73. Escobar-Henriques M, Anton F (2013) Mechanistic perspective of mitochondrial fusion: tubulation vs. fragmentation. Biochim Biophys Acta 1833: 162-175.

74. Fauquet CM, Fargette D (2005) International Committee on Taxonomy of Viruses and the 3,142 unassigned species. Virol J 2: 64.

75. Flick R, Neumann G, Hoffmann E, Neumeier E, Hobom G (1996) Promoter elements in the influenza vRNA terminal structure. RNA 2: 1046-1057.

76. Fodor E, Mingay LJ, Crow M, Deng T, Brownlee GG (2003) A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase promotes the generation of defective interfering RNAs. J Virol 77: 5017-5020.

77. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, et al. (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79: 2814-2822.

78. Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, et al. (2004) Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A 101: 1356-1361.

79. Fournie G, Guitian J, Desvaux S, Mangtani P, Ly S, et al. (2012) Identifying live bird markets with the potential to act as reservoirs of avian influenza A (H5N1) virus: a survey in northern Viet Nam and Cambodia. PLoS One 7: e37986.

80. Fricke J, Voss C, Thumm M, Meyers G (2004) Processing of a pestivirus protein by a cellular protease specific for light chain 3 of microtubule-associated proteins. J Virol 78: 5900-5912.

81. Frommhagen LH, Knight CA, Freeman NK (1959) The ribonucleic acid, lipid, and polysaccharide constituents of influenza virus preparations. Virology 8: 176-197.

82. Gabriel G, Herwig A, Klenk HD (2008) Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range of influenza A virus. PLoS Pathog 4: e11.

83. Gabriel G, Klingel K, Otte A, Thiele S, Hudjetz B, et al. (2011) Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus. Nat Commun 2: 156.

84. Gambaryan A, Webster R, Matrosovich M (2002) Differences between influenza virus receptors on target cells of duck and chicken. Arch Virol 147: 1197-1208.

85. Gannage M, Dormann D, Albrecht R, Dengjel J, Torossi T, et al. (2009) Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. Cell Host Microbe 6: 367-380.

86. Gao R, Cao B, Hu Y, Feng Z, Wang D, et al. (2013) Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med 368: 1888-1897.

87. Garcia-Sastre A (2001) Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology 279: 375-384.

88. Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, et al. (1998) Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 252: 324-330.

89. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, et al. (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325: 197-201.

90. Garten W, Klenk HD (1983) Characterization of the carboxypeptidase involved in the proteolytic cleavage of the influenza haemagglutinin. J Gen Virol 64 (Pt 10): 2127-2137.

91. Gastaminza P, Perales B, Falcon AM, Ortin J (2003) Mutations in the N-terminal region of influenza virus PB2 protein affect virus RNA replication but not transcription. J Virol 77: 5098-5108.

92. Gaydos JC, Hodder RA, Top FH, Jr., Allen RG, Soden VJ, et al. (1977) Swine influenza A at Fort Dix, New Jersey (January-February 1976). II. Transmission and morbidity in units with cases. J Infect Dis 136 Suppl: S363-368.

93. Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, et al. (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum Mol Genet 19: 4861-4870.

94. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, et al. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol 12: 119-131.

95. Gibbs JS, Malide D, Hornung F, Bennink JR, Yewdell JW (2003) The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. J Virol 77: 7214-7224.

96. Girard MP, Tam JS, Assossou OM, Kieny MP (2010) The 2009 A (H1N1) influenza virus pandemic: A review. Vaccine 28: 4895-4902.

97. Gomez-Puertas P, Albo C, Perez-Pastrana E, Vivo A, Portela A (2000) Influenza virus matrix protein is the major driving force in virus budding. J Virol 74: 11538-11547.

98. Gonzalez S, Ortin J (1999) Distinct regions of influenza virus PB1 polymerase subunit recognize vRNA and cRNA templates. EMBO J 18: 3767-3775.

99. Gorman OT, Bean WJ, Kawaoka Y, Donatelli I, Guo YJ, et al. (1991) Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol 65: 3704-3714.

100. Gorman OT, Bean WJ, Kawaoka Y, Webster RG (1990) Evolution of the nucleoprotein gene of influenza A virus. J Virol 64: 1487-1497.

101. Gottschalk A (1957) Neuraminidase: the specific enzyme of influenza virus and Vibrio cholerae. Biochim Biophys Acta 23: 645-646.

102. Graef KM, Vreede FT, Lau YF, McCall AW, Carr SM, et al. (2010) The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. J Virol 84: 8433-8445.

103. Greenbaum A, Quinn C, Bailer J, Su S, Havers F, et al. (2015) Investigation of an outbreak of variant influenza A (H3N2) virus associated with an agricultural fair - Ohio, August 2012. J Infect Dis.

104. Guan ZH, Zhang ML, Hou PL, Duan M, Cui YM, et al. (2012) Identification of cellular proteins interacting with influenza A virus PB1-F2 protein. Acta Virol 56: 199-207.

105. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, et al. (2005) Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 280: 5571-5580.

106. Gulati U, Hwang CC, Venkatramani L, Gulati S, Stray SJ, et al. (2002) Antibody epitopes on the neuraminidase of a recent H3N2 influenza virus (A/Memphis/31/98). J Virol 76: 12274-12280.

107. Guo CT, Takahashi N, Yagi H, Kato K, Takahashi T, et al. (2007) The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology 17: 713-724.

108. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, et al. (2004) Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119: 753-766.

109. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, et al. (2008) AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell 30: 214-226.

110. Hai R, Schmolke M, Varga ZT, Manicassamy B, Wang TT, et al. (2010) PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. J Virol 84: 4442-4450.

111. Halder UC, Bagchi P, Chattopadhyay S, Dutta D, Chawla-Sarkar M (2011) Cell death regulation during influenza A virus infection by matrix (M1) protein: a model of viral control over the cellular survival pathway. Cell Death Dis 2: e197.

112. Hale BG, Randall RE, Ortin J, Jackson D (2008) The multifunctional NS1 protein of influenza A viruses. J Gen Virol 89: 2359-2376.

113. Hatta M, Gao P, Halfmann P, Kawaoka Y (2001a) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293: 1840-1842.

114. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, et al. (2007) Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog 3: 1374-1379.

115. Hatta M, Neumann G, Kawaoka Y (2001b) Reverse genetics approach towards understanding pathogenesis of H5N1 Hong Kong influenza A virus infection. Philos Trans R Soc Lond B Biol Sci 356: 1841-1843.

116. Hause BM, Collin EA, Liu R, Huang B, Sheng Z, et al. (2014) Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. MBio 5: e00031-00014.

117. Hay AJ, Lomniczi B, Bellamy AR, Skehel JJ (1977) Transcription of the influenza virus genome. Virology 83: 337-355.

118. Heaton NS, Randall G (2010) Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe 8: 422-432.

119. Henkel M, Mitzner D, Henklein P, Meyer-Almes FJ, Moroni A, et al. (2010) The proapoptotic influenza A virus protein PB1-F2 forms a nonselective ion channel. PLoS One 5: e11112.

120. Henklein P, Bruns K, Nimtz M, Wray V, Tessmer U, et al. (2005) Influenza A virus protein PB1-F2: synthesis and characterization of the biologically active full length protein and related peptides. J Pept Sci 11: 481-490.

121. Herz C, Stavnezer E, Krug R, Gurney T, Jr. (1981) Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. Cell 26: 391-400.

122. Hirst M, Astell CR, Griffith M, Coughlin SM, Moksa M, et al. (2004) Novel avian influenza H7N3 strain outbreak, British Columbia. Emerg Infect Dis 10: 2192-2195.

123. Hoffman WH, Shacka JJ, Andjelkovic AV (2012) Autophagy in the brains of young patients with poorly controlled T1DM and fatal diabetic ketoacidosis. Exp Mol Pathol 93: 273-280.

124. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97: 6108-6113.

125. Holsinger LJ, Nichani D, Pinto LH, Lamb RA (1994) Influenza A virus M2 ion channel protein: a structure-function analysis. J Virol 68: 1551-1563.

126. Holsinger LJ, Shaughnessy MA, Micko A, Pinto LH, Lamb RA (1995) Analysis of the posttranslational modifications of the influenza virus M2 protein. J Virol 69: 1219-1225.

127. Honda A, Mizumoto K, Ishihama A (2002) Minimum molecular architectures for transcription and replication of the influenza virus. Proc Natl Acad Sci U S A 99: 13166-13171.

128. Horimoto T, Kawaoka Y (2001) Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev 14: 129-149.

129. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, et al. (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 20: 1981-1991.

130. Hsu MT, Parvin JD, Gupta S, Krystal M, Palese P (1987) Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc Natl Acad Sci U S A 84: 8140-8144.

131. Hu J, Mo Y, Wang X, Gu M, Hu Z, et al. (2015) PA-X decreases the pathogenicity of highly pathogenic H5N1 influenza A virus in avian species by inhibiting virus replication and host response. J Virol 89: 4126-4142.

132. Huang TS, Palese P, Krystal M (1990) Determination of influenza virus proteins required for genome replication. J Virol 64: 5669-5673.

133. Huber VC (2012) Can surveillance of the influenza virus PB1-F2 gene be used to predict the severity of secondary bacterial infections? Virulence 3: 523-524.

134. Hughey PG, Roberts PC, Holsinger LJ, Zebedee SL, Lamb RA, et al. (1995) Effects of antibody to the influenza A virus M2 protein on M2 surface expression and virus assembly. Virology 212: 411-421.

135. Hutchinson EC, von Kirchbach JC, Gog JR, Digard P (2010) Genome packaging in influenza A virus. J Gen Virol 91: 313-328.

136. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, et al. (2012) Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 486: 420-428.

137. Itakura E, Kishi C, Inoue K, Mizushima N (2008) Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell 19: 5360-5372.

138. Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, et al. (1998) Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 72: 7367-7373.

139. Iwai A, Shiozaki T, Kawai T, Akira S, Kawaoka Y, et al. (2010) Influenza A virus polymerase inhibits type I interferon induction by binding to interferon beta promoter stimulator 1. J Biol Chem 285: 32064-32074.

140. Jackson D, Hossain MJ, Hickman D, Perez DR, Lamb RA (2008) A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc Natl Acad Sci U S A 105: 4381-4386.

141. Jackson WT, Giddings TH, Jr., Taylor MP, Mulinyawe S, Rabinovitch M, et al. (2005) Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol 3: e156.

142. Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, et al. (2012) An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337: 199-204.

143. Jain MV, Paczulla AM, Klonisch T, Dimgba FN, Rao SB, et al. (2013) Interconnections between apoptotic, autophagic and necrotic pathways: implications for cancer therapy development. J Cell Mol Med 17: 12-29.

144. Jain S, Kamimoto L, Bramley AM, Schmitz AM, Benoit SR, et al. (2009) Hospitalized patients with 2009 H1N1 influenza in the United States, April-June 2009. N Engl J Med 361: 1935-1944.

145. Jin H, Leser GP, Zhang J, Lamb RA (1997) Influenza virus hemagglutinin and neuraminidase cytoplasmic tails control particle shape. EMBO J 16: 1236-1247.

146. Jin M, Liu X, Klionsky DJ (2013) SnapShot: Selective autophagy. Cell 152: 368-368 e362.

147. Johnson NP, Mueller J (2002) Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med 76: 105-115.

148. Jonges M, Bataille A, Enserink R, Meijer A, Fouchier RA, et al. (2011) Comparative analysis of avian influenza virus diversity in poultry and humans during a highly pathogenic avian influenza A (H7N7) virus outbreak. J Virol 85: 10598-10604.

149. Karasin AI, Olsen CW, Anderson GA (2000a) Genetic characterization of an H1N2 influenza virus isolated from a pig in Indiana. J Clin Microbiol 38: 2453-2456.

150. Karasin AI, Schutten MM, Cooper LA, Smith CB, Subbarao K, et al. (2000b) Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: evidence for wholly human and reassortant virus genotypes. Virus Res 68: 71-85.

151. Karasin AI, West K, Carman S, Olsen CW (2004) Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol 42: 4349-4354.

152. Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, et al. (2006) Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature 443: 578-581.

153. Kaushik S, Cuervo AM (2008) Chaperone-mediated autophagy. Methods Mol Biol 445: 227-244.

154. Kawaoka Y, Krauss S, Webster RG (1989) Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 63: 4603-4608.

155. Kawaoka Y, Webster RG (1988) Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A 85: 324-328.

156. Kenah E, Chao DL, Matrajt L, Halloran ME, Longini IM, Jr. (2011) The global transmission and control of influenza. PLoS One 6: e19515.

157. Khatchikian D, Orlich M, Rott R (1989) Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature 340: 156-157.

158. Khurana S, Suguitan AL, Jr., Rivera Y, Simmons CP, Lanzavecchia A, et al. (2009) Antigenic fingerprinting of H5N1 avian influenza using convalescent sera and monoclonal antibodies reveals potential vaccine and diagnostic targets. PLoS Med 6: e1000049.

159. Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J (2008) Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci U S A 105: 20567-20574.

160. Kim SJ, Khan M, Quan J, Till A, Subramani S, et al. (2013a) Hepatitis B virus disrupts mitochondrial dynamics: induces fission and mitophagy to attenuate apoptosis. PLoS Pathog 9: e1003722.

161. Kim SJ, Syed GH, Siddiqui A (2013b) Hepatitis C virus induces the mitochondrial translocation of Parkin and subsequent mitophagy. PLoS Pathog 9: e1003285.

162. Kim SJ, Syed GH, Khan M, Chiu WW, Sohail MA, et al. (2014) Hepatitis C virus triggers mitochondrial fission and attenuates apoptosis to promote viral persistence. Proc Natl Acad Sci U S A 111: 6413-6418.

163. Kirkin V, Lamark T, Sou YS, Bjorkoy G, Nunn JL, et al. (2009a) A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell 33: 505-516.

164. Kirkin V, McEwan DG, Novak I, Dikic I (2009b) A role for ubiquitin in selective autophagy. Mol Cell 34: 259-269.

165. Kissova I, Deffieu M, Manon S, Camougrand N (2004) Uth1p is involved in the autophagic degradation of mitochondria. J Biol Chem 279: 39068-39074.

166. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, et al. (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4: 151-175.

167. Knodler LA, Celli J (2011) Eating the strangers within: host control of intracellular bacteria via xenophagy. Cell Microbiol 13: 1319-1327.

168. Kobayashi M, Toyoda T, Adyshev DM, Azuma Y, Ishihama A (1994) Molecular dissection of influenza virus nucleoprotein: deletion mapping of the RNA binding domain. J Virol 68: 8433-8436.

169. Koopmans M, de Jong MD (2013) Avian influenza A H7N9 in Zhejiang, China. Lancet 381: 1882-1883.

170. Korolchuk VI, Mansilla A, Menzies FM, Rubinsztein DC (2009a) Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Mol Cell 33: 517-527.

171. Korolchuk VI, Menzies FM, Rubinsztein DC (2009b) A novel link between autophagy and the ubiquitin-proteasome system. Autophagy 5: 862-863.

172. Kosik I, Krejnusova I, Bystricka M, Polakova K, Russ G (2011) N-terminal region of the PB1-F2 protein is responsible for increased expression of influenza A viral protein PB1. Acta Virol 55: 45-53.

173. Kosik I, Krejnusova I, Praznovska M, Russ G (2013) The multifaceted effect of PB1-F2 specific antibodies on influenza A virus infection. Virology 447: 1-8.

174. Krejnusova I, Gocnikova H, Bystricka M, Blaskovicova H, Polakova K, et al. (2009) Antibodies to PB1-F2 protein are induced in response to influenza A virus infection. Arch Virol 154: 1599-1604.

175. Krossoy B, Hordvik I, Nilsen F, Nylund A, Endresen C (1999) The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. J Virol 73: 2136-2142.

176. Ku B, Woo JS, Liang C, Lee KH, Jung JU, et al. (2008) An insight into the mechanistic role of Beclin 1 and its inhibition by prosurvival Bcl-2 family proteins. Autophagy 4: 519-520.

177. Kubli DA, Gustafsson AB (2012) Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res 111: 1208-1221.

178. Kudchodkar SB, Levine B (2009) Viruses and autophagy. Rev Med Virol 19: 359-378.

179. Kuiken T, van den Brand J, van Riel D, Pantin-Jackwood M, Swayne DE (2010) Comparative pathology of select agent influenza a virus infections. Vet Pathol 47: 893-914.

180. Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, et al. (2008) Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood 112: 1493-1502.

181. Kurihara Y, Kanki T, Aoki Y, Hirota Y, Saigusa T, et al. (2012) Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. J Biol Chem 287: 3265-3272.

182. Kwan-Gett TS, Baer A, Duchin JS (2009) Spring 2009 H1N1 influenza outbreak in King County, Washington. Disaster Med Public Health Prep 3 Suppl 2: S109-116.

183. Kyei GB, Dinkins C, Davis AS, Roberts E, Singh SB, et al. (2009) Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. J Cell Biol 186: 255-268.

184. La Gruta NL, Thomas PG, Webb AI, Dunstone MA, Cukalac T, et al. (2008) Epitope-specific TCRbeta repertoire diversity imparts no functional advantage on the CD8+ T cell response to cognate viral peptides. Proc Natl Acad Sci U S A 105: 2034-2039.

185. Lai KY NG, Wong KF, Hung IFN, Hong JKF, Cheng FF, Chan JKC (2013) Human H7N9 avian influenza virus infection: a review and pandemic risk assessment. Emerging Microbes & Infections 2: e48.

186. Lakadamyali M, Rust MJ, Zhuang X (2004) Endocytosis of influenza viruses. Microbes Infect 6: 929-936.

187. Lakdawala SS, Lamirande EW, Suguitan AL, Jr., Wang W, Santos CP, et al. (2011) Eurasian-origin gene segments contribute to the transmissibility, aerosol release, and morphology of the 2009 pandemic H1N1 influenza virus. PLoS Pathog 7: e1002443.

188. Lamb RA, Choppin PW (1979) Segment 8 of the influenza virus genome is unique in coding for two polypeptides. Proc Natl Acad Sci U S A 76: 4908-4912.

189. Lamb RA, Takeda M (2001) Death by influenza virus protein. Nat Med 7: 1286-1288.

190. Lamb RA, Zebedee SL, Richardson CD (1985) Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell 40: 627-633.

191. Latham T, Galarza JM (2001) Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. J Virol 75: 6154-6165.

192. Laver WG (1984) Antigenic variation and the structure of influenza virus glycoproteins. Microbiol Sci 1: 37-43.

193. Lazarou M (2015) Keeping the immune system in check: a role for mitophagy. Immunol Cell Biol 93: 3-10.

194. Lazarowitz SG, Choppin PW (1975) Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 68: 440-454.

195. Le Goffic R, Bouguyon E, Chevalier C, Vidic J, Da Costa B, et al. (2010) Influenza A virus protein PB1-F2 exacerbates IFN-beta expression of human respiratory epithelial cells. J Immunol 185: 4812-4823.

196. Le Goffic R, Leymarie O, Chevalier C, Rebours E, Da Costa B, et al. (2011) Transcriptomic analysis of host immune and cell death responses associated with the influenza A virus PB1-F2 protein. PLoS Pathog 7: e1002202.

197. Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, et al. (2007) Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J Immunol 178: 3368-3372.

198. Leahy MB, Dessens JT, Nuttall PA (1997) Striking conformational similarities between the transcription promoters of Thogoto and influenza A viruses: evidence for intrastrand base pairing in the 5' promoter arm. J Virol 71: 8352-8356.

199. Leahy MB, Dobbyn HC, Brownlee GG (2001) Hairpin loop structure in the 3' arm of the influenza A virus virion RNA promoter is required for endonuclease activity. J Virol 75: 7042-7049.

200. Leahy MB, Pritlove DC, Poon LL, Brownlee GG (2001) Mutagenic analysis of the 5' arm of the influenza A virus virion RNA promoter defines the sequence requirements for endonuclease activity. J Virol 75: 134-142.

201. Lear JD (2003) Proton conduction through the M2 protein of the influenza A virus; a quantitative, mechanistic analysis of experimental data. FEBS Lett 552: 17-22.

202. Lee CC, Zhu H, Huang PY, Peng L, Chang YC, et al. (2014) Emergence and evolution of avian H5N2 influenza viruses in chickens in Taiwan. J Virol 88: 5677-5686.

203. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A (2007) Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 315: 1398-1401.

204. Lee JS, Li Q, Lee JY, Lee SH, Jeong JH, et al. (2009) FLIP-mediated autophagy regulation in cell death control. Nat Cell Biol 11: 1355-1362.

205. Levine B, Deretic V (2007) Unveiling the roles of autophagy in innate and adaptive immunity. Nat Rev Immunol 7: 767-777.

206. Levine B, Klionsky DJ (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell 6: 463-477.

207. Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132: 27-42.

208. Li ML, Rao P, Krug RM (2001) The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. EMBO J 20: 2078-2086.

209. Li WW, Li J, Bao JK (2012) Microautophagy: lesser-known self-eating. Cell Mol Life Sci 69: 1125-1136.

210. Liang Q, Seo GJ, Choi YJ, Kwak MJ, Ge J, et al. (2014) Crosstalk between the cGAS DNA sensor and Beclin-1 autophagy protein shapes innate antimicrobial immune responses. Cell Host Microbe 15: 228-238.

211. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, et al. (1998) Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. J Virol 72: 8586-8596.

212. Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, et al. (2010) Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. J Virol 84: 12069-12074.

213. Lupfer C, Thomas PG, Anand PK, Vogel P, Milasta S, et al. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. Nat Immunol 14: 480-488.

214. Ma W, Gramer M, Rossow K, Yoon KJ (2006) Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J Virol 80: 5092-5096.

215. Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR, et al. (2009) The role of swine in the generation of novel influenza viruses. Zoonoses Public Health 56: 326-337.

216. Manrubia SC, Escarmis C, Domingo E, Lazaro E (2005) High mutation rates, bottlenecks, and robustness of RNA viral quasispecies. Gene 347: 273-282.

217. Manuguerra JC, Hannoun C (1992) Natural infection of dogs by influenza C virus. Res Virol 143: 199-204.

218. Massey AC, Zhang C, Cuervo AM (2006) Chaperone-mediated autophagy in aging and disease. Curr Top Dev Biol 73: 205-235.

219. Masurel N, Marine WM (1973) Recycling of Asian and Hong Kong influenza A virus hemagglutinins in man. Am J Epidemiol 97: 44-49.

220. Matlin KS, Reggio H, Helenius A, Simons K (1981) Infectious entry pathway of influenza virus in a canine kidney cell line. J Cell Biol 91: 601-613.

221. Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, et al. (2000) Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 74: 8502-8512.

222. Matrosovich M, Zhou N, Kawaoka Y, Webster R (1999) The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 73: 1146-1155.

223. Matrosovich MN, Krauss S, Webster RG (2001) H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 281: 156-162.

224. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A 101: 4620-4624.

225. Matsuoka Y, Swayne DE, Thomas C, Rameix-Welti MA, Naffakh N, et al. (2009) Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. J Virol 83: 4704-4708.

226. Mazur I, Anhlan D, Mitzner D, Wixler L, Schubert U, et al. (2008) The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell Microbiol 10: 1140-1152.

227. McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, et al. (2010) PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. PLoS Pathog 6: e1001014.

228. McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, et al. (2007) Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host Microbe 2: 240-249.

229. McAuley JL, Tate MD, MacKenzie-Kludas CJ, Pinar A, Zeng W, et al. (2013) Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. PLoS Pathog 9: e1003392.

230. McAuley JL, Zhang K, McCullers JA (2010) The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. J Virol 84: 558-564.

231. McCullers JA, Van De Velde LA, Schultz RD, Mitchell CG, Halford CR, et al. (2011) Seroprevalence of seasonal and pandemic influenza A viruses in domestic cats. Arch Virol 156: 117-120.

232. Medina RA, Garcia-Sastre A (2011) Influenza A viruses: new research developments. Nat Rev Microbiol 9: 590-603.

233. Melen K, Fagerlund R, Franke J, Kohler M, Kinnunen L, et al. (2003) Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein. J Biol Chem 278: 28193-28200.

234. Mijaljica D, Prescott M, Devenish RJ (2011) Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy 7: 673-682.

235. Mikulasova A, Vareckova E, Fodor E (2000) Transcription and replication of the influenza a virus genome. Acta Virol 44: 273-282.

236. Mitnaul LJ, Castrucci MR, Murti KG, Kawaoka Y (1996) The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. J Virol 70: 873-879.

237. Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, et al. (2000) Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J Virol 74: 6015-6020.

238. Mitzner D, Dudek SE, Studtrucker N, Anhlan D, Mazur I, et al. (2009) Phosphorylation of the influenza A virus protein PB1-F2 by PKC is crucial for apoptosis promoting functions in monocytes. Cell Microbiol 11: 1502-1516.

239. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451: 1069-1075.

240. Mizushima N, Ohsumi Y, Yoshimori T (2002) Autophagosome formation in mammalian cells. Cell Struct Funct 27: 421-429.

241. Morens DM, Taubenberger JK, Fauci AS (2008) Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis 198: 962-970.

242. Morens DM, Taubenberger JK, Harvey HA, Memoli MJ (2010) The 1918 influenza pandemic: lessons for 2009 and the future. Crit Care Med 38: e10-20.

243. Morris S, Swanson MS, Lieberman A, Reed M, Yue Z, et al. (2011) Autophagy-mediated dendritic cell activation is essential for innate cytokine production and APC function with respiratory syncytial virus responses. J Immunol 187: 3953-3961.

244. Muramoto Y, Noda T, Kawakami E, Akkina R, Kawaoka Y (2013) Identification of novel influenza A virus proteins translated from PA mRNA. J Virol 87: 2455-2462.

245. Murti KG, Webster RG, Jones IM (1988) Localization of RNA polymerases on influenza viral ribonucleoproteins by immunogold labeling. Virology 164: 562-566.

246. Myers KP, Olsen CW, Gray GC (2007) Cases of swine influenza in humans: a review of the literature. Clin Infect Dis 44: 1084-1088.

247. Myers KP, Olsen CW, Setterquist SF, Capuano AW, Donham KJ, et al. (2006) Are swine workers in the United States at increased risk of infection with zoonotic influenza virus? Clin Infect Dis 42: 14-20.

248. Naffakh N, Massin P, van der Werf S (2001) The transcription/replication activity of the polymerase of influenza A viruses is not correlated with the level of proteolysis induced by the PA subunit. Virology 285: 244-252.

249. Naito T, Momose F, Kawaguchi A, Nagata K (2007) Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. J Virol 81: 1339-1349.

250. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, et al. (2011) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 12: 222-230.

251. Narendra D, Tanaka A, Suen DF, Youle RJ (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 183: 795-803.

252. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, et al. (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8: e1000298.

253. Nayak D, Shivakoti, S., Balogun, R. A., Lee, G., Zhou, Z. H. (2013) Structure, disassembly, assembly, and budding of influenza viruses. In: Webster RG, Monto, A. S., Braciale, T. J., Lamb, R. A., editor. Textbook of Influenza. 2nd ed: Wiley Blackwell.

254. Nayak DP, Balogun RA, Yamada H, Zhou ZH, Barman S (2009) Influenza virus morphogenesis and budding. Virus Res 143: 147-161.

255. Nayak DP, Hui EK, Barman S (2004) Assembly and budding of influenza virus. Virus Res 106: 147-165.

256. Nelson MI, Vincent AL, Kitikoon P, Holmes EC, Gramer MR (2012) Evolution of novel reassortant A/H3N2 influenza viruses in North American swine and humans, 2009-2011. J Virol 86: 8872-8878.

257. Neumann G, Kawaoka Y (1999) Genetic engineering of influenza and other negative-strand RNA viruses containing segmented genomes. Adv Virus Res 53: 265-300.

258. Neumann G, Kawaoka Y (2001) Reverse genetics of influenza virus. Virology 287: 243-250.

259. Neumann G, Kawaoka Y (2006) Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis 12: 881-886.

260. Neumann G, Noda T, Kawaoka Y (2009) Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 459: 931-939.

261. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, et al. (1999) Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 96: 9345-9350.

262. Neumann G, Zobel A, Hobom G (1994) RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 202: 477-479.

263. Nguyen DC, Uyeki TM, Jadhao S, Maines T, Shaw M, et al. (2005) Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. J Virol 79: 4201-4212.

264. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N (2013) Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta 1833: 3448-3459.

265. Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, et al. (1991) Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 182: 475-485.

266. Novel Swine-Origin Influenza AVIT, Dawood FS, Jain S, Finelli L, Shaw MW, et al. (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360: 2605-2615.

267. O'Neill RE, Talon J, Palese P (1998) The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. EMBO J 17: 288-296.

268. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, et al. (2006) Global patterns of influenza a virus in wild birds. Science 312: 384-388.

269. Olsen CW (2002) The emergence of novel swine influenza viruses in North America. Virus Res 85: 199-210.

270. Olsen CW, Carey S, Hinshaw L, Karasin AI (2000) Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States. Arch Virol 145: 1399-1419.

271. Olsen CW, Karasin A, Erickson G (2003) Characterization of a swine-like reassortant H1N2 influenza virus isolated from a wild duck in the United States. Virus Res 93: 115-121.

272. Orenstein SJ, Cuervo AM (2010) Chaperone-mediated autophagy: molecular mechanisms and physiological relevance. Semin Cell Dev Biol 21: 719-726.

273. Orvedahl A, Alexander D, Talloczy Z, Sun Q, Wei Y, et al. (2007) HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe 1: 23-35.

274. Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA (2000) Influenza B virus in seals. Science 288: 1051-1053.

275. Palese P, Shaw, M. L. (2007) Orthomyxoviridae: The Viruses and their Replication. In: Knipe DM, Griffin, D. E., Lamb, R. A., Martin, M A., Roiz-man, B., Straus, S. E., editor. Fields Virology. 5th ed: Lippincott Williams & Wilkins. pp. 1647-1690.

276. Palese P, Compans RW (1976) Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. J Gen Virol 33: 159-163.

277. Palese P, Schulman J (1974) Isolation and characterization of influenza virus recombinants with high and low neuraminidase activity. Use of 2-(3'-methoxyphenyl)-n-acetylneuraminic acid to identify cloned populations. Virology 57: 227-237.

278. Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, et al. (2005) Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 307: 593-596.

279. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, et al. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131-24145.

280. Pasick J, Handel K, Robinson J, Copps J, Ridd D, et al. (2005) Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol 86: 727-731.

281. Pasricha G, Mishra AC, Chakrabarti AK (2013) Comprehensive global amino acid sequence analysis of PB1F2 protein of influenza A H5N1 viruses and the influenza A

virus subtypes responsible for the 20th-century pandemics. Influenza Other Respir Viruses 7: 497-505.

282. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, et al. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122: 927-939.

283. Peiris JS, Poon LL, Guan Y (2009) Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. J Clin Virol 45: 169-173.

284. Pekosz A, He B, Lamb RA (1999) Reverse genetics of negative-strand RNA viruses: closing the circle. Proc Natl Acad Sci U S A 96: 8804-8806.

285. Pena L, Vincent AL, Loving CL, Henningson JN, Lager KM, et al. (2012) Restored PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in swine. J Virol 86: 5523-5532.

286. Pinto LH, Holsinger LJ, Lamb RA (1992) Influenza virus M2 protein has ion channel activity. Cell 69: 517-528.

287. Pleschka S, Jaskunas R, Engelhardt OG, Zurcher T, Palese P, et al. (1996) A plasmid-based reverse genetics system for influenza A virus. J Virol 70: 4188-4192.

288. Plotch SJ, Bouloy M, Ulmanen I, Krug RM (1981) A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23: 847-858.

289. Poole AC, Thomas RE, Yu S, Vincow ES, Pallanck L (2010) The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS One 5: e10054.

290. Presti RM, Zhao G, Beatty WL, Mihindukulasuriya KA, da Rosa AP, et al. (2009) Quaranfil, Johnston Atoll, and Lake Chad viruses are novel members of the family Orthomyxoviridae. J Virol 83: 11599-11606.

291. Pringle CR (1996) Virus taxonomy 1996 - a bulletin from the Xth International Congress of Virology in Jerusalem. Arch Virol 141: 2251-2256.

292. Pritlove DC, Fodor E, Seong BL, Brownlee GG (1995) In vitro transcription and polymerase binding studies of the termini of influenza A virus cRNA: evidence for a cRNA panhandle. J Gen Virol 76 (Pt 9): 2205-2213.

293. Pritlove DC, Poon LL, Devenish LJ, Leahy MB, Brownlee GG (1999) A hairpin loop at the 5' end of influenza A virus virion RNA is required for synthesis of poly(A)+ mRNA in vitro. J Virol 73: 2109-2114.

294. Rabadan R, Levine AJ, Robins H (2006) Comparison of avian and human influenza A viruses reveals a mutational bias on the viral genomes. J Virol 80: 11887-11891.

295. Reggiori F, Monastyrska I, Verheije MH, Cali T, Ulasli M, et al. (2010) Coronaviruses Hijack the LC3-I-positive EDEMosomes, ER-derived vesicles exporting short-lived ERAD regulators, for replication. Cell Host Microbe 7: 500-508.

296. Reid AH, Taubenberger JK, Fanning TG (2004) Evidence of an absence: the genetic origins of the 1918 pandemic influenza virus. Nat Rev Microbiol 2: 909-914.

297. Richardson JC, Akkina RK (1991) NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. Arch Virol 116: 69-80.

298. Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, et al. (2003) Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. J Clin Microbiol 41: 3198-3205.

299. Roberts PC, Lamb RA, Compans RW (1998) The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology 240: 127-137.

300. Robertson JS (1979) 5' and 3' terminal nucleotide sequences of the RNA genome segments of influenza virus. Nucleic Acids Res 6: 3745-3757.

301. Rodriguez-Enriquez S, Kim I, Currin RT, Lemasters JJ (2006) Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes. Autophagy 2: 39-46.

302. Rogers GN, Paulson JC (1983) Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 127: 361-373.

302. Roos R (2014) H5N2, H5N8 avian flu viruses surface in US. CIDRAP News. http://www.cidrap.umn.edu/news-perspective/2014/12/h5n2-h5n8-avian-flu-viruses-surface-us

303. Roos R (2015) Role of wild birds in US H5N2 outbreaks questioned. CIDRAP News. http://www.cidrap.umn.edu/news-perspective/2015/03/role-wild-birds-us-h5n2-outbreaks-questioned

303. Rossman JS, Lamb RA (2009) Autophagy, apoptosis, and the influenza virus M2 protein. Cell Host Microbe 6: 299-300.

304. Rothberg MB, Haessler SD (2010) Complications of seasonal and pandemic influenza. Crit Care Med 38: e91-97.

305. Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, et al. (2012) The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. Science 336: 1541-1547.

306. Rusten TE, Stenmark H (2010) p62, an autophagy hero or culprit? Nat Cell Biol 12: 207-209.

307. Samji T (2009) Influenza A: understanding the viral life cycle. Yale J Biol Med 82: 153-159.

308. Sanz-Ezquerro JJ, de la Luna S, Ortin J, Nieto A (1995) Individual expression of influenza virus PA protein induces degradation of coexpressed proteins. J Virol 69: 2420-2426.

309. Sato M, Sato K (2011) Degradation of paternal mitochondria by fertilization-triggered autophagy in C. elegans embryos. Science 334: 1141-1144.

310. Scalera NM, Mossad SB (2009) The first pandemic of the 21st century: a review of the 2009 pandemic variant influenza A (H1N1) virus. Postgrad Med 121: 43-47.

311. Schmid D, Munz C (2007) Innate and adaptive immunity through autophagy. Immunity 27: 11-21.

312. Schmitt AP, Lamb RA (2005) Influenza virus assembly and budding at the viral budozone. Adv Virus Res 64: 383-416.

313. Schmolke M, Manicassamy B, Pena L, Sutton T, Hai R, et al. (2011) Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. PLoS Pathog 7: e1002186.

314. Schnell JR, Chou JJ (2008) Structure and mechanism of the M2 proton channel of influenza A virus. Nature 451: 591-595.

315. Scholtissek C, Rohde W, Von Hoyningen V, Rott R (1978) On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87: 13-20.

316. Schroeder C, Heider H, Moncke-Buchner E, Lin TI (2005) The influenza virus ion channel and maturation cofactor M2 is a cholesterol-binding protein. Eur Biophys J 34: 52-66.

317. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, et al. (2007) NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci U S A 104: 19500-19505.

318. Scott I, Youle RJ (2010) Mitochondrial fission and fusion. Essays Biochem 47: 85-98.

319. Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, et al. (2004) Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 24: 8055-8068.

320. Selman M, Dankar, S.K., Forbes, N.E., Jia, J.J., Brown, E.G., (2012) Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. Emerging Microbes & Infections 1: e42.

321. Seo SH, Hoffmann E, Webster RG (2002) Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 8: 950-954.

322. Seth RB, Sun L, Ea CK, Chen ZJ (2005) Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 122: 669-682.

323. Sethi S (2002) Bacterial pneumonia. Managing a deadly complication of influenza in older adults with comorbid disease. Geriatrics 57: 56-61.

324. Shelly S, Lukinova N, Bambina S, Berman A, Cherry S (2009) Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity 30: 588-598.

325. Shi JZ LP, Zhou JP, Guan LZ, Li WH, Li XY, Guo J, Wang GJ, Fan J, Wang JL, Li YY, Jiang YP, Liu LL, Tian GB, Li CJ, Chen HL (2013) Isolation and characterization of H7N9 viruses from live poultry markets — Implication of the source of current H7N9 infection in humans. Chinese Science Bulletin 58: 1857-1863.

326. Shi L, Summers DF, Peng Q, Galarz JM (1995) Influenza A virus RNA polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. Virology 208: 38-47.

327. Shimbo K, Brassard DL, Lamb RA, Pinto LH (1996) Ion selectivity and activation of the M2 ion channel of influenza virus. Biophys J 70: 1335-1346.

328. Shrivastava S, Raychoudhuri A, Steele R, Ray R, Ray RB (2011) Knockdown of autophagy enhances the innate immune response in hepatitis C virus-infected hepatocytes. Hepatology 53: 406-414.

329. Simonsen L (1999) The global impact of influenza on morbidity and mortality. Vaccine 17 Suppl 1: S3-10.

330. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, et al. (2009) Autophagy regulates lipid metabolism. Nature 458: 1131-1135.

331. Sinha S, Colbert CL, Becker N, Wei Y, Levine B (2008) Molecular basis of the regulation of Beclin 1-dependent autophagy by the gamma-herpesvirus 68 Bcl-2 homolog M11. Autophagy 4: 989-997.

332. Sir D, Chen WL, Choi J, Wakita T, Yen TS, et al. (2008) Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. Hepatology 48: 1054-1061.

333. Sir D, Kuo CF, Tian Y, Liu HM, Huang EJ, et al. (2012) Replication of hepatitis C virus RNA on autophagosomal membranes. J Biol Chem 287: 18036-18043.

334. Sir D, Tian Y, Chen WL, Ann DK, Yen TS, et al. (2010) The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proc Natl Acad Sci U S A 107: 4383-4388.

335. Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69: 531-569.

336. Smith GJ, Bahl J, Vijaykrishna D, Zhang J, Poon LL, et al. (2009) Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A 106: 11709-11712.

337. Stegmann T (2000) Membrane fusion mechanisms: the influenza hemagglutinin paradigm and its implications for intracellular fusion. Traffic 1: 598-604.

338. Steinhauer DA (1999) Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology 258: 1-20.

339. Steinhauer DA, Domingo E, Holland JJ (1992) Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. Gene 122: 281-288.

340. Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, et al. (2004) Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 303: 1866-1870.

341. Suarez DL, Senne DA, Banks J, Brown IH, Essen SC, et al. (2004) Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg Infect Dis 10: 693-699.

342. Suarez DL, Woolcock PR, Bermudez AJ, Senne DA (2002) Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis 46: 111-121.

343. Subbarao EK, London W, Murphy BR (1993) A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 67: 1761-1764.

344. Suen DF, Narendra DP, Tanaka A, Manfredi G, Youle RJ (2010) Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. Proc Natl Acad Sci U S A 107: 11835-11840.

345. Sun MX, Huang L, Wang R, Yu YL, Li C, et al. (2012) Porcine reproductive and respiratory syndrome virus induces autophagy to promote virus replication. Autophagy 8: 1434-1447.

346. Suzuki T, Takahashi T, Guo CT, Hidari KI, Miyamoto D, et al. (2005) Sialidase activity of influenza A virus in an endocytic pathway enhances viral replication. J Virol 79: 11705-11715.

347. Takacs-Vellai K, Vellai T, Puoti A, Passannante M, Wicky C, et al. (2005) Inactivation of the autophagy gene bec-1 triggers apoptotic cell death in C. elegans. Curr Biol 15: 1513-1517.

348. Tal MC, Sasai M, Lee HK, Yordy B, Shadel GS, et al. (2009) Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. Proc Natl Acad Sci U S A 106: 2770-2775.

349. Tal R, Winter G, Ecker N, Klionsky DJ, Abeliovich H (2007) Aup1p, a yeast mitochondrial protein phosphatase homolog, is required for efficient stationary phase mitophagy and cell survival. J Biol Chem 282: 5617-5624.

350. Talloczy Z, Jiang W, Virgin HWt, Leib DA, Scheuner D, et al. (2002) Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. Proc Natl Acad Sci U S A 99: 190-195.

351. Tang Y, Lee CW, Zhang Y, Senne DA, Dearth R, et al. (2005) Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis 49: 207-213.

352. Tanida I, Fukasawa M, Ueno T, Kominami E, Wakita T, et al. (2009) Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles. Autophagy 5: 937-945.

353. Taubenberger JK, Kash JC (2010) Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe 7: 440-451.

354. Taylor R, Goldman SJ (2011) Mitophagy and disease: new avenues for pharmacological intervention. Curr Pharm Des 17: 2056-2073.

355. Thomas PG, Handel A, Doherty PC, La Gruta NL (2013) Ecological analysis of antigen-specific CTL repertoires defines the relationship between naive and immune T-cell populations. Proc Natl Acad Sci U S A 110: 1839-1844.

356. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, et al. (2012) A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A 109: 4269-4274.

357. Tong S, Zhu X, Li Y, Shi M, Zhang J, et al. (2013) New world bats harbor diverse influenza A viruses. PLoS Pathog 9: e1003657.

358. Treanor JJ, Snyder MH, London WT, Murphy BR (1989) The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys. Virology 171: 1-9.

359. Twig G, Hyde B, Shirihai OS (2008) Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta 1777: 1092-1097.

360. Twig G, Shirihai OS (2011) The interplay between mitochondrial dynamics and mitophagy. Antioxid Redox Signal 14: 1939-1951.

361. Ulmanen I, Broni B, Krug RM (1983) Influenza virus temperature-sensitive cap (m7GpppNm)-dependent endonuclease. J Virol 45: 27-35.

362. Uttenweiler A, Schwarz H, Neumann H, Mayer A (2007) The vacuolar transporter chaperone (VTC) complex is required for microautophagy. Mol Biol Cell 18: 166-175.

363. Varga ZT, Palese P (2011) The influenza A virus protein PB1-F2: killing two birds with one stone? Virulence 2: 542-546.

364. Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, et al. (2011) The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS Pathog 7: e1002067.

365. Vasin AV, Temkina OA, Egorov VV, Klotchenko SA, Plotnikova MA, et al. (2014) Molecular mechanisms enhancing the proteome of influenza A viruses: an overview of recently discovered proteins. Virus Res 185: 53-63.

366. Vazquez-Martin A, Cufi S, Corominas-Faja B, Oliveras-Ferraros C, Vellon L, et al. (2012) Mitochondrial fusion by pharmacological manipulation impedes somatic cell reprogramming to pluripotency: new insight into the role of mitophagy in cell stemness. Aging (Albany NY) 4: 393-401.

367. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA (2008) Swine influenza viruses a North American perspective. Adv Virus Res 72: 127-154.

368. Vreede FT, Jung TE, Brownlee GG (2004) Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates. J Virol 78: 9568-9572.

369. Wagner R, Matrosovich M, Klenk HD (2002) Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol 12: 159-166.

370. Wan H, Perez DR (2006) Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 346: 278-286.

371. Wang TT, Palese, P. (2013) Emergence and Evolution of the 1918, 1957, 1968, and 2009 pandemic virus strains. In: Webster RG, Monto, A. S., Braciale, T. J., Lamb, R. A., editor. Textbook of Influenza. 2nd ed: Wiley Blackwell.

372. Webby RJ, Rossow K, Erickson G, Sims Y, Webster R (2004) Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res 103: 67-73.

373. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, et al. (2000) Evolution of swine H3N2 influenza viruses in the United States. J Virol 74: 8243-8251.

374. Webby RJ, Webster RG (2003) Are we ready for pandemic influenza? Science 302: 1519-1522.

375. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992) Evolution and ecology of influenza A viruses. Microbiol Rev 56: 152-179.

376. Webster RG, Hinshaw VS, Laver WG (1982) Selection and analysis of antigenic variants of the neuraminidase of N2 influenza viruses with monoclonal antibodies. Virology 117: 93-104.

377. Webster RG, Laver WG, Air GM, Schild GC (1982) Molecular mechanisms of variation in influenza viruses. Nature 296: 115-121.

378. Wei P, Li W, Zi H, Cunningham M, Guo Y, et al. (2015) Epidemiological and molecular characteristics of the PB1-F2 proteins in H7N9 influenza viruses, Jiangsu. Biomed Res Int 2015: 804731.

379. West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, et al. (2015) Mitochondrial DNA stress primes the antiviral innate immune response. Nature 520: 553-557.

380. Westermann B (2010) Mitochondrial dynamics in model organisms: what yeasts, worms and flies have taught us about fusion and fission of mitochondria. Semin Cell Dev Biol 21: 542-549.

381. Whittaker G, Bui M, Helenius A (1996) The role of nuclear import and export in influenza virus infection. Trends Cell Biol 6: 67-71.

382. Wild P, Farhan H, McEwan DG, Wagner S, Rogov VV, et al. (2011) Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science 333: 228-233.

383. Wiley DC, Skehel JJ (1977) Crystallization and x-ray diffraction studies on the haemagglutinin glycoprotein from the membrane of influenza virus. J Mol Biol 112: 343-347.

384. Wise HM, Barbezange C, Jagger BW, Dalton RM, Gog JR, et al. (2011) Overlapping signals for translational regulation and packaging of influenza A virus segment 2. Nucleic Acids Res 39: 7775-7790.

385. Wise HM, Foeglein A, Sun J, Dalton RM, Patel S, et al. (2009) A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol 83: 8021-8031.

386. Wise HM, Hutchinson EC, Jagger BW, Stuart AD, Kang ZH, et al. (2012) Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. PLoS Pathog 8: e1002998.

387. Wright PF, Neumann, G., Kawaoka, Y. (2007) Orthomyxoviruses In: Knipe DM, Griffin, D. E., Lamb, R. A., Martin, M A., Roiz-man, B., Straus, S. E., editor. Fields Virology. 5th ed: Lippincott Williams & Wilkins. pp. 1691-1740.

388. Xia M, Meng G, Li M, Wei J (2014) Mitophagy in viral infections. DNA Cell Biol 33: 739-742.

389. Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9: 1102-1109.

390. Yamada H, Chounan R, Higashi Y, Kurihara N, Kido H (2004) Mitochondrial targeting sequence of the influenza A virus PB1-F2 protein and its function in mitochondria. FEBS Lett 578: 331-336.

391. Yang Z, Klionsky DJ (2010) Eaten alive: a history of macroautophagy. Nat Cell Biol 12: 814-822.

392. Yasuda J, Nakada S, Kato A, Toyoda T, Ishihama A (1993) Molecular assembly of influenza virus: association of the NS2 protein with virion matrix. Virology 196: 249-255.

393. Youle RJ, Narendra DP (2011) Mechanisms of mitophagy. Nat Rev Mol Cell Biol 12: 9-14.

394. Youle RJ, van der Bliek AM (2012) Mitochondrial fission, fusion, and stress. Science 337: 1062-1065.

395. Yuan P, Bartlam M, Lou Z, Chen S, Zhou J, et al. (2009) Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. Nature 458: 909-913.

396. Yuanji G, Desselberger U (1984) Genome analysis of influenza C viruses isolated in 1981/82 from pigs in China. J Gen Virol 65 (Pt 11): 1857-1872.

397. Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P (2005) Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog 1: e4.

398. Zamarin D, Ortigoza MB, Palese P (2006) Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J Virol 80: 7976-7983.

399. Zebedee SL, Lamb RA (1988) Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J Virol 62: 2762-2772.

400. Zebedee SL, Richardson CD, Lamb RA (1985) Characterization of the influenza virus M2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. J Virol 56: 502-511.

401. Zell R, Krumbholz A, Eitner A, Krieg R, Halbhuber KJ, et al. (2007) Prevalence of PB1-F2 of influenza A viruses. J Gen Virol 88: 536-546.

402. Zhang Q, Kang R, Zeh HJ, 3rd, Lotze MT, Tang D (2013) DAMPs and autophagy: cellular adaptation to injury and unscheduled cell death. Autophagy 9: 451-458.

403. Zhirnov OP, Poyarkov SV, Vorob'eva IV, Safonova OA, Malyshev NA, et al. (2007) Segment NS of influenza A virus contains an additional gene NSP in positive-sense orientation. Dokl Biochem Biophys 414: 127-133.

404. Zhou D, Spector SA (2008) Human immunodeficiency virus type-1 infection inhibits autophagy. AIDS 22: 695-699.

405. Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, et al. (1999) Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 73: 8851-8856.

406. Zhou R, Yazdi AS, Menu P, Tschopp J (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature 469: 221-225.

407. Zhou Y, Konig M, Hobom G, Neumeier E (1998) Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. Virology 246: 83-94.

408. Zhou Z, Jiang X, Liu D, Fan Z, Hu X, et al. (2009) Autophagy is involved in influenza A virus replication. Autophagy 5: 321-328.

409. Zhu B, Xu F, Li J, Shuai J, Li X, et al. (2012) Porcine circovirus type 2 explores the autophagic machinery for replication in PK-15 cells. Virus Res 163: 476-485.

410. Zhu H, Wang D, Kelvin DJ, Li L, Zheng Z, et al. (2013) Infectivity, transmission, and pathology of human-isolated H7N9 influenza virus in ferrets and pigs. Science 341: 183-186.

411. Zobel A, Neumann G, Hobom G (1993) RNA polymerase I catalysed transcription of insert viral cDNA. Nucleic Acids Res 21: 3607-3614.

2. PB1-F2 Protein does not impact the Virulence of Triple Reassortant H3N2 Swine Influenza Virus in Pigs but Alters the Pathogenicity and Transmission in Turkeys

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2.1 Abstract:

PB1-F2 protein, the 11th influenza A virus (IAV) protein, is considered to play an important role in primary influenza virus infection and post-influenza secondary bacterial pneumonia in mice. The functional role of PB1-F2 has been reported to be a strain-specific and host-specific phenomenon. Its precise contribution to the pathogenicity and transmission of influenza virus in mammalian host such as swine and avian hosts such as turkeys remain largely unknown. In this study, we explored the role of PB1-F2 protein of triple-reassortant (TR) H3N2 swine influenza virus (SIV) in pigs and turkeys. Using the eight-plasmid reverse genetics system, we rescued SIV A/swine/Minnesota/1145/2007 (H3N2) (SIV 1145) wild type (WT), PB1-F2 knock out mutant (SIV 1145-KO) and its N66S variant (SIV 1145-N66S). Ablation of PB1-F2 in SIV 1145 modulated early stage apoptosis in human monocytes but did not affect the viral replication. In pigs, PB1-F2 expression did not affect nasal shedding, lung viral load, immunophenotypes and lung pathology. On the other hand, in turkeys, deletion of PB1-F2 resulted in early induction of clinical disease and effective transmission among the turkey poults. Further, turkeys infected with SIV 1145-N66S displayed poor infectivity and transmissibility. The relative transmission advantage and higher immunogenicity observed in turkeys infected with SIV 1145-KO virus needs to be further explored. Taken together, these results emphasize the host-specific roles of PB1-F2 in the pathogenicity and transmission of IAV.

Importance:

Novel triple reassortant H3N2 swine influenza virus (TR H3N2 SIV) emerged in 1998 and spread rapidly among the North American swine population. Subsequently, it showed increased propensity to reassort generating a range of reassortants. Unlike

classical swine influenza virus, TR SIV produces a full-length PB1-F2 protein, which is considered an important virulence marker of IAV pathogenicity. Our study demonstrated that expression of PB1-F2 does not impact the pathogenicity of TR H3N2 SIV in pigs. On the other hand, deletion of PB1-F2 caused TR H3N2 SIV to induce clinical disease early and resulted in effective transmission among the turkey poults. Our study emphasizes the continuing need to better understand the virulence determinants for IAV in intermediate hosts such as swine and turkeys and highlights the host-specific role of PB1-F2 protein.

2.2 Introduction:

Influenza A virus (IAV) is zoonotic with a wide host range including humans, horses, pigs, dogs, sea mammals, and birds. It is responsible for annual seasonal epidemics in humans, which cause significant morbidity and socio-economic costs worldwide. Occasionally, it leads to pandemics as in the case of 1918 H1N1, 1957 H2N2 and 1986 H3N2 IAV outbreaks causing millions of death worldwide (1). Influenza in swine is an acute respiratory disease whose severity depends on many factors such as host age, virus strain, and secondary bacterial infections (2). Swine influenza virus (SIV) was first isolated in the year 1930 in USA (3). Until 1998, classical swine H1N1 (cH1N1) lineage with minimal changes was circulating in pigs (4). However, by late 1998, a novel triple reassortant (TR) H3N2 SIV emerged and become established in North American swine population. It possessed HA, NA, and PB1 gene segments from human IAVs, M, NS, and NP gene segments from cH1N1 SIVs and PA, and PB2 gene segments from avian IAVs (5, 6). Once established, the triple reassortant H3N2 viruses had undergone reassortment with cH1N1 SIV producing H1N2, reassortant H1N1 (rH1N1) and H3N1

SIVs (7-9). Currently, the H3N2, rH1N1 and H1N2 SIVs have become endemic and co-circulate in most North American swine population (10, 11). Reassortant SIVs that have become endemic in swine population contains triple-reassortant internal gene (TRIG) cassette comprising internal genes representing the PA and PB2 genes of avian origin, NS, NP and M genes of classical swine origin, and the PB1 gene of human origin (12, 13).

Host specificity of IAV host can be explained in part by the difference in receptor binding specificity of human and avian IAVs. Human IAV preferentially binds to α 2, 6 sialic acid (SA)-galactose (gal) receptors (α 2,6 SA-gal) present in respiratory tract while avian IAV preferentially binds to α 2,3 sialic acid (SA)-galactose (gal) receptors (α 2,3 SA-gal) present in the intestinal tract (14). Pig tracheal cells possess both α 2,6 SA-gal and α 2,3 SA-gal receptors and have been postulated to be a "mixing vessel" of IAVs from avian and human sources (15). Reassortment between avian, swine and human IAV occur in pigs as exemplified by periodical emergence of strains from pigs such as swine origin H1N1 pandemic virus in 2009 (16, 17).

Turkeys also possess α 2, 6 gal SA and α 2, 3 gal SA receptors in the tracheal epithelial cells (18). Turkeys are susceptible to wide range of IAVs and hence serve as an important intermediate host for IAVs (19). In 2003, some of the TR H3N2 SIVs crossed the species barrier and caused outbreaks in domestic turkey farms situated in Minnesota and North Carolina (20, 21) and is now an endemic virus in turkeys.

IAV PB1-F2 is a short non-structural accessory protein encoded by the alternate reading frame in the PB1 gene segment (22). It is an enigmatic protein with different lengths from 11 to 101 amino acids and with diverse functions attributed to it. The C-

terminal portion of PB1-F2 protein is predicted to target mitochondria, leading to apoptosis in immune cells as a result of interaction with the mitochondrial membrane associated proteins VDAC-1 and ANT-3 (23, 24). Expression of PB1-F2 protein has been shown to enhance viral pathogenicity in both primary influenza virus infection (25) and secondary bacterial infection in mice (26). PB1-F2 protein also inhibits type I Interferon induction by interfering with RIG-I/MAVS protein complex (27). Further, presence of serine instead of asparginine at position 66 is associated with increased lung immunopathology in a mouse model (28). It also increases viral polymerase activity *in vitro* by binding with PB1 subunit of polymerase complex (29). However, the precise function of PB1-F2 protein remains elusive and it appears that most of its functions are cell-type, virus strain, and even host-specific (30).

All three 20th century pandemic IAVs and highly pathogenic avian H5N1 IAVs produce full length PB1-F2 protein and considered to be one of the important virulence factors contributing to the pathogenesis of the IAV at least in a mouse model (31). During adaptation to humans, it appears that the PB1-F2 protein of many seasonal IAVs become truncated or the genetic markers for virulence in the C-terminal region are mutated (32). It is reasonable, therefore, to speculate that the truncated or mutated PB1-F2 may offer a survival advantage to the virus. Most avian IAVs express full-length PB1-F2 whereas it is truncated in cH1N1 SIVs due to the presence of in-frame stop codons after codons 11, 25 and 34 (33). PB1-F2 protein of TR H3N2, rH1N1, and H1N2 SIVs are highly similar to human seasonal H3N2 IAVs. To date, the precise contribution of PB1-F2 protein in the virulence of TR H3N2 SIV in swine and turkeys and its role in transmission between host species are not well understood. This is particularly important as several strains emerged

from pigs and crossed species to humans in the recent past including the swine origin H1N1 pandemic virus in 2009 (34) and variant H3N2 (35). In this study, we used the TR H3N2 SIV, A/Swine/Minnesota/1145/2007 (H3N2) (SIV 1145) to elucidate its role in hosts such as pigs and turkeys.

2.3 Materials and Methods:

Cells and Virus strains:

Madin-Darby canine kidney (MDCK) cells, human embryonic kidney epithelial (HEK 293T) cells, human alveolar basal epithelial cells (A549), and human monocytic (U937) cells were obtained from American Type Culture Collection (ATCC) and were grown either in Dulbecco's Minimal Essential Medium (DMEM) or in Minimal Essential Medium (MEM) (Invitrogen) supplemented with 10% heat inactivated Fetal Calf Serum (FCS) (Thermo Scientific) and 1% penicillin-streptomycin (Invitrogen). Influenza A/swine/Minnesota/1145/2007 (H3N2) (SIV 1145) strain originally obtained from Veterinary Diagnostic Laboratory, University of Minnesota and its complete genome was sequenced (GenBank accession # FJ410137-FJ410144) and described elsewhere (36). All experiments involving SIV 1145 virus were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee (IBC) and were conducted under biosafety level 2/animal biosafety level 2 conditions.

Constructs and cloning:

Eight gene segments of SIV 1145 were cloned into pHW 2000 vector as described earlier (37). The open reading frame for PB1-F2 was disrupted in SIV 1145-PB1 plasmid to create SIV 1145 Knock-Out (SIV 1145 KO) plasmid without affecting the reading frame of PB1 gene. Start codon was mutated from ATG to ACG (T120C) so that

translation will not initiate and two additional stop codons were introduced, one at position 12 (C153G) and one at position 58 (G291A) to ensure a complete knock-out using QuickChange II site-directed mutagenesis kit (Agilent Technologies). Asparagine is mutated to serine at position 66 to create SIV 1145-N66S plasmid. The sequences of each construct were confirmed by automated sequencing performed at Virginia bioinformatics institute (VBI) sequencing facility at Virginia Tech.

Rescue of recombinant SIV:

Recombinant SIV were rescued by transfecting co-cultured MDCK and HEK 293T cells with eight pHW 2000 vectors containing viral genomic RNA segments as described earlier (37). After 72 h, the supernatants were harvested and propagated in 10-day old embryonated specific pathogen free (SPF) chicken eggs and titrated on MDCK cells. The presence of the introduced mutations in the PB1 gene segment were confirmed by reverse transcriptase PCR (RT-PCR) and complete sequencing the PB1 gene segment.

Growth Kinetics of recombinant SIV:

To analyze the replication kinetics of recombinant SIV, A549 cells were infected at a multiplicity of infection (MOI) of 0.001. After 1 h incubation at 37 °C, the supernatants were removed, washed and cells were maintained in MEM containing 1 μg/mL of tosylsulphonyl phenalanyl chlrormethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich). Cell culture supernatants were collected at 24, 48, and 72 hours post-infection (hpi) and viral titers were determined by mean tissue culture infective dose ($TCID_{50}$) in MDCK cells. The $TCID_{50}$ per mL was calculated by Reed and Muench method (38).

Apoptosis assay:

Human monocytic cells, U937, were infected with 2 MOI of indicated recombinant swine influenza viruses. At 12 hpi, the cells were harvested, washed and resuspended in 100 µl of annexin binding buffer (Biolegend). Cells were stained with APC labeled annexin V^+ (Biolegend) for 20 minutes (min) and propidium iodide (18) (Biolegend) for 5 min. Cells were then acquired by FACS Aria flow cytometer and analyzed by FlowJo software version 7.6 (Tree star). Apoptotic cells were defined as annexin V+, while necrotic cellular events are defined as annexin V+ and PI+. Viable cells were considered as neither annexin V+ nor PI+.

Pathogenicity of recombinant SIV to pigs:

Twenty-seven 6-week-old outbred SPF pigs were obtained from the swine breeding facility at Virginia Tech. Animals were confirmed to be free of SIV-specific antibodies. All animal experiments were carried out in accordance with the approved protocols of IACUC at Virginia Tech. Pigs were randomly distributed into 3 groups of nine animals each. Each group was anesthetized with a mixture of Tiletamine and Zolazepam (Telazol, Fort Dodge Animal health) and Xylazine (Lloyd Laboratories) administered intramuscularly at 4.4 mg/kg of bodyweight. Subsequently, pigs were infected intranasally with 1 mL of $1x10^7$ plaque-forming unit (PFU) of respective recombinant SIV or mock-infected with 1 mL of phosphate buffered saline (PBS). Clinical signs exhibited by the experimental animals including lethargy, anorexia, nasal discharge, and rectal temperature was recorded daily. Nasal swabs were collected daily, and processed for viral titers by $TCID_{50}$ in MDCK cells. Three piglets per group were humanely euthanized on 3, 7, 14 days post infection (dpi) by intravenous injection of

sodium pentobarbital (Fatal-Plus, Vortech Pharmaceuticals) at 0.22-mL/kg bodyweight. After euthanasia, bronchoalveolar lavage fluid (BAL) was collected immediately. Fifty mL of cold PBS containing 0.3% EDTA was injected into the right and left anterior lobes of the lung using a sterile catheter and the BAL was aspirated immediately. Blood was collected into vacutainer tubes (Becton Dickinson). Lungs and trachea were also collected during necropsy.

Pathogenicity of recombinant SIV to turkeys:

Thirty-six 2-4 week-old SPF turkey poults were obtained from a commercial vendor. Animals were confirmed to be free of SIV-specific antibodies. All animal experiments were carried out in accordance with the approved protocols of IACUC at Virginia Tech. Birds were randomly distributed into 4 groups of six each. Each group was infected intranasally with $1x10^7$ PFU/50uL of respective recombinant SIV or mock infected with PBS. After 24 h, three age-matched turkey poults were introduced into the cage as in-contacts. Clinical signs including lethargy, anorexia, and diarrhea were monitored daily. Cloacal swabs were collected daily for 10 days to monitor viral shedding. After 10 dpi, birds were euthanized humanely and blood and lungs were collected for further examination.

Virus load in lungs:

For determination of viral loads in the lungs, lung samples were homogenized using a hand-held homogenizer. A 20% suspension of lung was prepared in MEM and clarified by centrifugation. Virus load was determined by $TCID_{50}$ in MDCK cells. Briefly, serial 10-fold dilutions of the 20% lung suspensions were overlaid on MDCK monolayers for 1 h at 37 °C. After 1 h, the inoculum was removed and the cell

monolayer was washed 2 x with PBS. Infected cells were incubated at 37 °C in MEM containing 1% Avicel (FMC Biopolymer) and 1 μg/mL TPCK-treated trypsin (Sigma-Aldrich). At 96 hpi, infected cell cultures were stained with 1% crystal violet in 10% buffered formalin. The $TCID_{50}$ per mL was calculated by Reed and Muench method (38).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR):

Lung samples from infected pigs were homogenized and resuspended in 1ml of sterile PBS. Total RNA was extracted from the lung samples using RNeasy kit (Qiagen). The cDNA was prepared by high capacity cDNA kit (Applied Biosystems) and tested for interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), GAPDH and 18S mRNA transcripts by real-time quantitative reverse transcription PCR (qRT-PCR) using Syber Green dye (Life technologies). Endogenous control genes GAPDH and 18S were used to normalize the target genes.

Flow cytometry:

Flow cytometric analysis of cell surface markers on cells in BAL was performed with primary monoclonal antibodies specific for porcine antigens raised in mouse. Fifty μL of $1x10^6$ cell suspension was prepared in FACS wash (0.05% sodium azide in PBS) buffer. Cells were incubated with mouse anti-porcine primary antibodies CD8-α, CD4, 2B11, CD21, CD14, MHC II, (VMRD, Pullman) and CD80 (Life Sciences) for 1 h at 4 °C. The cells were washed and incubated with respective fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen) for 30 min at 4 °C. The cells were then washed and fixed in 100 μL of 1% paraformaldehyde. Cells were then acquired in a 6-color FACS Calibur flow cytometer (BD biosciences) and analyzed using FlowJo software v7.6 (Tree star).

Lung pathology:

After euthanasia, lungs were removed immediately and examined for gross pathologic changes. Tissue samples from the trachea, lungs, and intestine were fixed in 10% neutral phosphate-buffered formalin, processed for histology and stained with hematoxylin and eosin (H&E). A board-certified veterinary pathologist who was blinded to the composition of the groups and the purpose of the study examined the samples histologically. Lung sections were examined for histopathologic changes and given a score of 0 to 3 to reflect the severity of inflammation and bronchointerstitial pneumonia as described previously (11).

Hemagglutination inhibition assays:

Hemagglutination inhibition (HI) assay was used to determine seroconversion of infected pigs and turkeys. Sera were heat-inactivated at 56 °C, treated with receptor-destroying enzyme (Denka Seiken) to remove non-specific inhibitors, and adsorbed with 0.5% swine red blood cells. Serial two-fold dilutions of sera were tested for HI antibodies.

Statistical analysis:

Analysis of variance (ANOVA) with Tukey-Kramer test was used to analyze data. Statistical analysis was done with the help of JMP (JMP Software, SAS Institute) and all the graphs were prepared using GraphPad Prism 5 (GraphPad Software).

2.4 Results:

Generation of SIV 1145 PB1-F2 mutant viruses

All classical SIV have a truncated PB1-F2 whereas triple reassortant North American H3N2 and H1N2 SIV encode a full length PB1-F2. We first established an

eight-plasmid reverse genetics system for the TR SIV 1145 wild type (SIV 1145-WT) and then generated two recombinant mutant SIV 1145 viruses for this study. To knockout PB1-F2 expression in SIV 1145 virus, we used site-directed mutagenesis to mutate start codon from ATG to ACG and introduced two stop codons at position 12 and 58 of PB1-F2 protein and generated recombinant SIV 1145 knock-out virus (SIV 1145-KO) by reverse genetics. In order to rescue recombinant SIV 1145-N66S, we engineered a point mutation in the SIV 1145 PB1-F2 protein to change the amino acid at position 66 from asparagine (N) to serine (S). All these mutations were silent in the PB1 gene segment and did not have any impact on amino acid sequence of the PB1 protein. Recombinant SIV were subsequently amplified in 10-day old SPF embryonated eggs and the identity of PB1 gene segments were confirmed through sequencing in both directions.

SIV 1145 PB1-F2 protein modulates early stage apoptosis but does not impact replication

To study the impact of N66S mutation and PB1-F2 knock out on SIV 1145 virus, we compared the replication of SIV-1145-KO and SIV-1145-N66S viruses with that of SIV-145-WT virus in A549 cells. All three viruses replicated to comparable titers in A549 cells without any significant difference at 24, 48 and 72 hpi (Figure 2.1A). The PB1-F2 of PR8 strain of IAV increases apoptosis in immune cells but not epithelial cells (22). Therefore, we examined whether SIV 1145-WT and mutant viruses modulate apoptosis in U937 human monocytic cells using annexin V-PI staining at 12 hpi. SIV 1145-KO virus induced significantly less apoptosis ($P<0.05$) when compared to SIV 1145-WT and SIV 1145-N66S virus (Figure 2.1B).

SIV 1145 PB1-F2 protein does not impact virulence in swine:

We intranasally inoculated 6-week old SPF pigs with SIV 1145-WT and SIV 1145-KO viruses and evaluated virus shedding in nasal swabs, virus load in lungs, expression of proinflammatory genes, lung immunophenotype and lung pathology. Transient rise in body temperature and mild respiratory signs such as nasal discharge were observed as early as 1 dpi in both virus-infected groups. Clinical signs including conjunctivitis, respiratory distress and nasal secretion lasted up to 4 dpi in both virus-infected groups. Nasal shedding of virus was detected in both virus-infected groups until 5 dpi and the virus titer peaked on 3 dpi but no significant differences were observed between the virus-infected groups (Figure 2.2A). Lung virus loads were determined on 3, 7, and 14 dpi. Comparable virus loads were observed in the lungs of pigs infected with SIV 1145-WT and SIV 1145-KO virus on 3 dpi (Figure 2.2B). Lung viral loads were undetectable in both virus-infected pigs on 7 and 14 dpi. To assess seroconversion, blood samples were collected on 7 and 14 dpi and examined using HI test. Comparable antibody titers were observed in both SIV 1145-WT and SIV 1145-KO infected pigs on 7 and 14 dpi (Figure 2.2C). Next, we assessed pro-inflammatory cytokine (IL-1β, IL-6, TNF-α, and IFN-γ) mRNA levels in the lungs of virus-infected pigs on 3 and 7 dpi by real time qRT-PCR. Only IL1-β and TNF-α were elevated in virus infected pig lungs. However, no statistically significant differences (P>0.05) in the mRNA levels of IL1-β and TNF-α were observed in the lungs of SIV 1145-WT and SIV 1145-KO infected groups (Figure 2.2D).

SIV 1145 PB1-F2 protein does not alter cellularity and lung pathology in pigs

To understand the role of SIV 1145 PB1-F2 protein in recruiting immune cells to the lungs after infection, we examined the immunophenotype of cells in the lungs. Pigs were inoculated with SIV 1145-WT and SIV 1145-KO virus and were euthanized on 3, 7, and 14 dpi and the lungs were collected and processed for flow cytometry. Early influx of CD80+ cells by 3 dpi followed by infiltration of CD14+, 2B11+ T cells and CD8+ T cells by 7 dpi were found. Statistically significant differences in the number of CD14+, CD80+, CD21+ B cells, 2B11+ T cells, CD4+ T cells and CD8+ T cell types between the lungs of virus-infected animals were not observed (Figure 2.3).

We next examined the effect of SIV 1145 PB1-F2 protein on pathologic changes in the lung. Three animals per group were sacrificed on 3, 7, and 14 dpi and lungs were examined. During necropsy, the macroscopic lesions were examined and mean pathological scores assigned. In general, the gross lesions including irregularly marked, plum-colored consolidated depressions were observed on individual lobes. Lesions were mostly located in the apical lobes with some involvement of caudal lobes. The mediastinal lymph nodes were usually enlarged and hyperemic. SIV 1145-WT and SIV 1145-KO virus infected groups demonstrated peak macroscopic lesions on 3 dpi which resolved by 14 dpi. No statistically significant differences were seen in the gross lesion scores between the virus-infected groups (data not shown). Trachea and lung histopathologic sections were examined microscopically. Bronchointerstitial pneumonia characterized by mild degeneration to necrosis of bronchial epithelium and accumulation of necrotic cellular debris within airway lumen was observed. In addition, moderate lymphocytic infiltration surrounding peribronchiolar and perivascular areas were

observed (Figure 2.4). In trachea, at 3 dpi, there were diffuse epithelial necrosis and loss of cilia in both virus-infected groups. However, no statistically significant differences were detected in histopathology scores of SIV 1145-WT and SIV 1145-KO in the trachea and lungs of virus-infected pigs (P>0.05).

SIV 1145-WT and SIV 1145-KO viruses induce clinical disease and efficiently transmit between turkey poults whereas SIV 1145-N66S virus has poor infectivity and transmissibility in turkey poults

In SIV 1145-WT infected groups, diarrhea was noticeable in two out of six animals from 5 dpi and two of the in-contacts also developed diarrhea from 7 dpi. On the other hand, in SIV 1145-KO infected groups, diarrhea was noticeable in two out of six animals as early as 4 dpi and two of the in-contacts also developed diarrhea by 4 dpi. SIV 1145-N66S infected animals displayed poor infectivity and no clinical signs were evident during the entire 10 days of observation. SIV 1145-WT and SIV 1145-KO viruses were re-isolated using MDCK cells from the cloacal swabs of infected poults but not in SIV 1145-N66S group. In histologic sections of intestines, focal perivascular heterophils and mild infiltration of lymphocytes was evident in the lamina propria of jejunum in all three virus-infected groups (Figure 2.5C). SIV 1145-KO infected groups had higher histopathological scores in intestine when compared to SIV 1145-WT and SIV 1145-N66S infected groups ($p<0.05$) (Figure 2.5A). All three virus-infected groups of turkeys seroconverted as shown by the HI titers at 10 dpi. SIV 1145-N66S infected groups showed poor HI titers when compared with SIV 1145-WT and SIV 1145-KO infected groups. Among the in-contacts, seroconversion was observed in SIV 1145-WT and SIV

1145-KO groups but not in SIV 1145-N66S, suggesting absence of transmission from infected poults to in-contacts (Table 2.1).

2.5 Discussion:

PB1-F2 is a small IAV accessory protein with diverse range of functions attributed to it (30, 39). It is considered an important marker of IAV pathogenicity in mice (25). Its functional roles appear to vary depending on the IAV strain and host species (40). TR SIVs contain internal genes from swine (M, NS, and NP), human (PB1), and avian (PA and PB2) IAV forming a constellation of genes called as TRIG cassette (12, 13). This specific internal gene combination is well conserved and provided a high level of fitness for the TR SIVs and they spread rapidly among the North American swine population. In subsequent years, TR SIVs showed increased propensity to reassort generating a range of reassortants (41). Unlike cH1N1 SIV, most of the TR SIVs produce a full-length PB1-F2 protein (42). In order to better understand the functional significance of PB1-F2 protein in TR H3N2 SIV, we designed experiments to characterize the disease phenotype associated with the presence or absence of PB1-F2 in SIV 1145 virus.

Expression of PB1-F2 and the presence of N66S mutation in TR SIV 1145 virus background did not impact virus replication in human respiratory epithelial cell line A549. The data obtained in A549 cells are in agreement with the recent report that showed PB1-F2 of TR H3N2 SIV has negligible effects on replication efficiency in porcine respiratory explants (43). IAV infects monocytes in humans (44-46) and induces apoptosis (47, 48). In pigs, *in vivo* depletion studies showed that monocyte-derived alveolar macrophages are critical for controlling IAV infection in the lungs (49). PB1-F2

has been reported to induce apoptosis in human monocytes and thereby play a vital role in down-regulation of host immune response to IAV infection (22, 24). *In vitro infection* of human monocytic cells with PB1-F2 recombinant viruses indicated that the presence of PB1-F2 and N66S mutation in SIV 1145 virus increased apoptotic cell death.

A recent report mapped the cell death phenotype of PB1-F2 to three amino acid motifs I68, L69, and V70 in the C-terminal region of the protein (32). However, SIV 1145 virus does not contain any of the identified cytotoxic residues but still causes immune cell apoptosis. The reason for this discrepancy might be due to differences in the virus strains and *in vitro* systems used for the study. Previous studies have shown that ablation of PB1-F2 start codon increases expression of N40 (50). It is not known whether decrease in immune cell apoptosis observed in SIV-1145-KO virus is due to absence of PB1-F2 expression or overexpression of N40 or a combination of both. These studies are beyond the scope of the current report and require further investigation. Nevertheless, there was no correlation between the ability of PB1-F2 recombinant viruses to induce apoptosis in monocytic cells and the virulence to pigs. Deletion of PB1-F2 expression had no effect on SIV 1145 virulence as measured by nasal shedding, lung viral load, lung immunophenotypes and lung pathology. These observations are consistent with the report by Pena et al, who also recorded minimal effects of PB1-F2 of TR H3N2 influenza viruses in swine host (43).

The pro-inflammatory cytokine response in the lungs is essential for recruitment of effector cells to the site of infection for virus clearance. It was shown that PB1-F2 modulates cytokine response in lungs and contributes to increased pathogenicity in mice (28, 31). *In vivo* studies in swine host showed that there is no difference in the expression

of proinflammatory cytokines and lung pathology between SIV 1145-WT and SIV 1145-KO viruses. These results are in disagreement with the findings of another study that reported ablation of PB1-F2 in TR H3N2 SIV leads to significant increase in the pulmonary levels of IFN-γ and lung pathology at 3 dpi in pigs (43). This discrepancy might be due to the difference in the route of inoculation used between the studies. In our study, we used intranasal inoculation route in pigs, which mimics the natural mode of infection, rather than the intratracheal route deployed in the other study that deposits the virus in the lower respiratory tract. McAuley et al, showed that PB1-F2 protein of H3N2 IAV differs markedly in their ability to induce inflammatory responses and lung pathology (31). Pro-inflammatory motifs are mapped to L62, R75, R79, and L82 amino acid residues in the C-terminal region of the PB1-F2 protein (51). The PB1-F2 sequence of SIV 1145 H3N2 virus contains a mixture of the pro-inflammatory residues (L62 and L82) and non-inflammatory motifs (H75 and Q79).

Turkeys carry both α 2,6 SA gal and α 2,3 SA gal receptors in their respiratory tract (18). Turkeys are highly susceptible to IAV from aquatic birds (51, 21) and with the introduction of SIV, there is an increasing chance for the emergence of novel reassortants with genes adapted for replication in pigs or even humans (20, 21). Hence, it is important to study the role of PB1-F2 in the pathogenesis and transmission of TR H3N2 SIV in turkeys. Turkey poults infected with SIV 1145-WT and SIV 1145-KO viruses developed clinical signs such as diarrhea and transmitted virus to in-contact groups efficiently as evidenced by their immunogenicity. SIV 1145-KO infected turkey poults and its in-contacts developed clinical signs earlier than SIV 1145-WT groups and also displayed higher histopathological scores. Despite the absence of clinical signs of respiratory

illness, virus infected poults revealed histologic lung damage suggesting respiratory and enteric tropism of TR H3N2 SIV in turkeys. Interestingly, SIV 1145-N66S virus infected turkey poults showed poor infectivity and not transmitted efficiently among the in-contacts. Most of the SIV outbreaks in turkeys in the United States occurred in older flocks and generally associated with drop in egg production (19, 21). Strong evidence of turkey-to-turkey transmission was also recorded (20). It appears that deletion of PB1-F2 confers selective advantage to the TR H3N2 SIV to infect and transmit among turkey poults. To our knowledge, this is the first report showing that the PB1-F2 modulates TR H3N2 SIV ability to induce clinical infection and bird-to-bird transmission among turkeys. Interestingly, the virulence associated 66S mutation in PB1-F2 abolished the ability of the TR H3N2 SIV to successfully infect turkeys and transmit to in-contacts. The reason for this is intriguing and requires further studies.

In summary, we found that the PB1-F2 protein in TR H3N2 SIV did not impact pathogenesis and virulence in swine but influences clinical disease and virus transmission among turkey poults. The 66S mutation in PB1-F2 of TR H3N2 SIV drastically reduced infectivity and abolished transmission in turkeys. Our study emphasizes the continuing need to better understand the virulence determinants for influenza virus in intermediate hosts such as swine and turkeys and highlights the strain- and species-specific role of PB1-F2 protein.

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2.7 References:

1. **Wright PF, Neumann, G., Kawaoka, Y.** 2007. Orthomyxoviruses p. 1691-1740. *In* Knipe DM, Griffin, D. E., Lamb, R. A., Martin, M A., Roiz-man, B., Straus, S. E. (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins.
2. **Easterday BC.** 1980. The epidemiology and ecology of swine influenza as a zoonotic disease. Comp Immunol Microbiol Infect Dis **3:**105-109.
3. **Shope RE.** 1931. The Etiology of Swine Influenza. Science **73:**214-215.
4. **Olsen CW.** 2002. The emergence of novel swine influenza viruses in North America. Virus Res **85:**199-210.
5. **Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG.** 1999. Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol **73:**8851-8856.
6. **Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG.** 2000. Evolution of swine H3N2 influenza viruses in the United States. J Virol **74:**8243-8251.
7. **Karasin AI, Olsen CW, Anderson GA.** 2000. Genetic characterization of an H1N2 influenza virus isolated from a pig in Indiana. J Clin Microbiol **38:**2453-2456.
8. **Webby RJ, Rossow K, Erickson G, Sims Y, Webster R.** 2004. Multiple lineages of antigenically and genetically diverse influenza A virus co-circulate in the United States swine population. Virus Res **103:**67-73.

9. **Ma W, Gramer M, Rossow K, Yoon KJ.** 2006. Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J Virol **80:**5092-5096.

10. **Choi YK, Goyal SM, Joo HS.** 2002. Prevalence of swine influenza virus subtypes on swine farms in the United States. Arch Virol **147:**1209-1220.

11. **Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, Webby RJ.** 2003. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. J Clin Microbiol **41:**3198-3205.

12. **Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR, Richt JA.** 2009. The role of swine in the generation of novel influenza viruses. Zoonoses Public Health **56:**326-337.

13. **Vincent AL, Ma W, Lager KM, Janke BH, Richt JA.** 2008. Swine influenza viruses a North American perspective. Adv Virus Res **72:**127-154.

14. **Matrosovich M, Matrosovich T, Uhlendorff J, Garten W, Klenk HD.** 2007. Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology **361:**384-390.

15. **Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol **72:**7367-7373.

16. **Novel Swine-Origin Influenza AVIT, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM.**

2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. NEJM **360:**2605-2615.

17. **Peiris JS, Poon LL, Guan Y.** 2009. Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. J Clin Virol **45:**169-173.

18. **Pillai SP, Pantin-Jackwood M, Yassine HM, Saif YM, Lee CW.** 2010. The high susceptibility of turkeys to influenza viruses of different origins implies their importance as potential intermediate hosts. Avian Dis **54:**522-526.

19. **Suarez DL, Woolcock PR, Bermudez AJ, Senne DA.** 2002. Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis **46:**111-121.

20. **Choi YK, Lee JH, Erickson G, Goyal SM, Joo HS, Webster RG, Webby RJ.** 2004. H3N2 influenza virus transmission from swine to turkeys, United States. Emergi Infect Dis **10:**2156-2160.

21. **Tang Y, Lee CW, Zhang Y, Senne DA, Dearth R, Byrum B, Perez DR, Suarez DL, Saif YM.** 2005. Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis **49:**207-213.

22. **Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW.** 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med **7:**1306-1312.

23. **Yamada H, Chounan R, Higashi Y, Kurihara N, Kido H.** 2004. Mitochondrial targeting sequence of the influenza A virus PB1-F2 protein and its function in mitochondria. FEBS Lett **578:**331-336.

24. **Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P.** 2005. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog **1:**e4.

25. **Zamarin D, Ortigoza MB, Palese P.** 2006. Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J Virol **80:**7976-7983.

26. **McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, Bennink J, Yewdell JW, McCullers JA.** 2007. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host Microbe **2:**240-249.

27. **Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, Fernandez-Sesma A, Palese P.** 2011. The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS Pathog **7:**e1002067.

28. **Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P.** 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog **3:**1414-1421.

29. **Mazur I, Anhlan D, Mitzner D, Wixler L, Schubert U, Ludwig S.** 2008. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cellular Microbiol **10:**1140-1152.

30. **Kosik I, Holly J, Russ G.** 2013. PB1-F2 expedition from the whole protein through the domain to aa residue function. Acta Virol **57:**138-148.

31. **McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, McCullers JA.** 2010. PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. PLoS Pathog **6:**e1001014.

32. **Alymova IV, Samarasinghe A, Vogel P, Green AM, Weinlich R, McCullers JA.** 2014. A novel cytotoxic sequence contributes to influenza A viral protein PB1-F2 pathogenicity and predisposition to secondary bacterial infection. J Virol **88:**503-515.

33. **Zell R, Krumbholz A, Eitner A, Krieg R, Halbhuber KJ, Wutzler P.** 2007. Prevalence of PB1-F2 of influenza A viruses. J Gen Virol **88:**536-546.

34. **Centers for Disease C, Prevention.** 2009. Swine influenza A (H1N1) infection in two children--Southern California, March-April 2009. MMWR **58:**400-402.

35. **Nelson MI, Vincent AL, Kitikoon P, Holmes EC, Gramer MR.** 2012. Evolution of novel reassortant A/H3N2 influenza viruses in North American swine and humans, 2009-2011. J Virol **86:**8872-8878.

36. **Kumar SR, Deflube L, Biswas M, Shobana R, Elankumaran S.** 2011. Genetic characterization of swine influenza viruses (H3N2) isolated from Minnesota in 2006-2007. Virus Genes **43:**161-176.

37. **Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. PNAS U S A **97:**6108-6113.

38. **Reed L, Muench, H.** 1938. A simple method of estimating fifty per cent endpoints. Am J Epidemiol **27:**493-497.

39. **Chakrabarti AK, Pasricha G.** 2013. An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. Virology **440:**97-104.

40. **Chen CJ, Chen GW, Wang CH, Huang CH, Wang YC, Shih SR.** 2010. Differential localization and function of PB1-F2 derived from different strains of influenza A virus. J Virol **84:**10051-10062.

41. **Ma W, Lager KM, Lekcharoensuk P, Ulery ES, Janke BH, Solorzano A, Webby RJ, Garcia-Sastre A, Richt JA.** 2010. Viral reassortment and transmission after co-infection of pigs with classical H1N1 and triple-reassortant H3N2 swine influenza viruses. J Gen Virol **91:**2314-2321.

42. **Pasricha G, Mishra AC, Chakrabarti AK.** 2013. Comprehensive global amino acid sequence analysis of PB1F2 protein of influenza A H5N1 viruses and the influenza A virus subtypes responsible for the 20th-century pandemics. Influenza Other Respir Viruses **7:**497-505.

43. **Pena L, Vincent AL, Loving CL, Henningson JN, Lager KM, Li W, Perez DR.** 2012. Strain-dependent effects of PB1-F2 of triple-reassortant H3N2 influenza viruses in swine. J Gen Virol **93:**2204-2214.

44. **Louie M, Yoo J, Moran T, Mayer L, Sperber K.** 1995. Impairment of monocytic function after influenza virus infection. Clin Diag Lab Immunol **2:**426-433.

45. **Hofmann P, Sprenger H, Kaufmann A, Bender A, Hasse C, Nain M, Gemsa D.** 1997. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. J Leukoc Biol **61:**408-414.

46. **Hoeve MA, Nash AA, Jackson D, Randall RE, Dransfield I.** 2012. Influenza virus A infection of human monocyte and macrophage subpopulations reveals increased susceptibility associated with cell differentiation. PloS One **7:**e29443.

47. **Brydon EW, Morris SJ, Sweet C.** 2005. Role of apoptosis and cytokines in influenza virus morbidity. FEMS Microbiol Rev**29:**837-850.

48. **Xie D, Bai H, Liu L, Xie X, Ayello J, Ma X, Zhang J.** 2009. Apoptosis of lymphocytes and monocytes infected with influenza virus might be the mechanism of combating virus and causing secondary infection by influenza. Int Immunol **21:**1251-1262.

49. **Kim HM, Lee YW, Lee KJ, Kim HS, Cho SW, van Rooijen N, Guan Y, Seo SH.** 2008. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. J Virol **82:**4265-4274.

50. **Tauber S, Ligertwood Y, Quigg-Nicol M, Dutia BM, Elliott RM.** 2012. Behaviour of influenza A viruses differentially expressing segment 2 gene products in vitro and in vivo. J Gen Virol **93:**840-849.

51. **Alymova IV, Green AM, van de Velde N, McAuley JL, Boyd KL, Ghoneim HE, McCullers JA.** 2011. Immunopathogenic and antibacterial effects of H3N2 influenza A virus PB1-F2 map to amino acid residues 62, 75, 79, and 82. J Virol **85:**12324-12333.

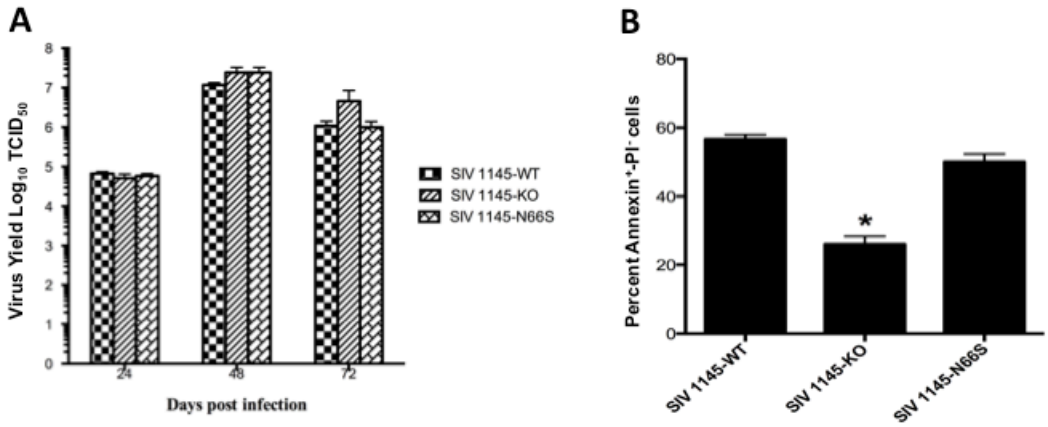

Figure 2.1: PB1-F2 of TR H3N2 SIV modulates immune cell apoptosis but does not impact virus replication (A) Replication kinetics. A549 cells were infected with indicated recombinant PB1-F2 influenza viruses at a MOI of 0.001. At 24, 48, and 72 hpi, supernatants were collected and titrated by $TCID_{50}$ in MDCK cells. Values obtained from the triplicates are shown as mean (expressed as $log_{10}TCID_{50}$/ml) ± standard deviation (SD). (B). Apoptosis assay. U937 cells were infected with 2 MOI of respective recombinant PB1-F2 influenza viruses or mock infected with PBS. After 12 hpi, cells were collected, washed, stained with Annexin V^+ and Propidium iodide (18) and analyzed by flow cytometry. Mean percentage of Annexin V^+-PI^- positive cells normalized over control from triplicates ± SD was shown (*=P<0.05).

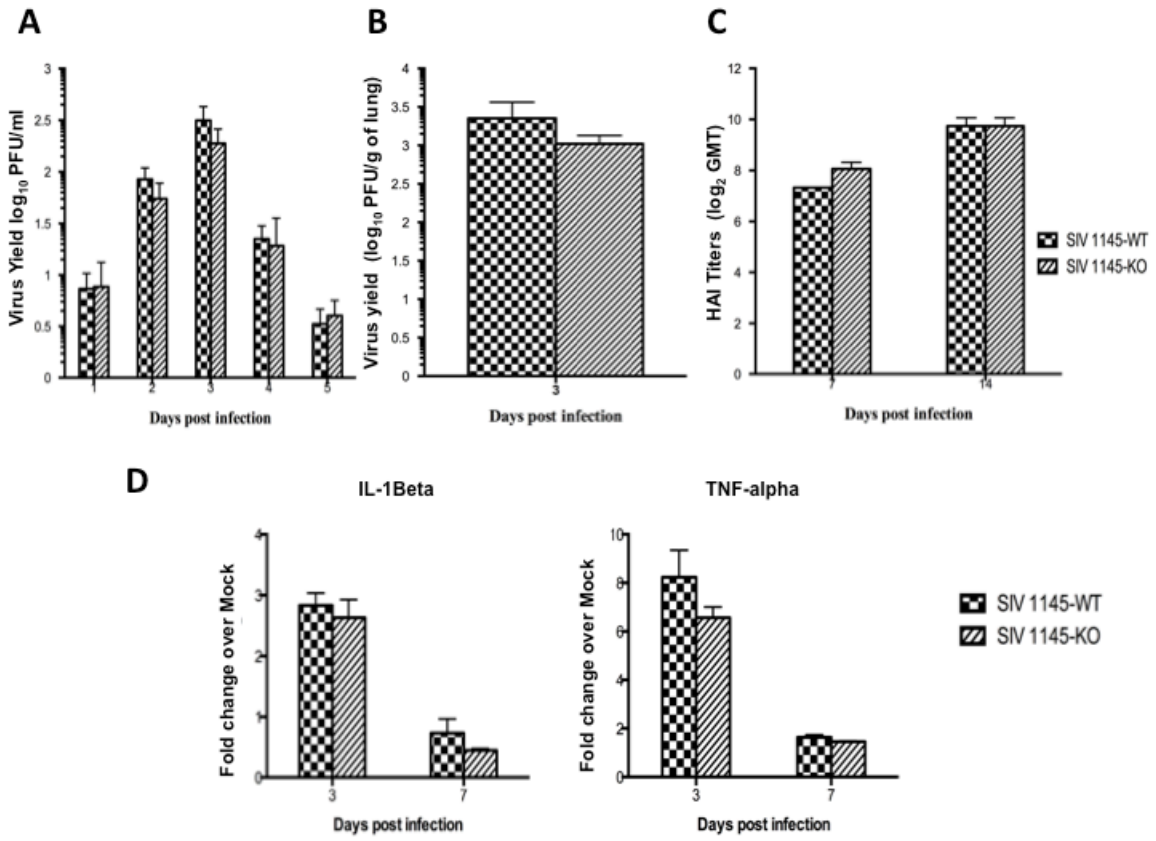

Figure 2.2: Replication and cytokine profile of TR H3N2 SIV in pigs. Groups of pigs (n=9) were infected intranasally with $1x10^7$ PFU of PB1-F2 recombinant SIV viruses or mock infected with PBS. Nasal swabs were collected daily for monitoring nasal shedding and at day 3, 7, and 14 dpi, three animals from each group were harvested. (A) Viral shedding in nasal secretions of infected pigs. (B) Viral load in the lungs of infected pigs. (A, B) Virus yield were determined by $TCID_{50}$ on MDCK cells. Mean viral titers expressed as $log_{10}TCID_{50}$/ml ± standard deviation (SD) are shown. (C) Hemagglutination inhibition (HAI) titer of convalescent sera samples collected from infected pigs. Values expressed as log_2 geometric mean titer (GMT) ± SD. (D) Cytokine levels in the supernatants of lung homogenates were determined using real-time qRT-PCR. Average cytokine levels over mock-infected group ± SD are shown.

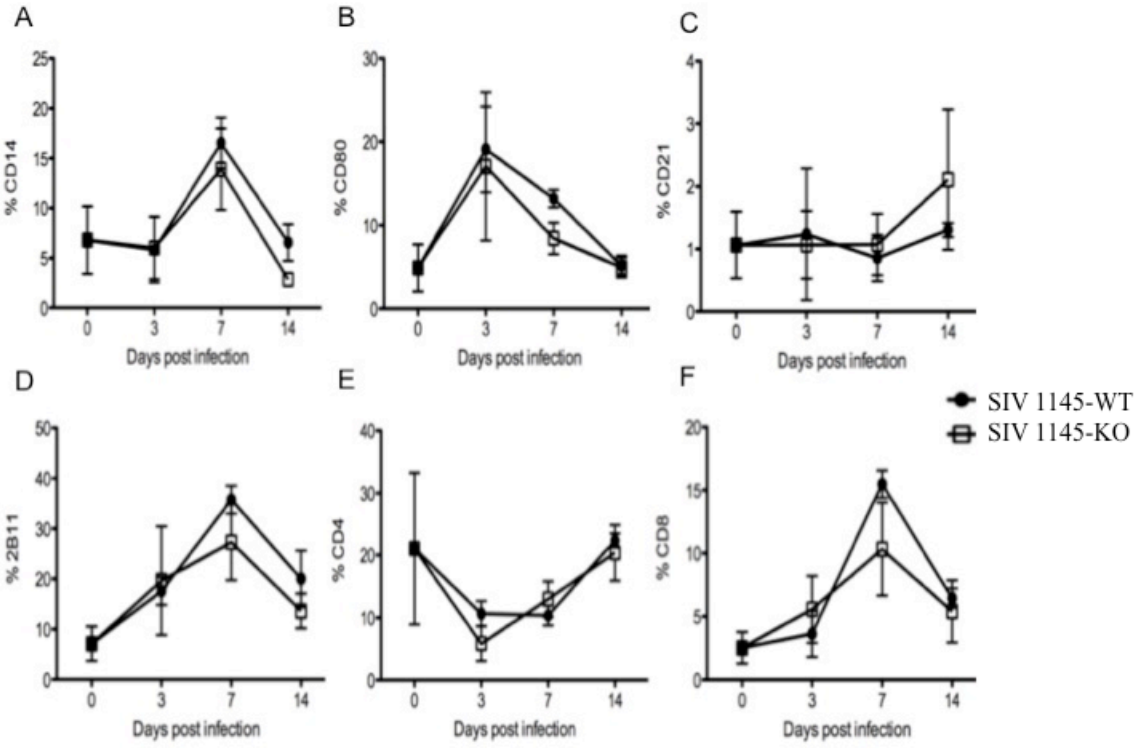

Figure 2.3: Immunophenotype of cells in BAL of TR H3N2 SIV in pigs. Nine animals per group were infected intranasally with $1x10^7$ PFU of PB1-F2 recombinant SIV viruses and three animals from each group were euthanized on day 3, 7, and 14 dpi. BAL was collected and analyzed by flow-cytometry for percentage of (A) CD14, (B) CD80, (C) CD21, (D) 2B11, (E) CD4, and (F) CD8 cells within the lymphocyte gate are shown. X-axis indicates days post infection and Y-axis indicates percentage positive cells. Error bars indicate standard deviation from the mean.

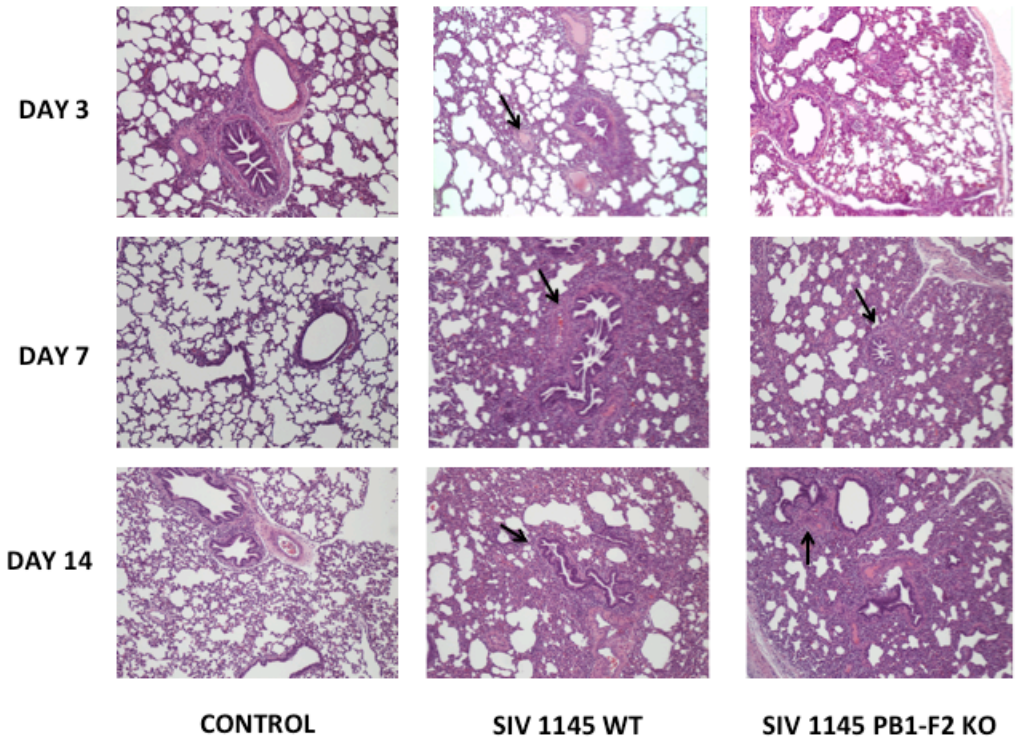

Figure 2.4: Histopathology of swine lungs infected with TR H3N2 SIV. Groups of pigs (n=9) were infected intranasally with $1x10^7$ PFU of PB1-F2 recombinant SIV viruses or mock infected with PBS and three animals from each group were harvested on day 3, 7, and 14 dpi. After euthanasia, lungs were collected and processed for histology. Representative images from swine infected with SIV 1145-WT, SIV 1145-KO and mock-infected groups are shown. Black arrows indicate areas of diffuse broncho-interstitial pneumonia and cellular infiltrates were evident in the lungs of pigs.

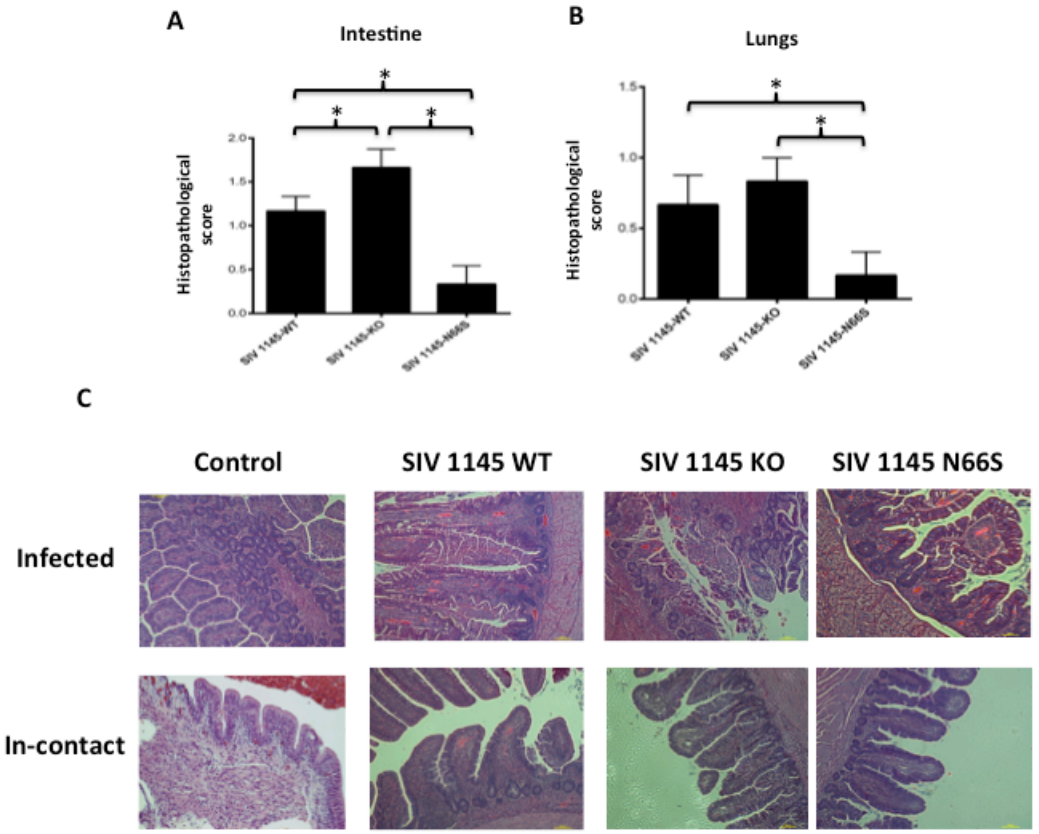

Figure 2.5: Histopathology of turkey intestine infected with TR H3N2 SIV. Groups of turkeys (n=6) were infected intranasally with $1x10^7$ PFU of PB1-F2 recombinant SIV viruses or mock infected with PBS and 24h later; three age-matched birds were introduced as in-contacts. After 10 dpi, birds were euthanized; lungs and jejunum were collected for histopathological examination. Histopathological scores in the (A) Intestine and (B) Lungs (*=P<0.05). (C) Photomicrographs representing perivascular cellular infiltrates in the intestines of virus infected and in-contact turkey poults.

Table 2.1: Seroconversion in turkey poults after exposure to TR H3N2 SIV

Virus	Geometric Mean Hemagglutinin Titer (Log_2)			
	Day 0		Day 10	
	Infected	In-contact	Infected	In-contact
SIV 1145-WT	<2	<2	6.5	4.0
SIV 1145-KO	<2	<2	5.66	2.33
SIV 1145-N66S	<2	<2	2.5	<2

3. Multiple C-terminal Amino Acids Together with 66S of PB1-F2 Protein Modulate the Pathogenicity of Pandemic H1N1 Influenza A Virus in Mice

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3.1 Abstract

The influenza A virus (IAV) protein PB1-F2 is considered to be a virulence factor in primary IAV infection and secondary bacterial pneumonia. Most pandemic and lethal IAV express a full-length PB1-F2 protein and the presence of serine at position 66 (66S) has been identified as a virulence marker. The 2009 pandemic swine origin H1N1 influenza virus (pdm09 H1N1) does not express a full-length PB1-F2 and restoring PB1-F2 expression or introducing 66S in pdm09 H1N1 impacted virulence minimally. We identified several residues in the C-terminus of PB1-F2 that were unique in pandemic H1N1 1918, H2N2 1957, and H3N2 1968, and highly pathogenic avian IAV strains. There exists a possibility that avian-like PB1-F2 with these unique residues may be acquired by circulating IAV either by mutation or genetic reassortment. We, therefore, hypothesized that specific residues in addition to 66S in the PB1-F2 may modulate virulence of IAV. We demonstrate that C-terminal residues 73K, 75R, and 79R together with 66S in pdm09 H1N1background increased virus replication, decreased type I interferon response and induced fulminant acute respiratory distress syndrome (ARDS) in mice with characteristic clinical and pathological features of acute lung injury (ALI). The lethal phenotypic mutants increased infiltration of neutrophils, inflammatory monocytes with the production of myeloperoxidase in the lungs consistent with ALI. Additional mutations at 74T, and 76V in PB1-F2 protein compensated the effects and alleviated ARDS. Our study suggests that these additional C-terminal residues together with 66S in pdm09 H1N1background play a role in pathogenicity and may serve as markers for predicting the virulence of IAV.

Author Summary: Seasonal epidemics and pandemic influenza claim multiple lives and increase disability adjusted life years (DALYs). The pdm09 H1N1 influenza is considered generally mild but globally circulating. PB1-F2 protein is considered a virulence marker expressed by all pandemic and highly pathogenic avian influenza viruses, however pdm09 H1N1 virus fails to express a functional PB1-F2 protein. Serine at position 66 at the C-terminus of PB1-F2 has been incriminated in virulence but the role of other amino acid residues is unknown. Restoring full-length PB1-F2 or introducing serine at 66 does not enhance pathogenicity of pdm09 H1N1. Our study demonstrated that specific C-terminal amino acid residues together with a serine at 66 dictate the pathogenicity of pdm09 H1N1 virus in mice. It is possible for the pdm09 H1N1 virus to acquire lethal PB1-F2 phenotype by mutations or natural ressortment with circulating influenza virus. Screening for highly virulent strains with specific mutations identified in this study, may help pandemic preparedness.

3.2 Introduction

Influenza A viruses (IAVs) infect a wide range of host species including avian, swine and humans. Annual seasonal epidemics and occasional pandemics due to IAV result in significant mortality and socio-economic costs [1]. IAV PB1-F2 is a non-structural accessory protein encoded by the alternate +1 open reading frame in the PB1 gene [2]. The PB1-F2 protein varies in size from 11 to 101 amino acids long with truncations at the amino (N) or carboxy (C) termini [3] and its precise function in the IAV life cycle remains unclear. It is a multifunctional and enigmatic protein with diverse functions attributed to it; such as immune cell apoptosis, inflammation, enhancing immunopathology, modulating innate immune response, influencing secondary bacterial

infections, and increasing viral polymerase activity in a strain and host-specific manner [4].

PB1-F2 has been shown to permeabilize mitochondrial membranes [5,6] resulting in loss of mitochondrial membrane potential and efflux of cytochrome complex that activates the caspase cascade and initiates apoptosis. The exact mechanism by which PB1-F2 induces apoptosis is not clear. Early studies demonstrated that PB1-F2 protein induces apoptosis by interacting with the inner mitochondrial membrane adenine nucleotide translocator 3 (ANT3) and the outer mitochondrial membrane voltage channel 1 (VDAC1) that is involved in the formation of the permeability-transition pore complex [7]. Later work proposed that PB1-F2 protein promotes a mitochondrial pathway of apoptosis in a strain-specific manner through the activation of the pro-apoptotic Bcl-2 family effector proteins BAX and BAK [8]. In addition, using structural studies, it is proposed that PB1-F2 protein self-oligomerizes resulting in the formation of amyloid-like fibers in infected cells that leads to permeabilization of cellular membranes and finally to cell death [9].

PB1-F2 protein plays an important role in the pathogenicity of primary influenza virus infection and post-influenza secondary bacterial pneumonia by modulating the inflammatory responses resulting in infiltration of immune cells and tissue damage in the lungs of infected mice [8,10,11]. In addition, the PB1-F2 protein with serine at position 66 instead of asparginine enhanced disease pathology in a mouse model [12]. The PB1-F2 protein is also reported to possess type I interferon (IFN) antagonistic activity by interfering with RIG-I/MAVS protein complex and activating IFN regulatory factor 3 [13-15]. In contrast, another group reported that PB1-F2 protein exacerbates IFN-β

expression through the activation of NF-kB pathway resulting in enhanced inflammatory response in lungs [16,17]. The PB1-F2 protein has been shown to increase viral polymerase activity *in vitro* by interacting with PB1 polymerase protein of polymerase complex [18]. However, the effect of PB1-F2 protein on viral polymerase activity appears to be strain specific and may not contribute to influenza virus pathogenesis *in vivo* [19].

IAV PB1-F2 protein is considered an important virulence factor, and reported to be associated with the pathogenicity of the 1997 H5N1 avian IAV and 1918 H1N1, 1957 H2N2, and the 1968 H3N2 pandemic (pdm) viruses and [4,8,13]. The 2009 swine origin pdm H1N1 (pdm09 H1N1) virus is highly transmissible but mild in pathogenicity [20]. All pandemic and most circulating avian IAV strains have the full-length PB1-F2 protein except pdm09 H1N1. However, restoration of PB1-F2 in pdm09 H1N1 expression had minimal effects on virulence in mouse and swine models [21,22]. Also, the N66S mutation recreated in PB1-F2 of pdm09 H1N1 failed to increase pathogenicity in mice and swine [21,22]. It is unclear why the PB1-F2 protein of certain pdm and avian strains are associated with virulence while most other full-length PB1-F2 proteins do not contribute to virulence. When we compared the amino acid sequences of PB1-F2 protein of different IAVs, we found that amino acid residues in C-terminus of restored PB1-F2 of pdm09 H1N1 virus differs significantly from other 20th century pdm viruses and highly pathogenic H5N1 avian influenza viruses. It has been reported that the C-terminal region of PB1-F2 protein plays an important role in IAV virulence by modulating the proinflammatory environment in the lungs of mice [8]. We hypothesized that specific C-terminus amino acid residues in addition to 66S may play a role in the virulence of

pdm09 H1N1. Here, we describe C-terminal residues 73K, 75R, and 79R of PB1-F2 protein in combination with 66S were responsible for the differential pathogenicity of mutant pdm09 H1N1 virus in a mouse model. We also show that these residues modulate acute lung injury leading to the acute respiratory distress syndrome (ARDS) induced by mutant pdm09 H1N1 virus in mice.

3.3 Materials and Methods:

Cells and Viruses:

Human alveolar basal epithelial cells (A549), human embryonic kidney epithelial cells (293T), Madin-Darby canine kidney cells (MDCK), and human monocytic cells (THP-1) were obtained from American Type Culture Collection (ATCC) and were maintained either in Dulbecco's minimal essential medium (DMEM) or in minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal calf serum (Thermo Scientific) and penicillin-streptomycin (Invitrogen). The IAV strain used in this study is A/California/04/2009 (H1N1) (CA/04), a kind gift from Dr. Terence Tumpey, CDC, Atlanta. All experiments involving CA/04 virus were approved by the Institutional Biosafety Committee (IBC) at Virginia Tech.

Construction of plasmids:

All the eight gene segments of CA/04 were cloned into pHW2000 vector [23]. The CA/04-PB1 plasmid was subjected to three rounds of site-directed mutagenesis using QuickChange II site-directed mutagenesis kit (Agilent Technologies). The stop codons in the open reading frame of PB1-F2 at positions 12, 58, and 88 were modified to code for serine, tryptophan, and tryptophan, respectively to create CA/04 Knock-in (CA-KI) PB1 plasmid without affecting the reading frame of PB1 gene. Asparagine is changed to serine

at position 66 for creating CA-KI-66S plasmid. For obtaining additional C-terminal mutants, arginine, isoleucine, histidine, alanine and glutamine at positions 73, 74, 75, 76, and 79 of PB1-F2 were changed to lysine, threonine, arginine, valine, and arginine, respectively in the plasmid CA-KI-66S (Figure 1). The sequences of each construct were confirmed by automated sequencing performed at Virginia Bioinformatics Institute (VBI) core sequencing facility.

Rescue of recombinant influenza A viruses:

Co-cultured MDCK and 293T cells were transfected with eight plasmids containing CA/04 viral genome segments as described previously [23]. The rescued viruses were plaque purified on MDCK cells and propagated once in 10-day old embryonated specific pathogen free (SPF) chicken eggs. The presence of the introduced mutations in the PB1 segments was confirmed by sequencing the viral genomic RNA (vRNA). The virus stocks were titrated and stored at -80°C until used.

Immunofluorescence assay:

A549 cells were grown in 8-well chamber slides (Sigma-Aldrich) and infected with respective recombinant IAV at a MOI of 1. After 36 hours post infection (hpi), cells were fixed and permeabilized with 1:1 acetone: methanol at -20°C. Cells were then blocked with 5% bovine serum albumin and incubated with primary rabbit antipeptide antibody raised against PR/08 H1N1 PB1-F2. For detection, Alexa Fluor 594-conjugated anti-rabbit secondary antibodies (Molecular Probes) were used. Cells were mounted using mounting medium containing DAPI (Vector Laboratories) and then observed under an Eclipse TE2000-PFS inverted microscope (Nikon Instruments).

Growth kinetics of recombinant viruses:

To analyze multicycle replication of recombinant viruses, A549 cells were infected at a MOI of 0.001. After 1 h incubation at 37°C, the inoculum was removed and cells were maintained with MEM containing 5% fresh allantoic fluid from 10 day-old SPF chicken embryos. Virus yield in infected cell culture supernatants at 24, 48, and 72 hpi were determined by plaque assays in MDCK cells. Briefly, serial 10-fold dilutions of the supernatants were overlaid on MDCK cells for 1 h at 37°C. After 1 h, the inoculum was removed and the cell monolayer was washed 2x with PBS. Infected cells were incubated at 37°C in MEM containing 1% Avicel (FMC Biopolymer) and 1 μg/mL tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich). After 72-96 hpi, infected cell cultures were stained with 1% crystal violet in 10% buffered formalin and plaques were counted. Virus titers were expressed as mean log pfu/mL ± standard deviation (SD).

Mini-replicon assay:

Mini-replicon assays to measure the influenza viral polymerase activity were carried out in 293T cells. Briefly, the segment 2 PB1 sequence of CA/04 mutated to express a full length PB1-F2 (CA-KI), or mutants of the knocked-in PB1-F2 (N66S, +2M, +3M or +5M) were cloned into the pCAGGS expression vector. The polymerase was then reconstituted by transfection of the pCAGGS PB1 plasmids along side pCAGGS encoding for the CA/04 NP, PA and PB2 proteins. A v-RNA like firefly luciferase reporter plasmid containing the UTR of influenza segments and an expression plasmid for Renilla luciferase as a transfection control were also included. After 24 hpi, the cells were lysed using passive lysis buffer (Promega) and the firefly and Renilla

luciferase activities were measured using a luminometer and the Stop and Glo dual luciferase kit (Promega).

Real time PCR:

Human monocytic (THP-1) cells differentiated with *phorbol myristate acetate* (100 ng/mL) and A549 cells were infected with respective recombinant IAV at a MOI of 1. At 12 hpi, cells were collected and total RNA extracted using RNeasy kit (Qiagen). The cDNA was prepared using high capacity cDNA kit (Applied Biosystems) and tested for IFN-beta, IP-10, and house keeping genes GAPDH, 18S transcripts by real time PCR using Syber Green dye (Life technologies). The mRNA levels of target genes were normalized to endogenous control GAPDH and18S.

Detection of mitochondrial superoxide production:

Mitochondrial superoxide production in live cells was detected by staining with MitoSOX Red (Life Technologies) and flow cytometric analysis as described earlier [24]. Briefly, A549 cells were infected with respective recombinant influenza viruses at a multiplicity of infection (MOI) of 1. After 16 hpi at 37°C, the supernatants were removed and cells were stained with MitoSOX Red at a final concentration of 5 μM. After 30 min incubation at 37°C, cells were trypsinized and washed with Hank's balanced salt solution containing calcium and magnesium (Invitrogen). After final wash, cells were resuspended in FACS buffer at a concentration of $1x10^6$ cells per 100 μl. The cells were acquired in a FACS Aria flow cytometer and analyzed using FlowJo software version 7.6.

Apoptosis assay:

Human monocytic (U937) cells were infected with indicated recombinant IAV at a MOI of 1. After 12 hpi incubation at 37°C, cells were trypsinized, washed and stained

with APC labeled Annexin V for 20 min and propidium Iodide (PI) for 5 min. (Biolegend). The cells were acquired in a FACS Aria flow cytometer and analyzed using FlowJo software version 7.6. Necrotic cellular events were defined as Annexin-V^+ and PI^+, whilst apoptotic events were Annexin-V^+ only. Viable cells were considered as Annexin-V^- and PI^-.

Mouse Pathogenicity:

All mouse studies were approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) at Virginia Tech. These animal studies were performed prior to the voluntary pause on gain-of-function studies. Six to eight week-old BALB/c mice were purchased from Charles River laboratories and housed in individual ventilated cages in our enhanced biosafety level-2 facility. Mice were randomly distributed into six groups of five animals. Each group were anesthetized and infected via intranasal route with $1x10^5$ plaque-forming units (PFU) of each recombinant IAV in 50 µl of phosphate buffered saline (PBS). Mice were weighed and observed daily for visual clinical signs like lethargy, shivering, ruffled fur, labored respiration, hunched posture, and huddling and eating/drinking behavior. Animals were euthanized humanely if they lost >20% of their original body weight as per the guidelines of VirginiaTech IACUC. In another experiment, fifteen mice were either mock infected with 50 µl of PBS or infected with $1x10^4$ plaque-forming units (PFU) of recombinant IAV in 50 µl of PBS via the intranasal route. Mice were euthanized on 1, 3, 5, 7, and 14 days post-infection (dpi) and the lung tissues were collected for determining the viral load, immune cell infiltration and histopathology.

Virus load in lungs:

For determination of viral loads, lungs from mock or virus infected mice were homogenized using a hand held homogenizer. A 20% suspension was prepared in MEM and clarified by centrifugation. Virus yields were then determined by a plaque assay in MDCK cells as described above.

Cytokine quantitation:

The levels of four different cytokines (interleukin-1 beta [IL-1β], interleukin-6 [IL-6], tumor necrosis factor alpha [TNF-α], and interferon gamma [IFN-γ]) in the supernatants of lung homogenates was determined by multiplex enzyme-linked immunosorbent assays (ELISAs) as per the manufacturer's instructions (Aushon BioSystems).

Flow cytometry:

For flow cytometry analysis, lung samples were finely minced in RPMI 1640 medium containing 1mg/ml of collagenase A (Sigma-Aldrich), 1% bovine serum albumin and 1000 units of DNAse I (Fermentas). Minced lung pieces were then incubated for 1h at 37°C with constant shaking and passed through a 100μm cell strainer (Falcon). Samples were then treated with RBC lysis buffer (BD Biosciences) for 10 min at room temperature in dark and washed with cold PBS supplemented with 10% FCS. Cell numbers and cell viability were assessed via trypan blue exclusion using Cellometer (Nexcelcom). Single cell suspensions were Fc blocked with purified rat anti-mouse CD16/CD32 (BD Biosciences) for 20 min at 4°C. The cell suspensions were stained with a cocktail of antibodies for CD3e (500A2), CD4 (RM4-5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), GR1 (RB6-8C5), MHC class II (M5/114.15.2), CD80 (16-10A1;

all from BD Biosciences) and F4/80 (BM8; Biolegend) for 30 min at 4°C. The cells were acquired in a FACS Aria flow cytometer and analyzed using FlowJo software version 7.6.

Histopathology and immunohistochemistry:

The lungs from euthanized mice were removed and fixed in 10% neutral buffered formalin, and processed for histology. The sections were then stained with hematoxylin and eosin (H&E) stain and evaluated by a board-certified pathologist who was blinded to the study.

In order to detect myeloperoxidase (MPO) release from neutrophils, serial lung sections were subjected to immunohistochemical staining. After quenching the endogenous peroxidase activity with 3% hydrogen peroxide in distilled water, the sections were incubated with rabbit anti-human myeloperoxidase (DAKO) for 30 min. The Vectastain ABC kit (Vector Laboratories) was then used in conjunction with NOVA-Red substrate (Vector Laboratories) according to the manufacturer's instructions to detect MPO. Sections were counterstained with hematoxylin (Vector Laboratories) and were observed under a bright field microscope (Nikon).

Statistical analysis:

Data sets were analyzed by analysis of variance (ANOVA) with Tukey-Kramer test. Survival data were analyzed with Kaplan-Meier survival plots, followed by the log rank test. JMP was used for all statistical analyses (JMP Software, SAS Institute). GraphPad Prism 5 (GraphPad Software) was utilized for preparation of all graphs.

3.4 Results:

Generation of PB1-F2 C-terminal mutants of CA/04 influenza viruses:

The pdm 1918 H1N1, 1957 H2N2, 1968 H3N2, and 1997 H5N1 avian IAV have a full-length PB1-F2 protein with unique C-terminal residues (R73K, I74T, H75R, A76V and Q79R) in addition to N66S. In human seasonal strains, these C-terminal residues were absent in H1N1 lineage or mutated in H3N2 lineage. On the contrary, 79.6% of H5N1 avian IAV strains and most recently emerged zoonotic avian IAV strains like H7N7, H7N9, and H9N2 viruses encode a fully functional PB1-F2 protein with unique C-terminal residues [25]. We first established an eight-plasmid reverse-genetics system for the CA/04 wild-type (CA-WT) virus as described earlier [23]. We then generated five recombinant mutant IAVs for this study. To restore the expression of full-length PB1-F2 protein, we used site-directed mutagenesis to remove stop codons at amino acid position 12, 58, and 88 of PB1-F2 protein of CA/04 virus and generated the CA/04 knock-in virus (CA-KI) by reverse genetics. Then, we engineered a recombinant CA/04 virus with a point mutation at position 66 (CA-N66S) of PB1-F2 protein that changes the asparagine (N) to serine (S). In addition to N66S, additional C-terminal mutants were created by changing arginine to lysine at position 73 (R73K), isoleucine to threonine at position 74 (I74T), histidine to arginine at position 75 (H75R), alanine to valine at position 76 (A76V) and glutamine to arginine at position 79 (Q79R) using site-directed mutagenesis in the full-length PB1 gene segment. CA-N66S-2M is a recombinant CA/04 virus that contains two point mutations R73K and H75R in addition to N66S. CA-N66S-3M contains three additional mutations over N66S i.e. R73K, H75R and Q79R whereas CA-N66S-5M contains five mutations i.e. R73K, I74T, H75R, A76V and Q79R in addition to

N66S (Figure 3.1). All these mutations did not have any impact on the amino acid sequence of the PB1 and N40. As the first mutation in the alternate reading frame of PB1-F2 is introduced only after the AUG of N40, its expression will not be affected. Rescued recombinant IAV were subsequently amplified in 10-day old embryonated eggs and the sequences of the PB1 gene segments were confirmed through sequencing in both directions. All the recombinant IAV have critical sequences for efficient PB1-F2 translation and the expression of PB1-F2 protein is confirmed by immunofluorescence staining using PB1-F2 antipeptide antibodies raised against PR/08 H1N1 IAV in rabbits (Figure 3.2).

Specific PB1-F2 C-terminal residues enhance virus replication through type I interferon antagonism:

To study the effects of C-terminal mutations in the CA-KI virus, we examined the multicycle replication of recombinant IAV in A549 cells, the CA-N66S-2M and CA-N66S-3M viruses exhibited significantly higher ($p<0.05$) peak titers than the CA-WT, CA-KI and CA-N66S viruses at all time points examined. CA-N66S-5M had a significantly higher ($p<0.05$) virus yield only at 24 hpi, but reduced to comparable levels of CA-WT, CA-KI and CA-N66S at 48 and 72 hpi (Figure 3.3A). Increased virus replication could be due to enhancement of polymerase activity by PB1-F2 mutants. To determine this, we measured polymerase activity by a dual luciferase reporter assay in 293T cells at 32°C and 37°C. No significant differences in polymerase activity between CA-WT, CA-KI and C-terminal mutants were seen in 293T cells (Figure 3.3B, 3.3C).

We next questioned, whether the increase in virus replication was due to increased type I IFN antagonism by the mutants. A549 cells and THP-1 cells were

infected with CA-WT and recombinant PB1-F2 mutant viruses and IFN-β and IP-10 mRNA levels were measured at 12 hpi by real time RT-PCR. The levels of IFN-β and IP-10 were significantly lower in CA-N66S, CA-N66S-2M, CA-N66S-3M and CA-N66S-5M mutant virus infected THP-1 human monocytic cells compared to CA-WT, and CA-KI viruses ($p<0.01$) but there was a significantly higher level of inhibition ($p<0.05$) in the latter mutants than CA-N66S (Figure 3.4A). A similar trend was observed in A549 cells, but the relative levels of IFN-β and IP-10 transcripts were several folds lower than in THP-1 cells (Figure 3.4B). There were no statistically significant differences ($p>0.05$) between the CA-WT and CA-KI viruses in the induction of type I IFN either in THP-1 or A549 cells (Figure 3.4A, 3.4B).

PB1-F2 C-terminal mutations increase mitochondrial superoxide production and do not enhance apoptosis:

Next, we examined the role of C-terminal mutations of PB1-F2 on mitochondrial damage indicated by superoxide production and apoptosis. A549 cells were infected with CA-WT and recombinant PB1-F2 mutant viruses and stained with MitoSOX Red after 16 hpi. CA-N66S, CA-N66S-2M, CA-N66S-3M and CA-N66S-5M viruses induced significantly higher mitochondrial superoxide when compared to CA-WT, and CA-KI viruses ($p<0.05$) and the latter three mutant viruses induced 1.44-1.59 fold higher superoxide than 66S (Figure 3.5A, 3.5B). Then, we examined whether these mutations also modulated apoptosis in U937 human monocytic cells using Annexin V-PI staining at 12 hpi. The WT, CA-KI and C-terminal mutant viruses induced apoptosis of U937 cells. However, no significant differences were observed between the WT and mutant viruses in inducing apoptosis (Figure 3.5C).

PB1-F2 C-terminal mutations enhance pathogenicity in mice:

As the *in vitro* data indicated that PB1-F2 C-terminal residues modulate virus replication by down regulating type I IFN signaling and enhanced mitochondrial superoxide production, we examined the role of these mutations *in vivo* in BALB/c mouse model. We inoculated 6-8 weeks old BALB/c mice with different recombinant viruses and evaluated weight loss, virus load in the lungs, expression of proinflammatory genes and lung pathology. Clinical signs including lethargy and respiratory signs were evident in virus-infected mice as early as 1 dpi. Mice infected with CA-KI showed mild clinical signs and weight loss until 2 dpi and gained body weight from there on and survived until 14 dpi. CA-N66S-2M and CA-N66S-3M viruses induced lethargy, inactivity, ruffled fur and pronounced loss of body weight as early as 2 dpi (Figure 3.6A). Disease severity increased and labored respiration and acute respiratory distress signs were observed on 3 dpi. By 5 dpi, the mice in these two groups lost >25% of body weight and had to be euthanized as per the IACUC guidelines of Virginia Tech. CA-N66S infected mice displayed clinical signs by 2 dpi and weight loss commenced from 3 dpi. Subsequently, the disease progression was similar to CA-N66S-2M and CA-N66S-3M and infected mice reached experimental end points by 7 dpi. In CA-N66S-5M infected mice, clinical signs were delayed and commenced only by 3 dpi and disease severity was less as compared to CA-N66S-2M and CA-N66S-3M. The CA-N66S-5M virus infected mice experienced moderate weight loss (12% of body weight) by 7 dpi but gained body weight between 7 and 14 dpi and all the mice survived until 14 dpi (Figure 3.6A, 3.6B).

PB1-F2 C-terminal mutations increase virus replication in the lungs:

In an independent experiment, we examined the temporal differences in virus replication, immunophenotype of cells, proinflammatory cytokine production and pathology in the lungs of infected mice to correlate disease severity. On 1 and 3 dpi, virus loads were significantly higher ($p<0.05$) in the lungs of CA-N66S-2M and CA-N66S-3M infected mice as compared to CA-KI (Fig 3.6C). Comparable virus loads were observed in the lungs of mice infected with CA-KI, CA-N66S and CA-N66S-5M on 1 and 3 dpi. Mice infected with CA-KI cleared virus from the lungs by 7 dpi while virus replication ranging from 10^4-10^5 $\log_{10}$ PFU/g of lung was still evident on 7 dpi with other mutants. Mice infected with CA-N66S-5M showed significant virus load on 5 dpi ($p<0.05$) and was cleared from the lungs by 14 dpi (Fig 3.6C).

PB1-F2 C-terminal mutations increased cellularity in the lungs of infected mice:

To understand the role of C-terminal residues of PB1-F2 in recruiting immune cells to the lungs after IAV infection, we examined the immunophenotype of cells in the lungs by flow cytometry. Mice were inoculated with recombinant IAV and were euthanized on 1, 3, 5, 7, and 14 dpi and the lungs were collected and processed for flow cytometry. We detected a decline in the resident alveolar macrophage (AM) population ($CD11c^{hi}/CD11b^{lo}/MHCII^{lo}$) in CA-N66S, CA-N66S-2M and CA-N66S-3M infected mice over the course of infection. On 3, 5 and 7 dpi, there was a statistically significant reduction ($p<0.05$) in resident AM population in mice infected with CA-N66S, CA-N66S-2M and CA-N66S-3M compared to CA-KI group (Figure 3.7A). On the other hand, comparable but increased levels of neutrophil ($F4\text{-}80^-/GR1^+/CD11b^-$) infiltration in the lungs were seen among the virus-infected groups on 1 dpi (Figure 3.7B). Lungs of

mice infected with CA-N66S, CA-N66S-2M and CA-N66S-3M had significantly higher neutrophil infiltration when compared to CA-KI and CA-N66S-5M on 3 dpi ($p<0.05$). On 5 dpi, significantly increased neutrophil infiltration was observed only in the lungs of mice infected with CA-N66S-3M when compared with other groups ($p<0.05$), whereas the CA-N66S-2M and CA-N66S-3M virus infected lungs had significantly high neutrophil infiltration on 7 dpi (Figure 3.7B) compared to other groups ($p<0.05$).

During the course of infection, lungs of mice infected with mutant viruses had elevated levels of monocytes ($CD11c^{+}/CD11b^{+}$) (Figure 3.7C). On 3, 5 and 7 dpi, mice infected with CA-N66S, CA-N66S-2M and CA-N66S-3M viruses had a significant increase in monocyte infiltration in the lungs ($p<0.05$). On 1 and 3 dpi, we found comparable levels of inflammatory monocytes ($CD11c^{-}/MHCII^{-}/CD11b^{+}$) in the lungs of virus-infected mice. Increase in inflammatory monocytes was statistically significant ($p<0.05$) in CA-N66S-3M infected mice on 3, 5 and 7 dpi whereas in CA-N66S-2M infected mice only on 7 dpi compared to other groups (Figure 3.7D).

We observed a decline in dendritic cells (DCs) ($CD11c^{hi}/MHC\ II^{+}/CD11b^{-}$) in CA-N66S-2M and CA-N66S-3M over the course of infection (Figure 3.7E). Lungs of mice infected with mutant viruses (CA-N66S, CA-N66S-2M, CA-N66S-3M and CA-N66S-5M) showed significant decrease in DCs when compared with CA-KI on 3 dpi ($p<0.05$) but not on 5 dpi. On 7 dpi, the DCs were significantly low in lungs of mice infected with CA-N66S-2M and CA-N66S-3M when compared with other groups ($p<0.05$). Interestingly, the TNF-α/inducible nitric oxide synthase producing DCs (tipDCs) were significantly elevated ($p<0.05$) only in CA-N66S group on 5 and 7 dpi and CA-N66S-5M group on 7 dpi (Figure 3.7F).

PB1-F2 mutants induce a pro-inflammatory milieu in the lung:

Having determined that PB1-F2 drives an influx of inflammatory cells into the lungs, we next assessed pro-inflammatory cytokines levels in the lungs of virus-infected mice on 3 and 5 dpi by ELISA. Significantly elevated levels of IL-6 were observed in the lungs of CA-N66S-2M and CA-N66S-3M infected mice only on 3 dpi ($p<0.05$) when compared with other groups. Levels of IL-1β, TNF-α and IFN-γ were increased on 3 or 5 dpi in some groups but no statistically significant differences ($p>0.05$) were found between the groups (Figure 3.8A).

We next examined the impact of this enhanced inflammatory response on pathologic changes in the lung tissue. The overall gross and histologic findings in mice infected with C-terminal mutants indicated that there was acute lung injury (ALI) and severe pneumonia leading to fulminant acute respiratory distress syndrome (ARDS). Lungs of CA-KI infected animals were unremarkable except on 1 dpi when mild hemorrhage was recorded. As expected, the highest degree of lung pathology was observed in CA-N66S-2M and CA-N66S-3M virus infected mice. In these animals, pneumonia with extensive areas of hemorrhage and consolidation on 3 dpi were evident. By 5 dpi, pneumonia and hemorrhage spread to all the lung lobes. In CA-N66S infected mice, pneumonia and hemorrhagic lesions were noticed by 3 dpi. Both were widespread by 5 dpi and spread to the whole lung by 7 dpi. Lungs of mice infected with CA-N66S-5M displayed focal areas of hemorrhage and consolidation on 3 dpi, which were minimal by 7 dpi (data not shown).

A board-certified veterinary pathologist who was blinded to the purpose of the study and the composition of the groups examined lung histopathologic sections. Mild to

moderate multifocal intra-alveolar hemorrhage with various degrees of neutrophilic and lymphocytic infiltration were seen in all infected mice. Mice infected with CA-KI showed mild intra-alveolar hemorrhage with lymphocytic infiltration as early as 1 dpi, which resolved over the course of infection. Mice infected with CA-N66S, CA-N66S-2M and CA-N66S-3M showed moderate intra-alveolar hemorrhage with widespread infiltration of neutrophils and lymphocytes covering 50% of the tissue sections as early as 1 dpi, which sustained over the course of infection. Mild to moderate amounts of fibrin and hyaline membranes were also observed on 3 and 5 dpi. Mice infected with CA-N66S-5M showed mild intra-alveolar hemorrhage with neutrophilic and monocytic infiltration on 1 and 3 dpi. Alveolar hemorrhage and inflammatory cell infiltration progressed to moderate levels by 7 dpi and resolved by 14 dpi. Representative histologic lesions on 3 dpi for the experimental groups are shown (Figure 3.8B).

In order to understand whether the increased neutrophilic influx seen in the lungs of infected mice contributed to pathology, we stained the lung sections for myeloperoxidase (MPO). MPO staining was significantly more intense in the lungs of mice infected with CA-N66S, CA-N66S-2M and CA-N66S-3M viruses when compared to CA-KI (Figure 3.8C) confirming extensive degranulation of granulocytes in the lungs of mice infected with these recombinant viruses.

Discussion:

PB1-F2 is a small protein with multiple functions. Since its discovery in 2001 [2], a range of functions for PB1-F2 from a pro-apoptotic protein to an interferon antagonist has been attributed [4]. Interestingly, many IAV do not express a full-length PB1-F2 protein. However, the PB1-F2 is highly conserved in 95% avian influenza viruses and all

of the 20th century pdm IAVs [3]. On the other hand, the 2009 pdm09 H1N1 virus does not express a full-length PB1-F2 protein and causes a relatively mild disease. In many studies, the C-terminal region of PB1-F2 protein has been shown to be involved in the virulence of IAV [10,12,19]. Highly pathogenic IAV and 1918 H1N1 have highly conserved mutations in the C-terminus of the protein and in particular the N66S mutation, which has been correlated to their virulence [12,13]. Surprisingly, reconstituting PB1-F2 in pdm09 H1N1 did not enhance virulence in mice, ferrets or swine [21,22] and N66S mutation in the restored PB1-F2 also failed to increase virulence but increased proinflammatory cytokines in animals and enhanced virus replication in A549 cells [21]. This raises an interesting question as to whether there are additional mutations acting in concert with the N66S in other pandemic strains to increase virulence. We found highly conserved additional mutations in the C-terminus of PB1-F2 of lethal strains and explored their role in the pathogenicity of IAV.

The C-terminal region of reconstituted PB1-F2 of pdm09 H1N1 virus is unique and remarkably different from the 20th century pdm strains and highly pathogenic avian H5N1 and lacked 66S and all the molecular signatures of virulence identified in this study. It is highly similar to recent H3N2 and variant H3N2 that are circulating in pigs and humans. It appears that during adaptation to humans, the PB1-F2 of many seasonal and non-avian IAV become truncated or mutated the genetic markers for virulence. It is reasonable, therefore, to speculate that the truncated or mutated PB1-F2 may offer a survival advantage to the virus. A recent study demonstrated that the C-terminal residues 62L, 66S, 75R, 79R and 82L of IAV PB1-F2 are associated with enhanced severity of secondary-bacterial infections, in a bacterium specific manner, in a Gram-positive

bacterial superinfection model [26]. The pdm09 H1N1 virus has now become a seasonal virus in many countries around the world. It is quite possible that the circulating pdm09 H1N1 strain can acquire avian-like PB1-F2 through mutation or genetic reassortment. In fact, the polymerase subunit PB1 is the only gene of avian origin other than the surface glycoproteins in the 1957 H2N2 and 1968 H3N2 pdm IAV [27]. In pdm1918 H1N1, the PB1 gene played a vital role for its high virulence [28]. To understand the consequences of such genetic changes in pdm09 H1N1 virus, we evaluated the mechanism(s) and disease phenotype associated with specific C-terminal amino acid mutations that were found to be conserved in pathogenic IAV.

The mutant pdm09 H1N1 viruses CA-N66S-2M and CA-N66S-3M replicated several logs higher than other mutant viruses in human A549 respiratory epithelial cells and in the lungs of BALB/c mice early in the course of infection and show delayed clearance. Increased virus replication could either be due to enhanced polymerase function [18,22,29] or increased interferon antagonism [15,30]. Viral polymerase activity using dual luciferase reporter assay in 293T cells indicated that there were no significant differences in polymerase activity between CA-WT, CA-KI and C-terminal mutant viruses. However, the C-terminal mutants displayed potent type I IFN antagonistic function in epithelial (A549) and immune (THP-1) cells. The CA-WT virus did not show any enhanced type I IFN response compared to CA-KI virus suggesting type I IFN antagonism is sequence dependent. We argue that type I IFN antagonistic signature in PB1-F2 is 66S and additive mutations such as 73K, 75R, and 79R further enhance this phenotype. Addition of 74T and 76V to this pool may compensate this effect while still remaining type I IFN antagonistic. PB1-F2 has been shown to bind at the level of MAVS

adapter protein in mitochondria and acts as an interferon antagonist [14,15,30,31]. Type I IFN is an important antiviral defense mechanism of the host and subverting it would allow enhanced virus replication. The structural basis for this enhanced type I IFN antagonism by specific amino acid residues in PB1-F2 requires future studies.

PB1-F2 is considered a pro-apoptotic protein [7]. Further PB1-F2 was reported to induce cell death only in immune cells [2,10] in a strain dependent manner [19]. Although apoptosis was observed in infected human immune cells, no significant differences in the magnitude of apoptosis could be discerned between different mutant viruses suggesting that cell death mechanisms between these mutants might be different. Superoxide is the primary reactive oxygen species (ROS) produced by the mitochondria in response to damage. The CA-N66S-2M, CA-N66S-3M and CA-N66S-5M mutants enhanced the release of mitochondrial superoxide compared to the CA-WT and CA-KI viruses suggesting oxidative stress to the cell. Sudden burst of ROS and oxidative stress can alter the mitochondrial membrane potential [32,33] leading to cell death by a variety of mechanisms and possibly also can alter innate immune response through the RIG-I pathway [31]. Many viruses have been shown to induce oxidative stress to the cells including human adenovirus 5 [34], hepatitis C virus [35], human immunodeficiency virus [36] and Epstein-Barr virus [37].

We found that the specific C-terminal residues in the pdm09 H1N1 virus exerted significant effects on disease pathogenesis. In BALB/c mice, severe clinical disease and mortality were noticed when infected with CA-N66S-3M, CA-N66S-2M, CA-N66S, CA-N66S-5M mutants in that order compared to CA-KI viruses. The mutants CA-N66S-3M and CA-N66S-2M caused fulminant ARDS in infected mice and resulting in death in a

short duration from onset. The pdm09 H1N1 has been shown to cause ARDS in humans under specific conditions [38]. Pathologically, the ARDS was evident as difuse alvelolar hemorrhage, fibrinous exudates and hylain membrane formation as seen in acute lung injury (ALI) in mice infected with CA-N66S-3M and CA-N66S-2M mutants. The ALI was mild to moderate in other mutants. Enhanced levels of proinflammatory cytokines such as IL-6 detected in the lungs are essential for the recruitment and activation of immune cells in infected tissues [39] and to alter the composition of claudin in lung tight junctions leading to leakage of fluids [40,41]. The increased infiltration of neutrophils and inflammatory monocytes observed in the lungs of mice infected with CA-N66S-3M and CA-N66S-2M viruses are consistent with the requirement of neutrophils in influenza virus infection for virus clearance as neutrophil depletion leads to lethal viremia in influenza-infected mice [42]. Besides, massive influx of neutrophils has also been reported in lethal influenza virus infections [43-46]. Neutrophils have been shown to play a major role in ALI in influenza virus infections [47-49]. Neutrophils have been shown to be responsible for virus clearance by inactivating viral proteins with the production of ROS mediated by MPO contained in the azurophil granules [43,50]. Higher viral titers in the lungs of mice infected with CA-N66S-3M, CA-N66S-2M mutants was observed as early as 1 DPI than other mutants and all of these mutants except CA-KI were unable to clear the virus until 7 dpi. The intense neutrophilic MPO activity could have also contributed to the increased pathology in the lungs of mice infected with CA-N66S-2M and CA-N66S-3M mutants. Neutrophil MPO has also been shown to play a role in inflammatory lung damage by altering certain claudins leading tight junction leakage, and also by influencing virus replication and spread during influenza infection [51]. In fact,

neutrophil depleted mice showed mild pathology in lungs [49]. MPO and superoxide generated after infection with CA-N66S-3M and CA-N66S-2M mutants suggest these may lead to the formation of neutrophil extracellular traps (NETs) and mediate endothelial damage [49]. NET formation has been shown to be abrogated by inhibitors of redox enzymes (MPO and superoxides), implicating their role in NET formation [49]. Although, we did not directly show NET formation in infected mice, the damage to pulmonary vascular bed evident by excessive alveolar hemorrhage, fibrin deposition and hylaine membrane formation and increased MPO activity in the lungs of infected mice and superoxide generation in human lung epithelial (A549) cells indicate that certain amino acid residues in PB1-F2 may modulate NET formation. Additional studies are required to confirm this.

Macrophages in the lung contribute to virus clearance by phagocytosis of virus infected cells and also by maintaining the integrity of alevolar epothelium [52,53]. The rapid destruction of resident alveolar macrophages by CA-N66S-3M and CA-N66S-2M mutants that we observed is consistent with lethal influenza virus infections [54]. The depletion of alveolar macrophages may enhance ARDS as has been shown with macrophage depleted with mice infected with sub lethal doses of pdm09 H1N1 and PR8 [49]. The CA-KI, CA-N66S-5M and CA-66S mutants showed mild lung injury consistent with mild to moderate neutrophil infiltration, alveolar macrophage depletion and redox enzyme production in contrast to the CA-N66S-3M and CA-N66S-2M mutants. It would be interesting to study in future whether the AM depletion in the lungs is by direct virus-induced cell death. A previous study implicated tipDC recruitment in lethal influenza [55]. Here we found that tipDCs were recruited in significantly high numbers in mice

infected with CA-N66S and CA-N66S-5M viruses. The tipDCs have been shown to play dual functions: immunopathology and antigen specific CD8+ T cell responses [55].

It is intriguing that very few residues in the C-terminus of PB1-F2 together with 66S mediate ARDS induced by pdm09 H1N1 with minimal structural alteration. Four amino acid residues in the C-terminal region of PB1-F2 protein (62L, 75R, 79R, and 82L) enhance inflammation in the lungs of mice [56] and three residues (68I, 69L and 70V) enhance cytotoxicity [57] and these inflammatory and cytotoxic residues contribute to secondary bacterial infection [56,57]. Although the inflammatory residues were contained in our lethal mutants in addition to 66S, our CA-N66S-2M and CA-N66S-3M mutants did not possess the cytotoxic mutations.

All 20th century pdm IAV and highly pathogenic avian influenza viruses possess a PB1-F2 with unique C-terminal residues that may contribute to their pathogenicity [8]. Recent analysis indicates that many non-human and non-avian IAVs including the Eurasian swine influenza virus lineage, equine influenza and canine influenza viruses possess inflammatory and cytotoxic residues in their PB1-F2 and can effectively transmit between mammalian hosts [57]. Specific residues in the PB1-F2 protein are proposed as genetic markers for secondary bacterial infection and surveillance for these residues in circulating IAV has been suggested [26,58].

In many instances the pdm09 H1N1 induced influenza rapidly evolved into ARDS in humans [45,59-62]. In one study, a D222G HA mutant of swine origin H1N1 (A/swine/Shandong/731/2009) with a non-functional PB1-F2 induced ARDS in a mouse model and the ability to induce ARDS was ascribed to the HA D222G mutation [63]. However, most studies that documented ARDS in humans unfortunately failed to verify

the expression of PB1-F2 and therefore, direct comparisons could not be made.

In summary, our study reveals three major findings. Firstly, two to three amino acid residues in combination with 66S in the C-terminus of PB1-F2 protein may predict the pathogenicity of IAV and may serve as markers for potential lethality of IAV strains. Another study also demonstrated this possibility in a bacterial superinfection model in mice independent of subtype of swine derived IAV [26]. We identified specific residues (66S, 73K, 75R and 79R) dictate the development of fulminant ARDS with rapid destruction of alveolar macrophages, excessive neutrophil infiltration, destruction of the pulmonary vasculature, and ineffiecient virus clearance mediated by a combination of of neutrophilic redox enzymes and enhanced virus replication and spread. Second, our data provides the first evidence that the above C-terminal residues in PB1-F2 mediate excessive neutrophil infux, MPO production and ALI in influenza virus pneumonia. Third, we show that compensatory mutations (74T and 76V) in the PB1-F2 C-terminus may marginally alleviate the ALI induced by pdm09 H1N1 with 66S, 73K, 75R and 79R residues. It could be speculated that these residues are most likely to enhance post-influenza secondary pneumonia [26], which await future studies. The present findings enhance our understanding of PB1-F2 protein as a virulence factor in influenza virus infection and provide new insights into the impact of acquired genetic changes on the virulence of the pdm09 H1N1 virus. With increasing number of outbreaks due to pdm09 H1N1 in many countries, it is imperative to understand the role that specific proteins play in imparting pathogenicity. Our results, therefore, highlight the importance of increased influenza virus surveillance to identify viral genotypes that would help us predict lethal phenotypes.

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3.7 References:

1. Wright PF, Neumann, G., Kawaoka, Y. (2007) Orthomyxoviruses In: Knipe DM, Griffin, D. E., Lamb, R. A., Martin, M A., Roiz-man, B., Straus, S. E., editor. Fields Virology. 5th ed: Lippincott Williams & Wilkins. pp. 1691-1740.
2. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306-1312.
3. Pasricha G, Mishra AC, Chakrabarti AK (2013) Comprehensive global amino acid sequence analysis of PB1F2 protein of influenza A H5N1 viruses and the influenza A virus subtypes responsible for the 20th-century pandemics. Influenza Other Respir Viruses 7: 497-505.
4. Kosik I, Holly J, Russ G (2013) PB1-F2 expedition from the whole protein through the domain to aa residue function. Acta Virol 57: 138-148.
5. Chanturiya AN, Basanez G, Schubert U, Henklein P, Yewdell JW, et al. (2004) PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. J Virol 78: 6304-6312.
6. Henkel M, Mitzner D, Henklein P, Meyer-Almes FJ, Moroni A, et al. (2010) The proapoptotic influenza A virus protein PB1-F2 forms a nonselective ion channel. PLoS One 5: e11112.

7. Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P (2005) Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog 1: e4.
8. McAuley JL, Chipuk JE, Boyd KL, Van De Velde N, Green DR, et al. (2010) PB1-F2 proteins from H5N1 and 20 century pandemic influenza viruses cause immunopathology. PLoS Pathog 6: e1001014.
9. Chevalier C, Al Bazzal A, Vidic J, Fevrier V, Bourdieu C, et al. (2010) PB1-F2 influenza A virus protein adopts a beta-sheet conformation and forms amyloid fibers in membrane environments. J Biol Chem 285: 13233-13243.
10. Zamarin D, Ortigoza MB, Palese P (2006) Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J Virol 80: 7976-7983.
11. McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, et al. (2007) Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host Microbe 2: 240-249.
12. Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P (2007) A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog 3: 1414-1421.
13. Conenello GM, Tisoncik JR, Rosenzweig E, Varga ZT, Palese P, et al. (2011) A single N66S mutation in the PB1-F2 protein of influenza A virus increases virulence by inhibiting the early interferon response in vivo. J Virol 85: 652-662.
14. Dudek SE, Wixler L, Nordhoff C, Nordmann A, Anhlan D, et al. (2011) The influenza virus PB1-F2 protein has interferon antagonistic activity. Biol Chem 392: 1135-1144.

15. Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, et al. (2011) The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS Pathog 7: e1002067.
16. Le Goffic R, Bouguyon E, Chevalier C, Vidic J, Da Costa B, et al. (2010) Influenza A virus protein PB1-F2 exacerbates IFN-beta expression of human respiratory epithelial cells. J Immunol 185: 4812-4823.
17. Le Goffic R, Leymarie O, Chevalier C, Rebours E, Da Costa B, et al. (2011) Transcriptomic analysis of host immune and cell death responses associated with the influenza A virus PB1-F2 protein. PLoS Pathog 7: e1002202.
18. Mazur I, Anhlan D, Mitzner D, Wixler L, Schubert U, et al. (2008) The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell Microbiol 10: 1140-1152.
19. McAuley JL, Zhang K, McCullers JA (2010) The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. J Virol 84: 558-564.
20. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, et al. (2009) In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature 460: 1021-1025.
21. Hai R, Schmolke M, Varga ZT, Manicassamy B, Wang TT, et al. (2010) PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. J Virol 84: 4442-4450.

22. Pena L, Vincent AL, Loving CL, Henningson JN, Lager KM, et al. (2012) Restored PB1-F2 in the 2009 pandemic H1N1 influenza virus has minimal effects in swine. J Virol 86: 5523-5532.
23. Hoffmann E, Neumann, G., Kawaoka, Y., Hobom, G., Webster, R. G. (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proceedings of the National Academy of Sciences 97: 6108-6113.
24. Mukhopadhyay P, Rajesh M, Hasko G, Hawkins BJ, Madesh M, et al. (2007) Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. Nat Protoc 2: 2295-2301.
25. Alymova IV, York IA, McCullers JA (2014) Non-avian animal reservoirs present a source of influenza A PB1-F2 proteins with novel virulence-enhancing markers. PLoS One 9: e111603.
26. Weeks-Gorospe JN, Hurtig HR, Iverson AR, Schuneman MJ, Webby RJ, et al. (2012) Naturally occurring swine influenza A virus PB1-F2 phenotypes that contribute to superinfection with Gram-positive respiratory pathogens. J Virol 86: 9035-9043.
27. Kawaoka Y, Krauss S, Webster RG (1989) Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 63: 4603-4608.
28. Pappas C, Aguilar PV, Basler CF, Solorzano A, Zeng H, et al. (2008) Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus. Proc Natl Acad Sci U S A 105: 3064-3069.

29. Chen CJ, Chen GW, Wang CH, Huang CH, Wang YC, et al. (2010) Differential localization and function of PB1-F2 derived from different strains of influenza A virus. J Virol 84: 10051-10062.
30. Varga ZT, Palese P (2011) The influenza A virus protein PB1-F2: killing two birds with one stone? Virulence 2: 542-546.
31. Yoshizumi T, Ichinohe T, Sasaki O, Otera H, Kawabata S, et al. (2014) Influenza A virus protein PB1-F2 translocates into mitochondria via Tom40 channels and impairs innate immunity. Nat Commun 5: 4713.
32. Huttemann E, Jung A, van Hout N, Sakka SG (2006) Effects of propofol and methohexital on neutrophil function in cardiac surgical patients. Ann Card Anaesth 9: 126-131.
33. Hsieh HL, Wang HH, Wu WB, Chu PJ, Yang CM (2010) Transforming growth factor-beta1 induces matrix metalloproteinase-9 and cell migration in astrocytes: roles of ROS-dependent ERK- and JNK-NF-kappaB pathways. J Neuroinflammation 7: 88.
34. McGuire KA, Barlan AU, Griffin TM, Wiethoff CM (2011) Adenovirus type 5 rupture of lysosomes leads to cathepsin B-dependent mitochondrial stress and production of reactive oxygen species. J Virol 85: 10806-10813.
35. Nishina S, Hino K, Korenaga M, Vecchi C, Pietrangelo A, et al. (2008) Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. Gastroenterology 134: 226-238.

36. Kruman, II, Nath A, Mattson MP (1998) HIV-1 protein Tat induces apoptosis of hippocampal neurons by a mechanism involving caspase activation, calcium overload, and oxidative stress. Exp Neurol 154: 276-288.
37. Gargouri B, Van Pelt J, El Feki Ael F, Attia H, Lassoued S (2009) Induction of Epstein-Barr virus (EBV) lytic cycle in vitro causes oxidative stress in lymphoblastoid B cell lines. Mol Cell Biochem 324: 55-63.
38. Nakajima N, Hata S, Sato Y, Tobiume M, Katano H, et al. (2010) The first autopsy case of pandemic influenza (A/H1N1pdm) virus infection in Japan: detection of a high copy number of the virus in type II alveolar epithelial cells by pathological and virological examination. Jpn J Infect Dis 63: 67-71.
39. Wareing MD, Lyon AB, Lu B, Gerard C, Sarawar SR (2004) Chemokine expression during the development and resolution of a pulmonary leukocyte response to influenza A virus infection in mice. J Leukoc Biol 76: 886-895.
40. Wray RJ, Buskirk TD, Jupka K, Lapka C, Jacobsen H, et al. (2009) Influenza vaccination concerns among older blacks: a randomized controlled trial. Am J Prev Med 36: 429-434 e426.
41. Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, et al. (2002) Regulation of airway tight junctions by proinflammatory cytokines. Mol Biol Cell 13: 3218-3234.
42. Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y (2007) Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. J Immunol 178: 2448-2457.

43. Fujisawa H (2001) Inhibitory role of neutrophils on influenza virus multiplication in the lungs of mice. Microbiol Immunol 45: 679-688.

44. Crowe CR, Chen K, Pociask DA, Alcorn JF, Krivich C, et al. (2009) Critical role of IL-17RA in immunopathology of influenza infection. J Immunol 183: 5301-5310.

45. Jaber S, Conseil M, Coisel Y, Jung B, Chanques G (2010) [ARDS and influenza A (H1N1): patients' characteristics and management in intensive care unit. A literature review]. Ann Fr Anesth Reanim 29: 117-125.

46. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM (2008) H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog 4: e1000115.

47. Xu T, Qiao J, Zhao L, Wang G, He G, et al. (2006) Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. Am J Respir Crit Care Med 174: 1011-1017.

48. Deng J, Zheng Y, Li C, Ma Z, Wang H, et al. (2010) Plastic bronchitis in three children associated with 2009 influenza A(H1N1) virus infection. Chest 138: 1486-1488.

49. Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, et al. (2011) Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. Am J Pathol 179: 199-210.

50. Yamamoto K, Miyoshi-Koshio T, Utsuki Y, Mizuno S, Suzuki K (1991) Virucidal activity and viral protein modification by myeloperoxidase: a candidate

for defense factor of human polymorphonuclear leukocytes against influenza virus infection. J Infect Dis 164: 8-14.

51. Sugamata R, Dobashi H, Nagao T, Yamamoto K, Nakajima N, et al. (2012) Contribution of neutrophil-derived myeloperoxidase in the early phase of fulminant acute respiratory distress syndrome induced by influenza virus infection. Microbiol Immunol 56: 171-182.
52. Herold S, Steinmueller M, von Wulffen W, Cakarova L, Pinto R, et al. (2008) Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. J Exp Med 205: 3065-3077.
53. Narasaraju T, Ng HH, Phoon MC, Chow VT (2010) MCP-1 antibody treatment enhances damage and impedes repair of the alveolar epithelium in influenza pneumonitis. Am J Respir Cell Mol Biol 42: 732-743.
54. Schneider C, Nobs SP, Heer AK, Kurrer M, Klinke G, et al. (2014) Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. PLoS Pathog 10: e1004053.
55. Aldridge JR, Jr., Moseley CE, Boltz DA, Negovetich NJ, Reynolds C, et al. (2009) TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. Proc Natl Acad Sci U S A 106: 5306-5311.
56. Alymova IV, Green AM, van de Velde N, McAuley JL, Boyd KL, et al. (2011) Immunopathogenic and antibacterial effects of H3N2 influenza A virus PB1-F2 map to amino acid residues 62, 75, 79, and 82. J Virol 85: 12324-12333.
57. Alymova IV, Samarasinghe A, Vogel P, Green AM, Weinlich R, et al. (2014) A novel cytotoxic sequence contributes to influenza A viral protein PB1-F2

pathogenicity and predisposition to secondary bacterial infection. J Virol 88: 503-515.

58. Huber VC (2012) Can surveillance of the influenza virus PB1-F2 gene be used to predict the severity of secondary bacterial infections? Virulence 3: 523-524.
59. Homsi S, Milojkovic N, Homsi Y (2010) Clinical pathological characteristics and management of acute respiratory distress syndrome resulting from influenza A (H1N1) virus. South Med J 103: 786-790; quiz 791-782.
60. Kato Y (2010) [Pneumonia and acute respiratory distress syndrome due to pandemic influenza H1N1 2009]. Nihon Rinsho 68: 1666-1670.
61. Chowell G, Echevarria-Zuno S, Viboud C, Simonsen L, Grajales Muniz C, et al. (2012) Recrudescent wave of pandemic A/H1N1 influenza in Mexico, winter 2011-2012: Age shift and severity. PLoS Curr 4: RRN1306.
62. Witczak A, Prystupa A, Kurys-Denis E, Borys M, Czuczwar M, et al. (2013) Acute respiratory distress syndrome (ARDS) complicating influenza A/H1N1v infection--a clinical approach. Ann Agric Environ Med 20: 820-822.
63. Zhang Y, Sun H, Fan L, Ma Y, Sun Y, et al. (2012) Acute respiratory distress syndrome induced by a swine 2009 H1N1 variant in mice. PLoS One 7: e29347.

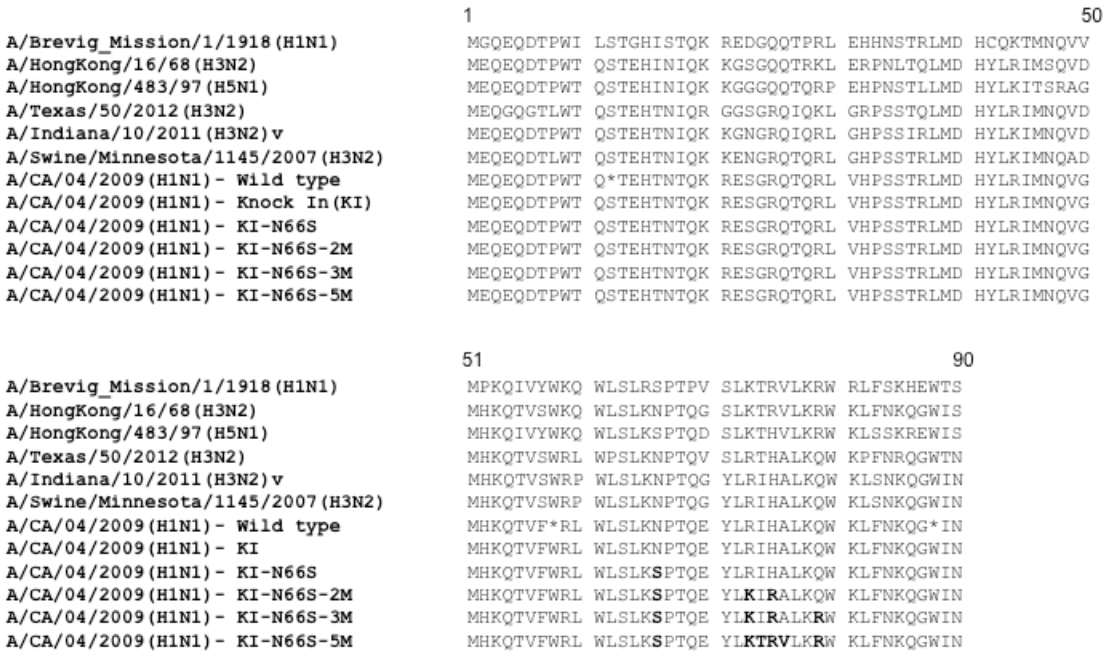


Figure 3.1: Sequence comparison of PB1-F2 of pandemic and highly pathogenic avian influenza viruses and PB1-F2 mutants generated for this study. The coding regions for the PB1-F2 amino acid sequences were aligned by Clustalw program of DNASTAR (Lasergene). The mutations in the C-terminal region of the PB1-F2 proteins are indicated.

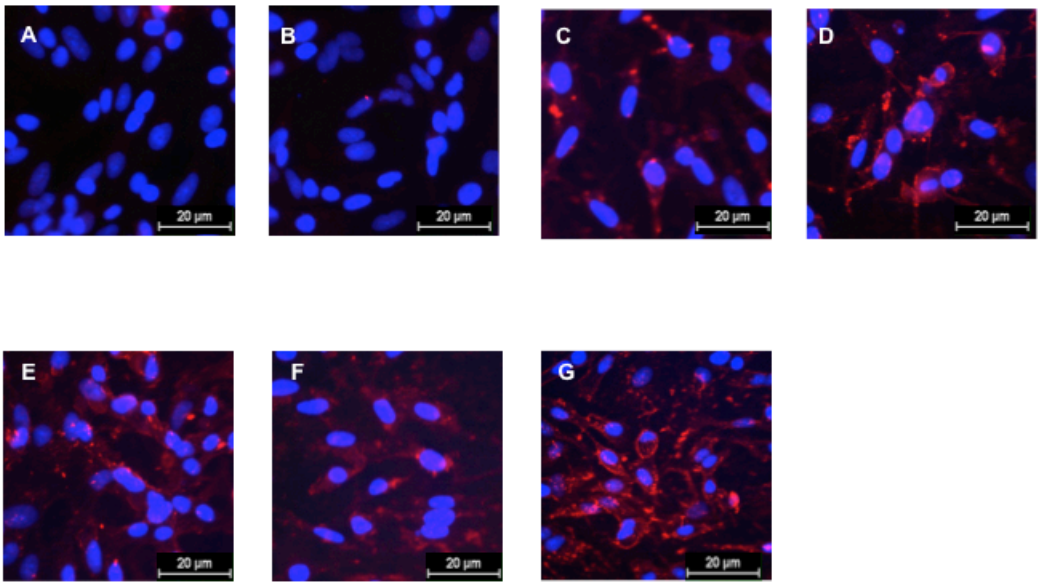


Figure 3.2: Expression of PB1-F2 protein by recombinant 2009 pandemic H1N1 CA/04 and PB1-F2 mutant viruses. A549 cells were infected with respective recombinant viruses for 36 h, the cells were then fixed, permeabilized and labeled with rabbit antipeptide antibody raised against PR/08 H1N1 PB1-F2 and Alexa Fluor 594-conjugated anti-rabbit secondary antibodies. Cells were mounted using DAPI mounting medium and images obtained using an inverted epifluorescence Nikon microscope. (A) Mock, (B) CA-WT, (C) CA-KI, (D) CA-N66S, (E) CA-N66S-2M, (F) CA-N66S-3M, and (G) CA-N66S-5M. Scale bars are in micrometers.

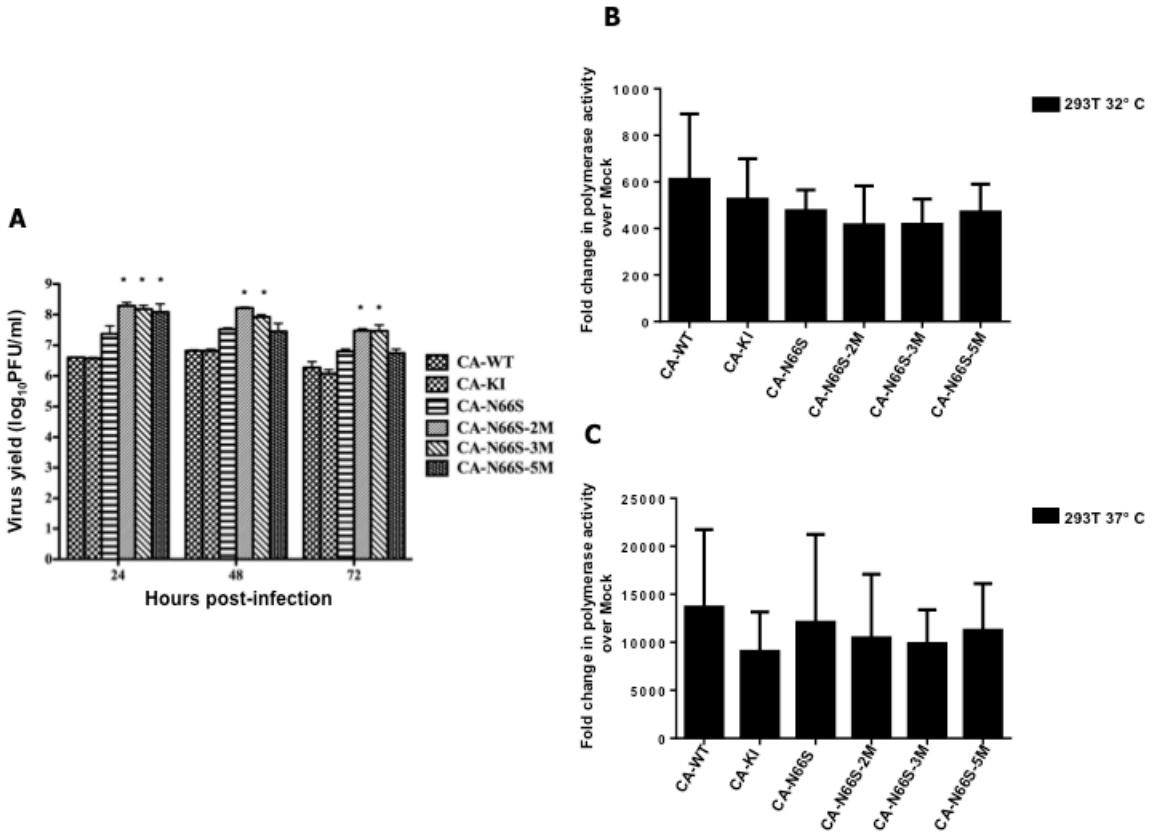


Figure 3.3: Increased virus replication in PB1-F2 mutants was not due to enhanced polymerase activity. (A) A549 cells were infected with the indicated recombinant CA/04 viruses (MOI, 0.001). Virus titers in the supernatants were determined by plaque assays on MDCK cells at the indicated time points. Mean virus yield +/- standard deviation (SD) from triplicate experiments are shown. An asterisk (*) indicates a significant difference when compared with CA-KI ($p<0.05$). (B) and (C) Viral polymerase assay. Mini-replicon assay to measure the influenza viral polymerase activity was carried out in 293T cells. Firefly luciferase levels were normalized for in sample Renilla luciferase activity and the average per condition was used to determine fold change in polymerase activity over the mock. Error bars indicate SD from the three replicates.

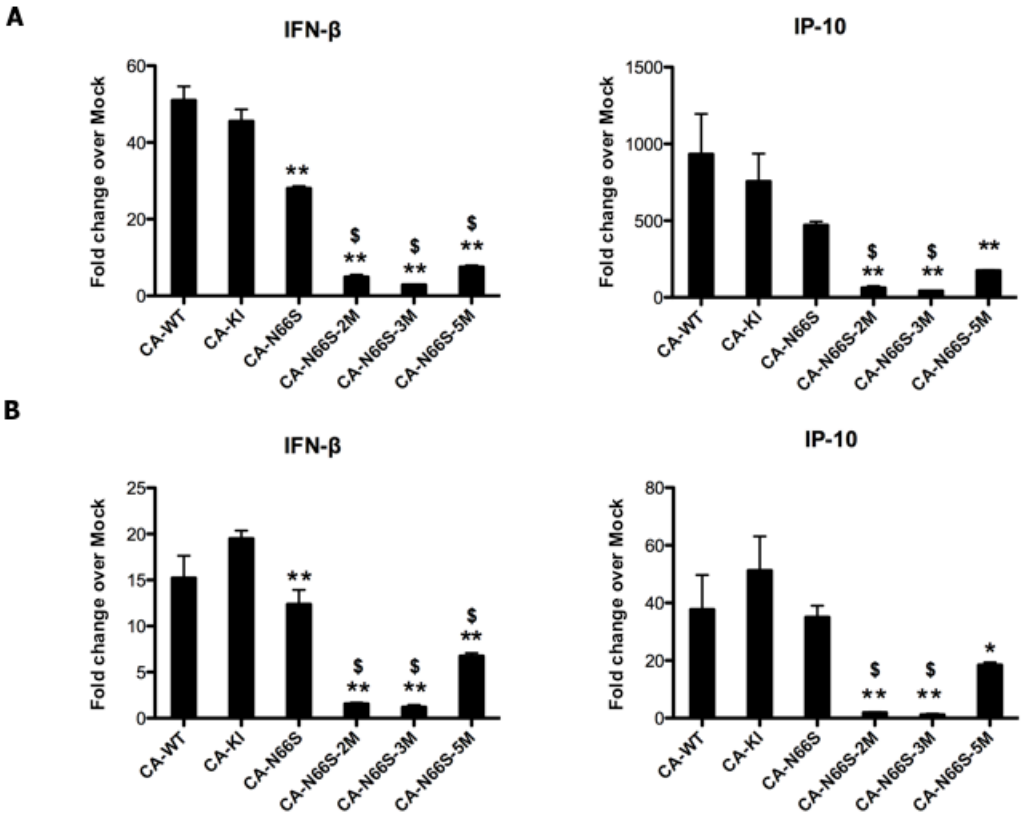


Figure 3.4: PB1-F2 C-terminal mutations down regulate IFN-β and IP-10. (A) THP-1 cells are differentiated with PMA (100ng/ml). After 48 h, cells were either infected with 1 MOI of indicated virus or mock infected with PBS. (B) A549 cells were either infected with 1 MOI of indicated virus or mock infected with PBS. Samples were collected 12 hpi from THP-1 and A549 cells. The mRNA transcripts for GAPDH, 18S, IFN-β, IP-10 were quantitated by real time RT-PCR using Syber Green dye. Mean fold change over mock +/- standard deviation (SD) from triplicate experiments are plotted. An asterisk (*) indicates a significant difference when compared with CA-KI (* = $p<0.05$; ** = $p<0.01$). A dollar ($) indicates a significant difference when compared with CA-N66S ($p<0.05$).

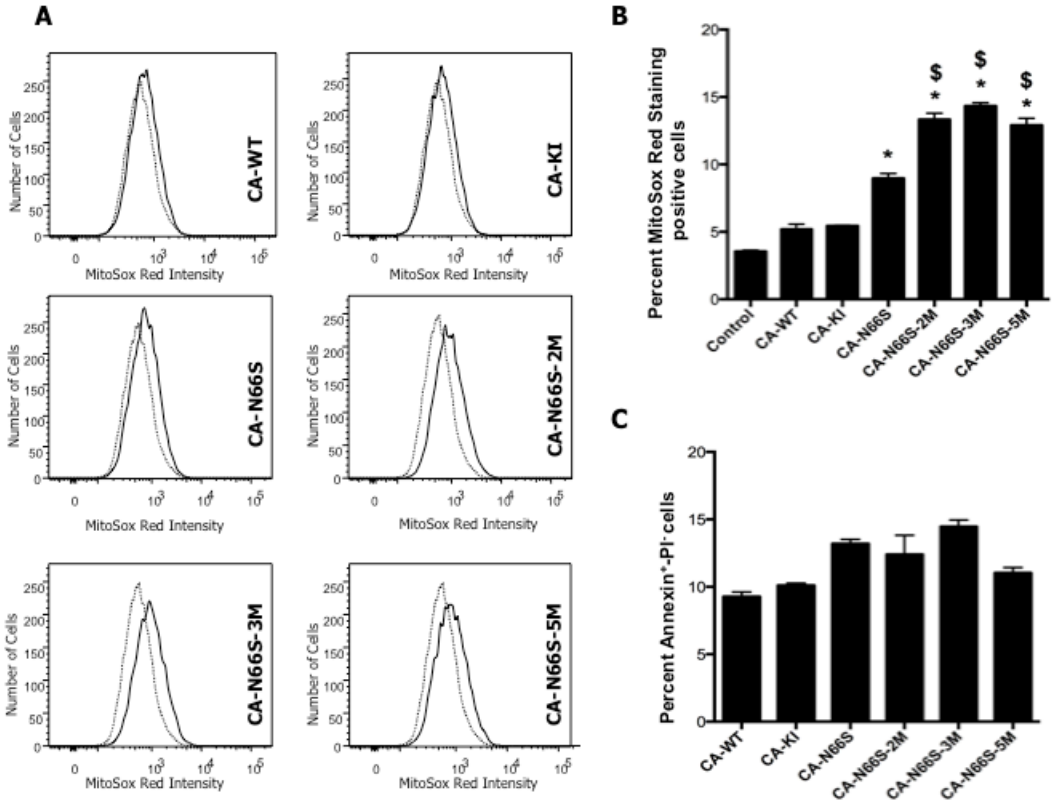


Figure 3.5: Mitochondrial superoxide production and apoptosis by PB1-F2 mutants. (A) A549 cells were infected with indicated recombinant CA/04 viruses at a multiplicity of infection (MOI) of 1. After 16 hpi at 37°C, cells were stained with MitoSOX red and analyzed by flow cytometry. Representative plots were shown. (B) Mean percentage of MitoSox red positive cells +/- standard deviation (SD) from triplicate experiments are displayed. An asterisk (*) indicates a significant difference when compared with CA-KI ($p<0.05$). A dollar ($) indicates a significant difference when compared with CA-N66S ($p<0.05$). (C) U937 cells were infected with the indicated recombinant CA/04 viruses (MOI 2). At 12 hpi, cells were stained with annexin V and propidium iodide (PI). Average percentage of apoptotic cells normalized over control +/- SD in triplicate experiments is shown.

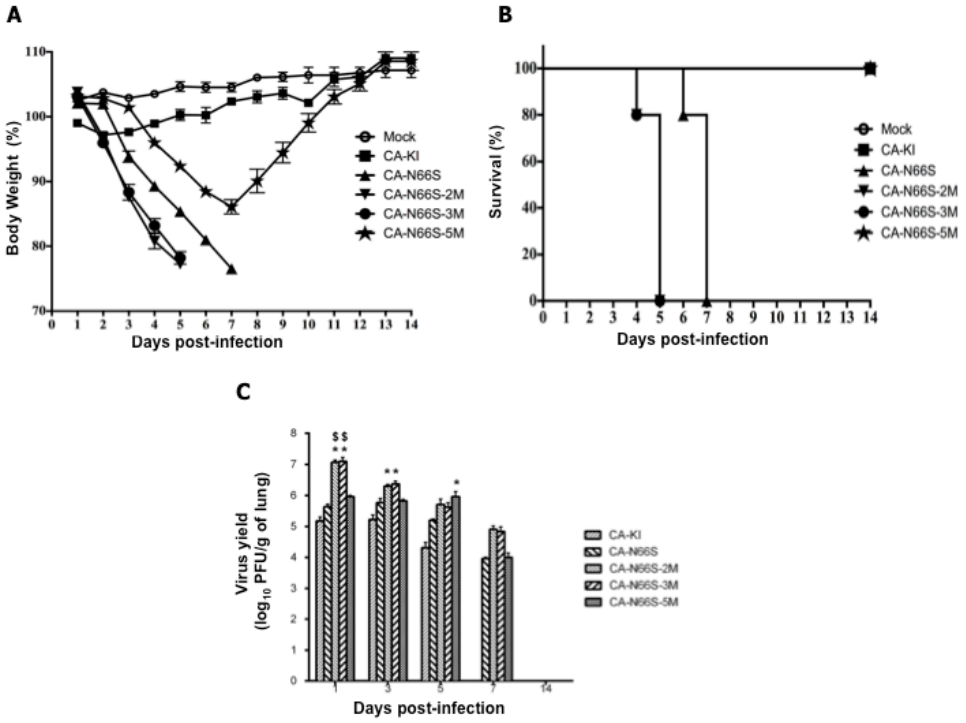


Figure 3.6: **Pathogenicity of CA/04 PB1-F2 mutant viruses in BALB/c mice**. (A). Eight-week-old female BALB/c mice (five animals/group) were infected via intranasal route with 10^5 PFU of the indicated recombinant CA/04 viruses. Following viral infection, mice were weighed daily, and the average body weights +/- standard deviations (SD) of mice in each group up to 14 dpi are shown as percentages of the original body weights. (B) Kaplan-Meier survival curve was plotted to show the percentage of mice that survived. (C) Eight-week-old female BALB/c mice were infected via intranasal route with 10^4 PFU of the indicated recombinant CA/04 viruses. On day 1, 3, 5, 7, and 14 dpi, three mice per group were sacrificed, and the virus titers in the lungs were determined from the lung supernatants. Average lung titers +/- SD is shown. An asterisk (*) indicates a significant difference compared to animals infected with CA-KI ($p<0.05$). A dollar ($) indicates a significant difference compared to animals infected with CA-N66S ($p<0.05$). Error bars indicate the standard deviation from the mean.

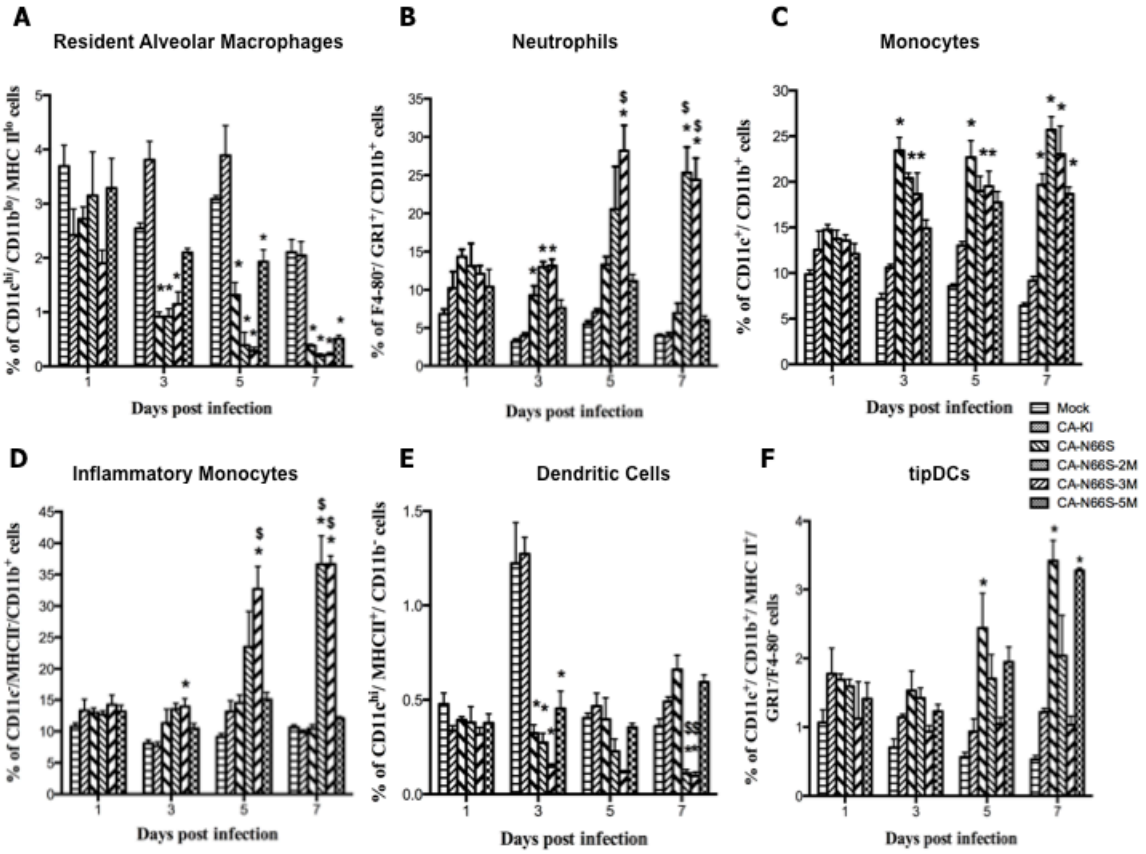
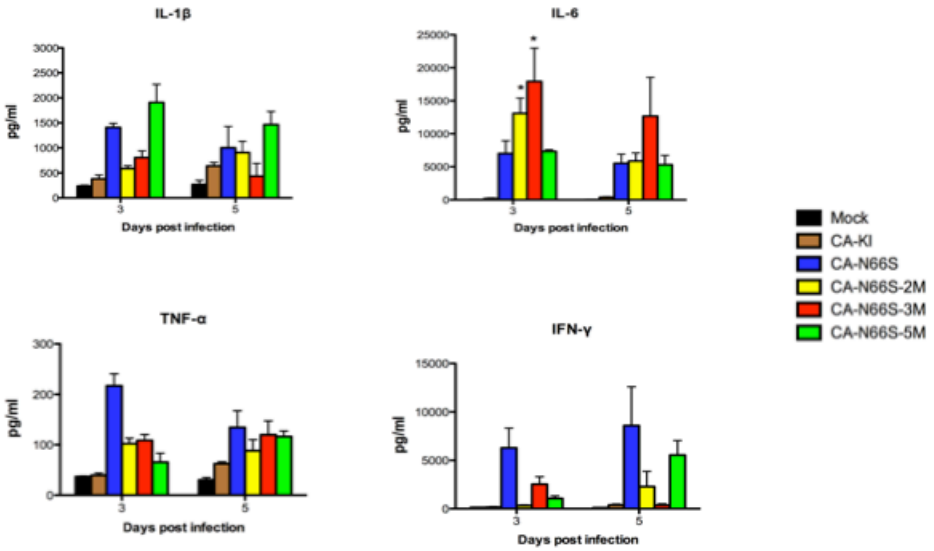
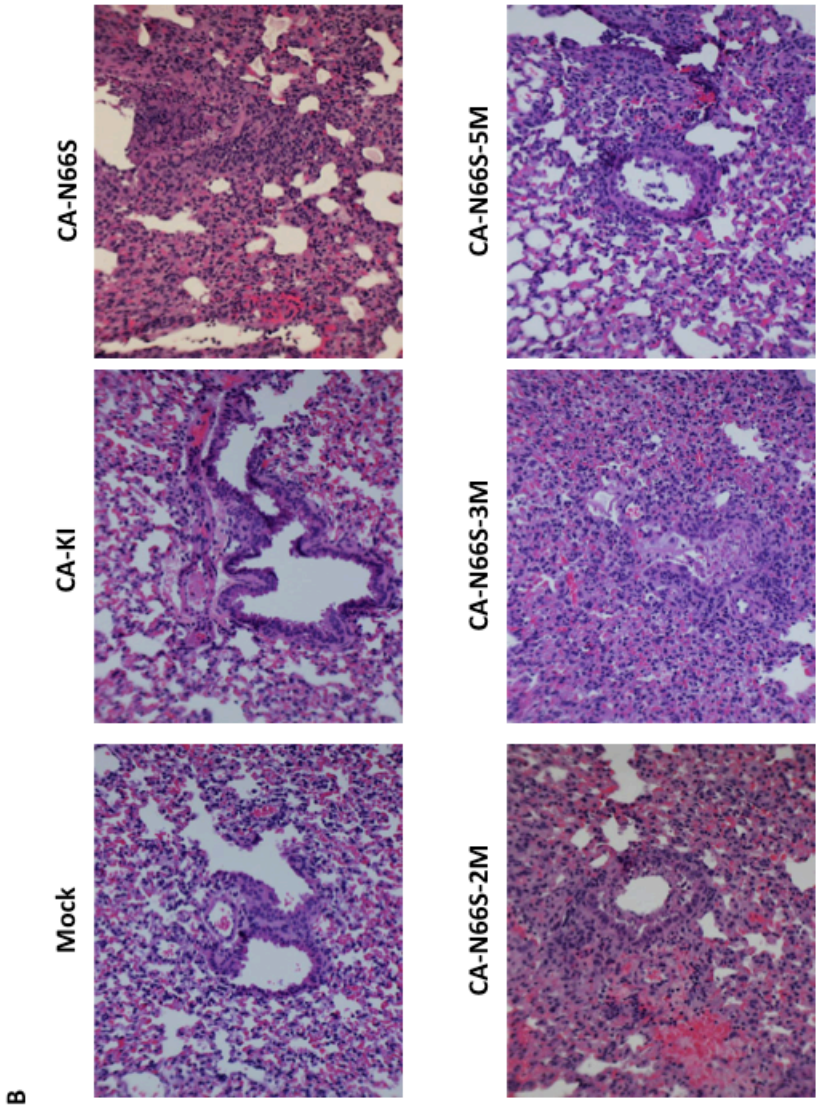


Figure 3.7: The C-terminal mutants of CA/04 PB1-F2 enhance lung inflammation. Groups of mice were exposed to recombinant CA/04 viruses as indicated and euthanized on day 1, 3, 5, 7, and 14 dpi. Lung samples were collected, treated enzymatically and assayed by flow-cytometry for the mean number of (A) resident alveolar macrophages, (B) neutrophils, (C) monocytes, (D) inflammatory monocytes, (E) dendritic cells, and (F) tipDCs. An asterisk (*) indicates a significant difference compared to animals infected with CA-KI ($p<0.05$). A dollar ($) indicates a significant difference compared to animals infected with CA-N66S ($p<0.05$). Error bars indicate the standard deviation from the mean.

A


IL-1β
pg/ml
Days post infection
IL-6
pg/ml
Days post infection
TNF-α
pg/ml
Days post infection
IFN-γ
pg/ml
Days post infection
Mock
CA-KI
CA-N66S
CA-N66S-2M
CA-N66S-3M
CA-N66S-5M


B
Mock
CA-KI
CA-N66S
CA-N66S-2M
CA-N66S-3M
CA-N66S-5M

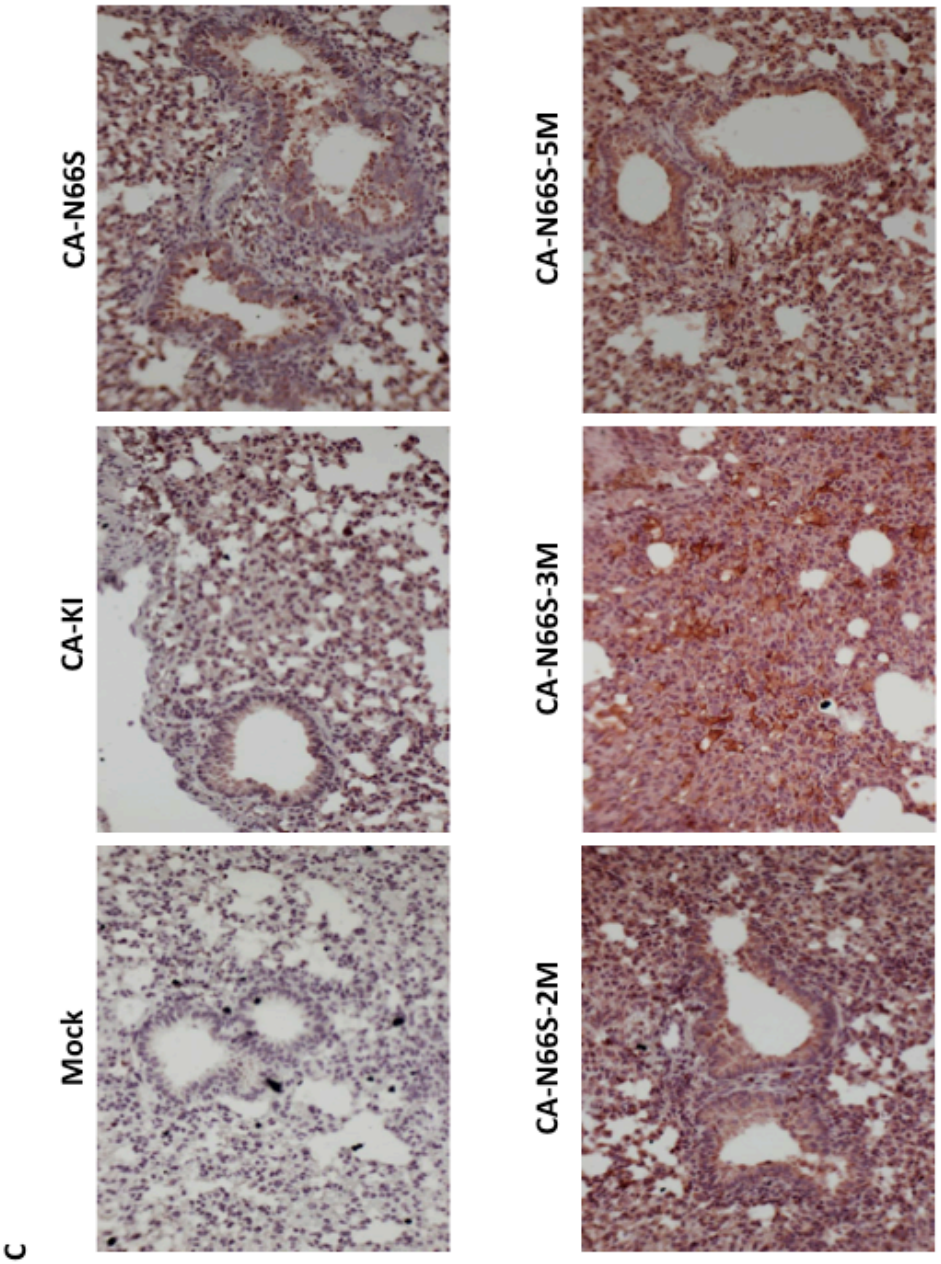

C
Mock
CA-KI
CA-N66S
CA-N66S-2M
CA-N66S-3M
CA-N66S-5M

Figure 3.8: C-terminal mutants of CA/04 PB1-F2 cause inflammation and lung pathology. (A) Groups of mice were exposed to indicated recombinant CA/04 viruses and euthanized on 1, 3, 5, and 7 dpi. Cytokine levels in the supernatants of lung homogenates were detected by multiplex ELISA at 3 and 5 dpi. Average cytokine levels +/- standard deviations are shown. An asterisk (*) indicates a significant difference compared to animals infected with CA-KI ($p<0.05$). (B) Moderate intra-alveolar hemorrhage with widespread infiltration of neutrophils and lymphocytes in the lungs of mice infected with CA-N66S, CA-N66S-2M and CA-N66S-3M when compared to mock infected and CA-KI infected mice. (C) Lungs were stained for neutrophil myeloperoxidase (MPO) release by immunohistochemistry. Note intense MPO staining in CA-N66S, CA-N66S-2M and CA-N66S-3M groups.

4. Influenza A Virus PB1-F2 Protein C-terminal Amino Acid Residues Interfere with Parkin-mediated Mitophagy and Enhance Accumulation of Damaged Mitochondria

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Manuscript in preparation

4.1 Abstract:

Mitochondria play an essential role in a number of cellular processes such as energy production, apoptosis and innate immune signaling. The sequestration and degradation of damaged mitochondria through a selective autophagy, known as mitophagy, is critical for maintaining cell viability. During influenza A virus (IAV) infection, oxidative stress results in significantly increased levels of reactive oxygen species (ROS) contributing to enhanced cell death and disease pathogenicity. However, the precise molecular mechanism of ROS-mediated pathogenicity during IAV infection is not yet fully understood. PB1-F2 is a small accessory IAV protein that predominantly localizes to mitochondria, interacts with mitochondrial proteins and causes depolarization of mitochondrial transmembrane potential. Here, we investigated the role of PB1-F2 in the selective removal of damaged mitochondria in IAV-infected lung epithelial cells. We found that recombinant 2009 H1N1 IAV PB1-F2 C-terminal amino acid residues (66S, 73K, 74T, 75R, 76V, and 79R) in combination block Parkin-mediated degradation of damaged mitochondria resulting in the accumulation of dysfunctional mitochondria in infected cells. Taken together, our results suggest that PB1-F2 protein inhibits mitophaghic degradation of damaged mitochondria in a sequence-specific manner, which may contribute to the increased pathogenicity of virulent IAV strains that possess these conserved C-terminal amino acid residues.

4.2 Introduction:

Mitochondria are essential cellular organelles that play a vital role in many important cellular functions such as energy production, cell death mechanism and innate immune signaling (Kubli et al, 2012). Influenza A virus (IAV) infection causes an

imbalance of cellular redox state resulting in the generation of reactive oxygen species (ROS). IAV-mediated oxidative stress plays an essential role in pathogenesis of IAV in respiratory cells and tissues (Akaike et al, 1996; Bove et al, 2006). Accumulation of ROS can trigger collapse of mitochondrial transmembrane potential and damage mitochondria (Kim et al, 2013). Dysfunctional mitochondria are a major source of ROS triggering a vicious cycle of subsequent damage to healthy mitochondria culminating in cell death (Kurihara et al, 2012). Mitophagy, an organelle specific form of autophagy, mediates the selective removal of damaged mitochondria (Bhatia-Kissova et al, 2012). Mitophagy acts as quality control process thereby dysfunctional mitochondria are selectively engulfed for subsequent lysosomal degradation (Taylor et al, 2011; Ding et al, 2012; Jin et al, 2013).

The phosphatase and tensin homolog-induced putative kinase 1 (PINK1)/Parkin pathway plays an important role in regulating mitophagy (Kubli et al, 2012). Under normal condition, PINK1 are regularly cleaved by mitochondrial proteases and found at very low levels in mitochondria. Parkin is an E3 ubiquitin ligase, which is predominantly present in the cytoplasm under basal conditions. Damage to mitochondria and subsequent loss of mitochondrial transmembrane potential leads to the accumulation of PINK1 on the outer mitochondrial membrane and subsequent translocation of Parkin to mitochondria (Narendra et al, 2008; Suen et al, 2010; Narendra et al, 2010). This process promotes ubiquitination of broad range of mitochondrial proteins such as mitofusin (Mfn) 1 & 2 and voltage dependent anion channel 1 (VDAC1) (Geisler et al, 2010; Gegg et al, 2010; Poole et al, 2010). Ubiquitination of mitochondrial proteins serve as a signal for autophagic degradation of impaired mitochondria (Pankiv et al, 2007; Kirkin et al, 2009). The proteolytic cleavage of microtubule-associated light chain 3 (LC3) protein (LC3-I)

and its lipidation with phosphatidylethanolamine (LC3-PE or LC3-II) is essential for autophagosome formation around damaged mitochondria (Kroemer et al, 2010). Autophagy adaptor protein Sequestosome 1 (SQSTM1/p62) binds to LC3 on the autophagosome via its LC3-interacting domain and to ubiquitinated proteins via its ubiquitin-associated region (Seibenhener et al 2004; Pankiv et al, 2007). Binding of SQSTM1 to ubiquitinated mitochondrial proteins tethers the damaged mitochondria to the LC3-positive autophagosome for engulfment and subsequent lysosomal degradation (Kubli et al, 2012).

Given the central importance of mitophagy to host defense against cell death, it is imperative to explore how IAV viral modulates mitophagy. Deletion of receptor interacting kinase 2 (RIPK-2) results in impaired mitophagy and accumulation of damaged mitochondria (Lupfer et al, 2013). Until now, the role of different IAV viral proteins in mitophagy has never been explored. PB1-F2 protein is an accessory protein encoded by alternative +1 open reading frame of IAV PB1 gene segment. It predominantly localizes to mitochondria in IAV infected cells (Chen et al, 2001). It has been shown that PB1-F2 protein self-oligomerizes resulting in the formation of non-selective protein pores in planar lipid membranes. These non-selective pores disrupt inner mitochondrial membrane (IMM) and depolarize mitochondrial transmembrane potential (Chanturiya et al, 2004; Henkel et 2010). PB1-F2 protein also indirectly interacts with mitochondrial membrane proteins such as adenine nucleotide translocator 3 (ANT3) and VDAC-1 present on mitochondrial membranes. This interaction leads to the formation of the permeability-transition pore complex (PTPC), which plays an important role in the permeabilization of the mitochondria (Zamarin et al, 2005; Danishuddin et al, 2010). So

far, it is unknown whether mitochondrial targeting and transmembrane potential depolarization property of PB1-F2 modulates the process of mitophagy. We hypothesized that PB1-F2 protein may interfere with mitophagy in a sequence specific manner. We found that in pandemic H1N1 backbone, PB1-F2 specific C-terminal amino acid residues induce mitochondrial damage resulting in ROS production and promote parkin-mediated mitophagy but suppress the autophagic degradation of damaged mitochondria in the infected epithelial cells.

4.3 Materials and Methods:

Cells, Viruses and Reagents:

Human alveolar basal epithelial (A549) cells was obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen), supplemented with 10% fetal calf serum (Thermo Scientific) and 1% penicillin-streptomycin (Invitrogen). Porcine kidney epithelial (PK-15) cells stably transfected with green fluorescent (GFP)-LC3 was a kind gift from Dr. X. J. Meng, VirginiaTech. Protease inhibitors (pepstatin and E64d), rapamycin and 3-methylyadenine (3-MA) were purchased from Santa Cruz, whereas carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was purchased from Sigma-Aldrich.

Construction of plasmids:

The IAV strain used in this study is A/California/04/2009 (H1N1) (CA/04), a kind gift from Dr. Terence Tumpey, CDC, Atlanta. All the eight gene segments of CA/04 were cloned into pHW2000 vector. The CA/04-PB1 plasmid was subjected to three rounds of site-directed mutagenesis using QuickChange II site-directed mutagenesis kit (Agilent Technologies). The stop codons in the open reading frame of PB1-F2 at positions 12, 58,

and 88 were modified to code for serine, tryptophan, and tryptophan, respectively to create CA/04 Knock-in (CA-KI) PB1 plasmid without affecting the reading frame of PB1 gene. Asparagine is changed to serine at position 66 for creating CA-KI-66S plasmid. For obtaining additional C-terminal mutants, arginine, isoleucine, histidine, alanine and glutamine at positions 73, 74, 75, 76, and 79 of PB1-F2 were changed to lysine, threonine, arginine, valine, and arginine, respectively in the plasmid CA-KI-66S. The sequences of each construct were confirmed by automated sequencing performed at Virginia Bioinformatics Institute (VBI) core sequencing facility.

Rescue of recombinant influenza A viruses:

Co-cultured MDCK and 293T cells were transfected with eight plasmids containing CA/04 viral genome segments to recover recombinant IAV designated CA-KI, CA-N66S, CA-N66S-3M, and CA-N66S-5M (Hoffmann et al, 2000). The rescued viruses were plaque purified on MDCK cells and propagated once in 10-day old embryonated specific pathogen free (SPF) chicken eggs. The presence of the introduced mutations in the PB1 segments was confirmed by sequencing the viral genomic RNA (vRNA). The virus stocks were titrated and stored at -80°C until used.

Electron Microscopy:

A549 cells were infected with PB1-F2 recombinant viruses or mock infected at a multiplicity of infection (MOI) of 1. After 24 hours (h), cells were harvested and fixed with 2.5% gluteraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h in room temperature. Cell pellets were then post-fixed with 2% osmium tetraoxide for 2 h and dehydrated with an acetone series. Ultrathin sections of cells were placed on Formvar-carbon-coated electron microscope grids (Electron Microscopy

Sciences) and negatively stained with 1% sodium phosphotungstic acid for 30 seconds. JEOL 1400 transmission electron microscope (JOEL) at an accelerating voltage of 80kVA was used to examine the morphologic characteristics of infected cells.

LC3 puncta:

PK-15 cells stably transfected with GFP-LC3 were grown in 8-well chamber slides (Sigma-Aldrich) and infected with recombinant PB1-F2 C-terminal mutant viruses at a MOI of 1. After 24 h post infection (hpi), cells were fixed and permeabilized with ice-cold 1:1 acetone: methanol for 10 minutes (min) at -20 °C. Cells were blocked with 5% bovine serum albumin (BSA) for 30 min and then washed 3x with phosphate buffered saline (PBS). Cells were mounted using a mounting medium containing DAPI (Vector Laboratories) and then visualized under a 100x oil immersion objective of Eclipse TE2000-PFS inverted microscope (Nikon Instruments).

Western blot analysis:

For immunoblotting, whole cell lysates were harvested from recombinant viruses or mock-infected A549 cells at 24 hpi and lysed with 100 µl of radioimmunoprecipitation assay (RIPA) lysis buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) for 30 min at 4 °C. The supernatants were then collected by centrifugation at 10,000 x g for 30 min at 4 °C. Total proteins were quantified using the Bradford assay (Bio-Rad). Equal amounts of protein were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) and electrophoretically transferred on to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). After blocking with 5% BSA, the blot was incubated with a rabbit anti-LC3-II (Sigma-Aldrich), a mouse anti-parkin (Cell Signaling), a rabbit anti-SQSTM1/p62 (Cell

Signaling), or a goat anti-actin antibody (Santa Cruz). After incubation with the primary antibody, the blot was washed and incubated with respective HRP-conjugated anti-IgG secondary antibodies (Santa Cruz) and images were taken using myECL imager (Thermo Scientific).

Statistical Analysis:

Quantitative values from triplicate experiments are expressed as mean ± standard deviation. Student's t-test was used to analyze data sets. $P < 0.01$ was considered significant. Statistical analysis was done with the help of JMP (JMP Software, SAS Institute) and the graphs were prepared using GraphPad Prism 5 (GraphPad Software).

4.4 Results:

PB1-F2 C-terminal Mutations induce autophagosomes with accumulation of damaged mitochondria:

PB1-F2 C-terminal mutant viruses triggered an increased mitochondrial superoxide production in respiratory epithelial cells (Chapter 3). We used transmission electron microscopy to observe the mitochondrial damage in A549 cells infected with PB1-F2 recombinant viruses. Significant accumulation of damaged mitochondria and numerous large, double-membrane autophaghic vacuoles indicating autophagosome formation were found in cells infected with CA-N66S, CA-N66S-3M, and CA-N66S-5M when compared to CA-KI virus and mock-infected control (Figure 4.1).

Conversion ratio of LC3-II/LC3-I is regarded as an accurate indicator of autophagosome formation (Kudchodkar and Levine, 2009). We performed Western blot analysis of A549 cells infected with PB1-F2 recombinant viruses to confirm this. Significantly enhanced conversion ratio of LC3-II/LC3-I in CA-N66S, CA-N66S-3M and

CA-N66S-5M mutant viruses was observed than in CA-KI (Figure 4.2). Cells stably transfected with GFP-LC3 can act as a fluorescent marker of autophagosome formation (Mizushima et al, 2004). The CA-N66S, CA-N66S-3M and CA-N66S-5M mutant virus infection showed increased accumulation of GFP-LC3 puncta when compared with CA-KI virus- and mock-infected PK-15 cells (Figure 4.3). Taken together, these results demonstrate that PB1-F2 C-terminal mutations significantly enhance mitochondrial damage and induce autophagosome formation in respiratory epithelial cells.

PB1-F2 C-terminal Mutations promote Parkin-dependent type-II Mitophagy:

Parkin translocates to damaged mitochondria and initiates the process of the mitophagy (Youle et al, 2011). Hence, we next examined whether there are any differences in the expression of Parkin between PB1-F2 recombinant viruses. Lysates collected from A549 cells infected with PB1-F2 recombinant viruses were probed for Parkin protein levels via immunoblotting. CCCP, a potent mitochondrial oxidative phosphorylation uncoupler is used as a positive control. CA-N66S-3M and CA-N66S-5M mutant viruses induced significantly higher levels of Parkin when compared to CA-KI and CA-N66S (Figure 4.4A). These results suggest that PB1-F2 C-terminal mutations initiate mitophagy inside infected epithelial cells.

In order to distinguish between type I and type II mitophagy, we treated PB1-F2 recombinant virus infected epithelial cells with 3-MA that inhibits phagophore formation (Seglen et al, 1982). 3-MA, a phosphatidylinositol 3-kinase (PI3K) inhibitor, blocks Beclin 1 mediated type I mitophagy (Lemasters, 2014). In the presence of 3-MA, CA-KI and CA-N66S virus infected cells displayed lower levels of LC3-II protein expression. In contrast, CA-N66S-3M and CA-N66S-5M infected cells showed significantly increased

LC3-II protein accumulation (Figure 4.4B). This shows that PB1-F2 C-terminal mutations induce Parkin-mediated type-II mitophagy independent of Beclin 1.

PB1-F2 C-terminal mutations inhibit autophagic degradation of damaged mitochondria:

Next, we analyzed the levels of p62 protein in A549 cells infected with PB1-F2 recombinant viruses. SQSTM1/p62 recruits ubiquitinated cargo into autophagosomes by binding to LC3-II (Geisler et al, 2010). After the fusion of autophagosomes with lysosomes, p62 gets degraded indicating the completion of autophagy process (Bjorkoy et al, 2005). Hence, accumulation of p62 inside host cell is associated with a deficit in autophagic degradation activity (Hara et al, 2014). A549 cells were infected with PB1-F2 recombinant viruses and the levels of p62 protein were analyzed at 24 hpi by immunoblotting. CA-N66S-3M and CA-N66S-5M showed greater accumulation of p62 when compared with CA-KI and CA-N66S mutant viruses (Figure 4.4C). These results suggest that the PB1-F2 C-terminal mutants suppress autophagic degradation of damaged mitochondria that may lead to the accumulation of damaged mitochondria in the infected cells.

4.5 Discussion:

Mammalian mitochondria form a highly dynamic reticular network, which undergo cycles of fission and fusion to preserve proper mitochondrial function (Scott et al, 2010). Imbalance in the fission/fusion equilibrium or mitochondrial damage can lead to selective removal of mitochondria by mitophagy (Twig et al, 2008). Damaged mitochondria have detrimental effect on cellular homeostasis and host immune system function (Lazarou et al, 2015). In order to prevent apoptosis, impaired mitochondria are

sequestered selectively and degraded via mitophagy (Youle et al, 2011). Viral pathogens have developed strategies to overcome this important host defense mechanism against cell death. In a series of experiments, Kim et al. showed that hepatitis B and hepatitis C viruses subvert mitophagy for virus replication and also to promote viral persistence in infected hepatocytes (Kim et al, 2013; Kim et al, 2014). Here, for the first time, we demonstrated that PB1-F2 inhibits Parkin-dependent mitophaghic removal of damaged mitochondria resulting in the increased accumulation of dysfunctional mitochondria in IAV-infected cells. The ion channel protein M2 has been shown to block autophagosome fusion with lysosomes resulting in enhanced apoptosis (Gannage et al, 2009; Rossman et al, 2009). It was also shown that M2 via its LC3 interacting motif relocalizes LC3 to the plasma membrane of IAV-infected cells at the time of virus budding and thereby facilitates viral replication (Beale et al, 2014). Incidentally, PB1-F2 protein also induces apoptosis and enhances viral replication in a sequence specific manner (Chakrabarti et al, 2013). It would be interesting to study whether M2 and PB1-F2 protein act in concert or individually. Interestingly, PB1-F2 proteins of neither low pathogenic nor lethal IAV strains possess a LC3 interacting motif or region.

Translocation of Parkin to mitochondria is considered a hallmark of mitophagy (Youle et al, 2011). We found a significant stimulation of Parkin in cells infected with PB1-F2 C-terminal mutants. PI3K inhibitor inhibits Beclin 1/PI3K pathway and can thus be used to distinguish nutrient deprivation-mediated type-I mitophagy from mitochondrial depolarization-induced type II mitophagy (Lemasters, 2014). Here, we show that PB1-F2 C-terminal mutants increased LC3-II accumulation in the presence of 3-MA. This indicates that PB1-F2 induces depolarization-induced type II mitophagy

independent of Beclin 1. PI3K-independent pathway of mitochondrial damage-induced mitophagy has been described in neuronal cell death and neurodegeneration (Chu et al, 2007).

SQSTM1/p62 contains both ubiquitin-associated region and LC3-interacting domain. It is recruited to mitochondria in a Parkin-dependent manner. Small interfering RNA (siRNA) mediated knockdown of p62 substantially inhibits mitophagy (Geisler et al, 2010; Ding et al, 2010). Here, we found that PB1-F2 C-terminal mutant virus infection results in an enhanced accumulation of damaged mitochondria indicating suppression of autophagic degradation of these mitochondria. Interestingly, PB1-F2 C-terminal mutants used in this study contain proposed inflammatory motifs (Alymova et al, 2011) and show enhanced immunopathology and acute lung injury in a mouse model (Chapter 3). It is intriguing how a specific constellation of amino acids commonly conserved in the PB1-F2 protein of lethal IAV strains evades mitophagy. A recent study suggested NOD2-RIPK-2 signaling protected against IAV-induced immunopathology by enhancing ULK-1 dependent mitophagy (Lupfer et al, 2013). NLRP3 inflammasome can sense dysfunctional mitochondria and trigger inflammatory responses within the host (Ding and Yin, 2012). Accumulation of damaged mitochondria in the absence of RIPK-2 leads to increased ROS production, activation of NLRP3 inflammasome, IL-18 induction culminating in enhanced lung immunopathology (Lupfer et al, 2013). Therefore, evasion of mitophagy could lead to enhanced immunopathology as seen with these mutations in a mouse . Interestingly, these C-terminal mutations also enhanced virus replication suggesting there could be cross talk between innate immune signaling and mitophagic pathways. However, the mechanism by which PB1-F2 protein prevents the interaction of

Parkin and LC3-II or NOD2-RIPK-2 pathway to interfere with mitophagy is unclear. With the help of protein homology recognition engine (Phyre) algorithm protein (Kelly et al, 2015), we identified that introduction of specific lethality associated amino acid residues in the PB1-F2 protein of 2009 pandemic H1N1 virus creates a F-Box domain in the protein. F-box domains are linked to the Skp1 protein and the core of Skp1-cullin-F-box protein ligase (SCF) complexes. SCF complexes constitute a new class of E3 ligases (Jia et al, 2011). It is tempting to speculate that through F-box domain interactions with SCF complexes, PB1-F2 may promote the degradation of key proteins involved in mitophagy such as RIPK-2 and Drp1.

Kim et al. recently reported that hepatitis C virus core protein interacts with the Parkin and inhibits its translocation to the damaged mitochondria. This interaction prevents the Parkin-mediated ubiquitination of mitochondrial proteins such as Mfn 1, Mfn 2 and VDAC1 and subsequent mitophagy (Kim et al, 2014). PB1-F2 protein was reported to interact indirectly with VDAC-1 present on mitochondrial membranes (Zamarin et al, 2005). VDAC-1 interacts with PINK1-Parkin complex. Therefore, it is possible that interaction of PB1-F2 with mitochondrial proteins inhibits translocation of Parkin to the mitochondria resulting in the failure of mitochondrial ubiquitination and autophagic degradation of mitochondria. Accumulation of cytosolic Parkin in infected cells lends credence to this supposition. Additional studies may provide insights into these mechanisms.

In conclusion, our results indicate that PB1-F2 protein induces mitochondrial damage and promotes Parkin-mediated mitophagy but suppresses the autophagic degradation of damaged mitochondria in a sequence dependent manner. Accumulated

dysfunctional mitochondria can aggravate host cell death and inflammatory responses. These findings provide an impetus to our understanding of host pathogenesis of virulent influenza strains through its multifunctional PB1-F2 protein.

4.6 References:

1. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, et al. (1996) Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. Proc Natl Acad Sci U S A 93: 2448-2453.
2. Alymova IV, Green AM, van de Velde N, McAuley JL, Boyd KL, et al. (2011) Immunopathogenic and antibacterial effects of H3N2 influenza A virus PB1-F2 map to amino acid residues 62, 75, 79, and 82. J Virol 85: 12324-12333.
3. Beale R, Wise H, Stuart A, Ravenhill BJ, Digard P, et al. (2014) A LC3-interacting motif in the influenza A virus M2 protein is required to subvert autophagy and maintain virion stability. Cell Host Microbe 15: 239-247.
4. Bhatia-Kissova I, Camougrand N (2013) Mitophagy: a process that adapts to the cell physiology. Int J Biochem Cell Biol 45: 30-33.
5. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, et al. (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171: 603-614.
6. Bove MJ, Kansal S, Rosen CA (2008) Influenza and the vocal performer: update on prevention and treatment. J Voice 22: 326-332.

7. Chakrabarti AK, Pasricha G (2013) An insight into the PB1F2 protein and its multifunctional role in enhancing the pathogenicity of the influenza A viruses. Virology 440: 97-104.
8. Chanturiya AN, Basanez G, Schubert U, Henklein P, Yewdell JW, et al. (2004) PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. J Virol 78: 6304-6312.
9. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306-1312.
10. Danishuddin M, Khan SN, Khan AU (2010) Molecular interactions between mitochondrial membrane proteins and the C-terminal domain of PB1-F2: an in silico approach. J Mol Model 16: 535-541.
11. Ding WX, Ni HM, Li M, Liao Y, Chen X, et al. (2010) Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. J Biol Chem 285: 27879-27890.
12. Ding WX, Yin XM (2012) Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem 393: 547-564.
13. Gannage M, Dormann D, Albrecht R, Dengjel J, Torossi T, et al. (2009) Matrix protein 2 of influenza A virus blocks autophagosome fusion with lysosomes. Cell Host Microbe 6: 367-380.

14. Gegg ME, Cooper JM, Chau KY, Rojo M, Schapira AH, et al. (2010) Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum Mol Genet 19: 4861-4870.
15. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, et al. (2010) PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat Cell Biol 12: 119-131.
16. Hara Y, Yanatori I, Ikeda M, Kiyokage E, Nishina S, et al. (2014) Hepatitis C virus core protein suppresses mitophagy by interacting with parkin in the context of mitochondrial depolarization. Am J Pathol 184: 3026-3039.
17. Henkel M, Mitzner D, Henklein P, Meyer-Almes FJ, Moroni A, et al. (2010) The proapoptotic influenza A virus protein PB1-F2 forms a nonselective ion channel. PLoS One 5: e11112.
18. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 97: 6108-6113
19. Jia L, Sun Y (2011) SCF E3 ubiquitin ligases as anticancer targets. Curr Cancer Drug Targets 11: 347-356.
20. Jin M, Liu X, Klionsky DJ (2013) SnapShot: Selective autophagy. Cell 152: 368-368 e362.
21. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015) The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 10: 845-858.

22. Kim SJ, Khan M, Quan J, Till A, Subramani S, et al. (2013) Hepatitis B virus disrupts mitochondrial dynamics: induces fission and mitophagy to attenuate apoptosis. PLoS Pathog 9: e1003722.
23. Kim SJ, Syed GH, Siddiqui A (2013) Hepatitis C virus induces the mitochondrial translocation of Parkin and subsequent mitophagy. PLoS Pathog 9: e1003285.
24. Kirkin V, McEwan DG, Novak I, Dikic I (2009) A role for ubiquitin in selective autophagy. Mol Cell 34: 259-269.
25. Kubli DA, Gustafsson AB (2012) Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res 111: 1208-1221.
26. Kudchodkar SB, Levine B (2009) Viruses and autophagy. Rev Med Virol 19: 359-378.
27. Kurihara Y, Kanki T, Aoki Y, Hirota Y, Saigusa T, et al. (2012) Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. J Biol Chem 287: 3265-3272.
28. Lazarou M (2015) Keeping the immune system in check: a role for mitophagy. Immunol Cell Biol 93: 3-10.
29. Lemasters JJ (2014) Variants of mitochondrial autophagy: Types 1 and 2 mitophagy and micromitophagy (Type 3). Redox Biol 2: 749-754.
30. Lupfer C, Thomas PG, Anand PK, Vogel P, Milasta S, et al. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. Nat Immunol 14: 480-488.

31. Mizushima N (2004) Methods for monitoring autophagy. Int J Biochem Cell Biol 36: 2491-2502.

32. Narendra D, Tanaka A, Suen DF, Youle RJ (2008) Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J Cell Biol 183: 795-803.

33. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, et al. (2010) PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8: e1000298.

34. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, et al. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131-24145.

35. Poole AC, Thomas RE, Yu S, Vincow ES, Pallanck L (2010) The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. PLoS One 5: e10054.

36. Rossman JS, Lamb RA (2009) Autophagy, apoptosis, and the influenza virus M2 protein. Cell Host Microbe 6: 299-300.

37. Scott I, Youle RJ (2010) Mitochondrial fission and fusion. Essays Biochem 47: 85-98.

38. Seglen PO, Gordon PB (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci U S A 79: 1889-1892.

39. Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, et al. (2004) Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 24: 8055-8068.

40. Suen DF, Narendra DP, Tanaka A, Manfredi G, Youle RJ (2010) Parkin overexpression selects against a deleterious mtDNA mutation in heteroplasmic cybrid cells. Proc Natl Acad Sci U S A 107: 11835-11840.
41. Taylor R, Goldman SJ (2011) Mitophagy and disease: new avenues for pharmacological intervention. Curr Pharm Des 17: 2056-2073.
42. Twig G, Hyde B, Shirihai OS (2008) Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta 1777: 1092-1097.
43. Youle RJ, Narendra DP (2011) Mechanisms of mitophagy. Nat Rev Mol Cell Biol 12: 9-14.
44. Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P (2005) Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog 1: e4.

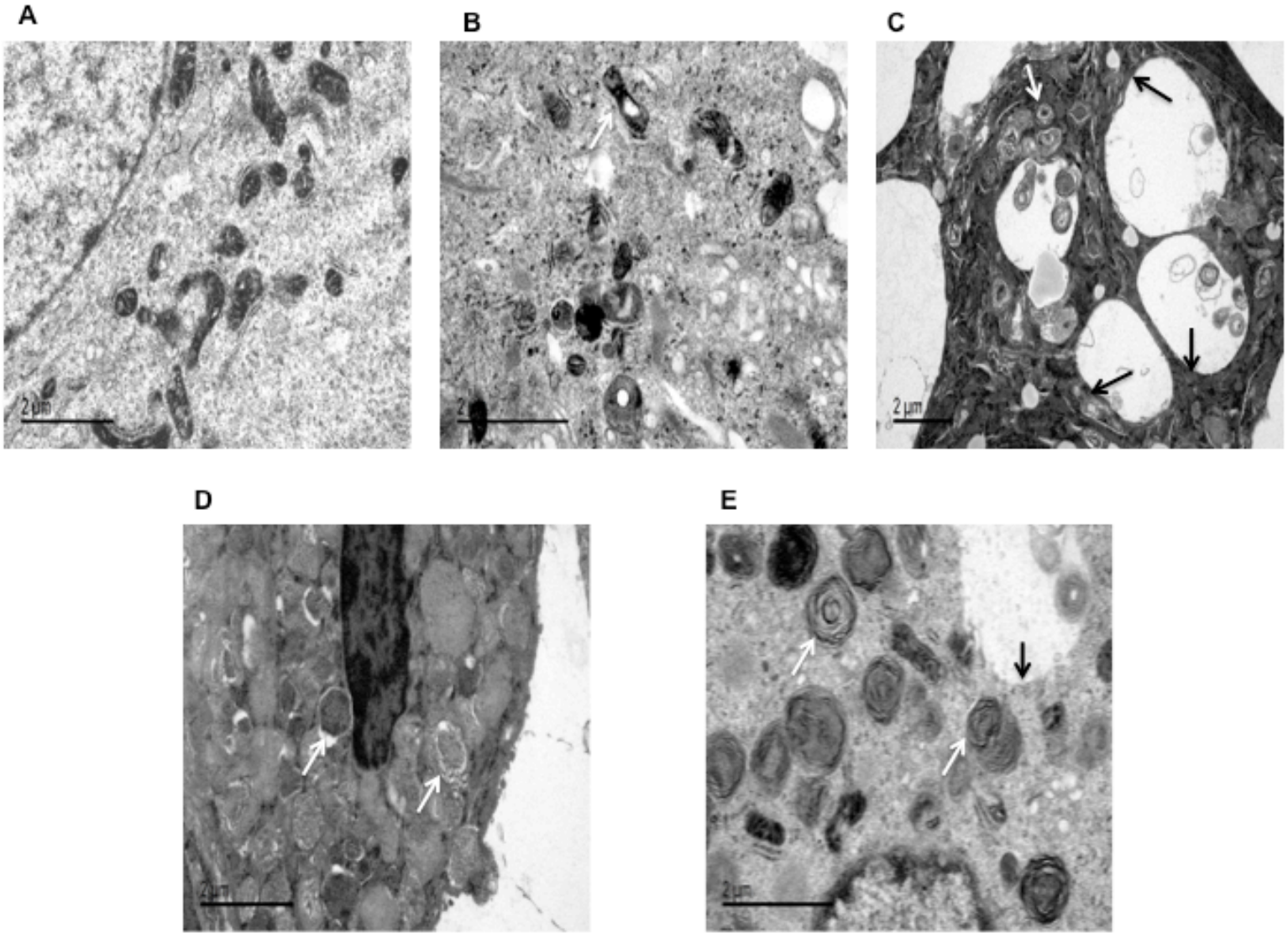
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Figure 4.1: Ultrastructural morphology of PB1-F2 C-terminal mutant virus infected cells showing accumulation of autophagosomes with damaged mitochondria. Mock-infected or PB1-F2 recombinant virus-infected cells were processed to examine morphologic characteristics of the cell via electron microscopy. Scale bar, 2 uM. Representative images were shown: (A) Mock, (B) CA-KI, (C) CA-N66S, (D) CA-N66S-3M, (E) CA-N66S-5M. Black arrows indicate autophagosomes and white arrows indicate damaged mitochondria.

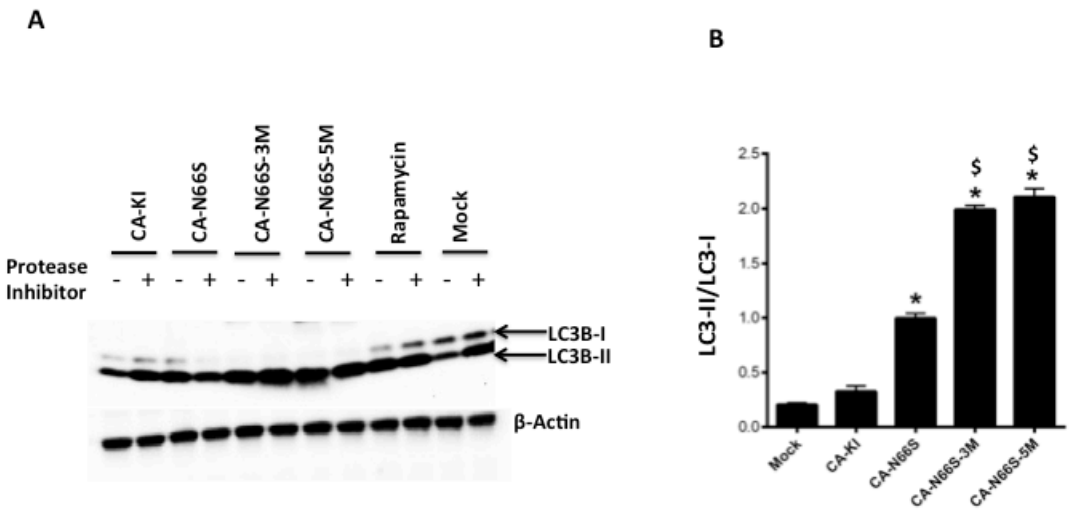

Figure 4.2: PB1-F2 C-terminal mutant viruses increases autophagosomes formation. A549 cells were mock-infected or infected with PB1-F2 recombinant viruses, At 24 hpi, whole cell lysates were collected and analyzed by immunoblotting using rabbit anti-LC3B and goat anti-actin antibodies. Pepstatin and E64d were used as protease inhibitors at the concentration of 10 uM. Rapamycin treatment at 5 uM concentration was used as positive control. β-Actin was used as an internal loading control. (A) Immunoblots for LC3-II (B) The intensity of the band was quantified using ImageJ and the fold change of LC3-II is calculated as the ratio of the intensity of LC3-II to LC3- I band normalized to that of actin. An asterisk (*) indicates a significant difference when compared with CA-KI ($p<0.05$). A dollar ($) indicates a significant difference when compared with CA-N66S ($p<0.05$).

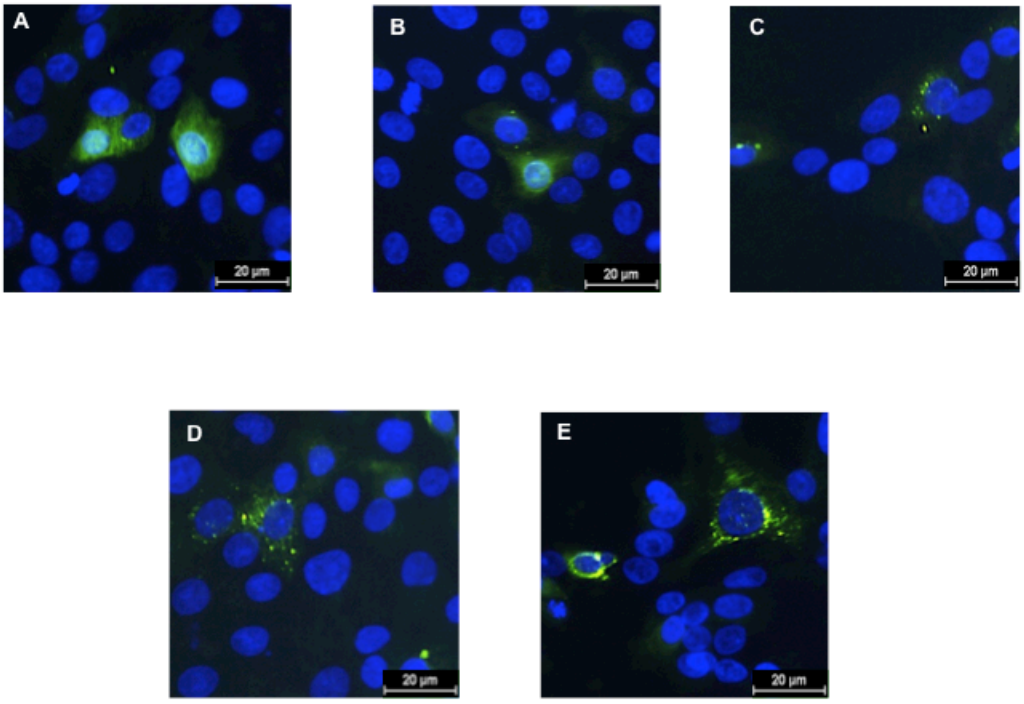

Figure 4.3: PB1-F2 C-terminal mutant viruses induces LC3 puncta formation. PK-15 stably transfected with GFP-LC3 cells were mock-infected or infected with respective recombinant PB1-F2 viruses (MOI, 1) for 24 h. The cells were then processed for the analysis of GFP-LC3 puncta formation using immunofluorescence microscopy. DAPI (blue) was used to stain nuclear DNA. Scale bar, 20 uM. Representative images were shown: (A) Mock, (B) CA-KI, (C) CA-N66S, (D) CA-N66S-3M, (E) CA-N66S-5M.

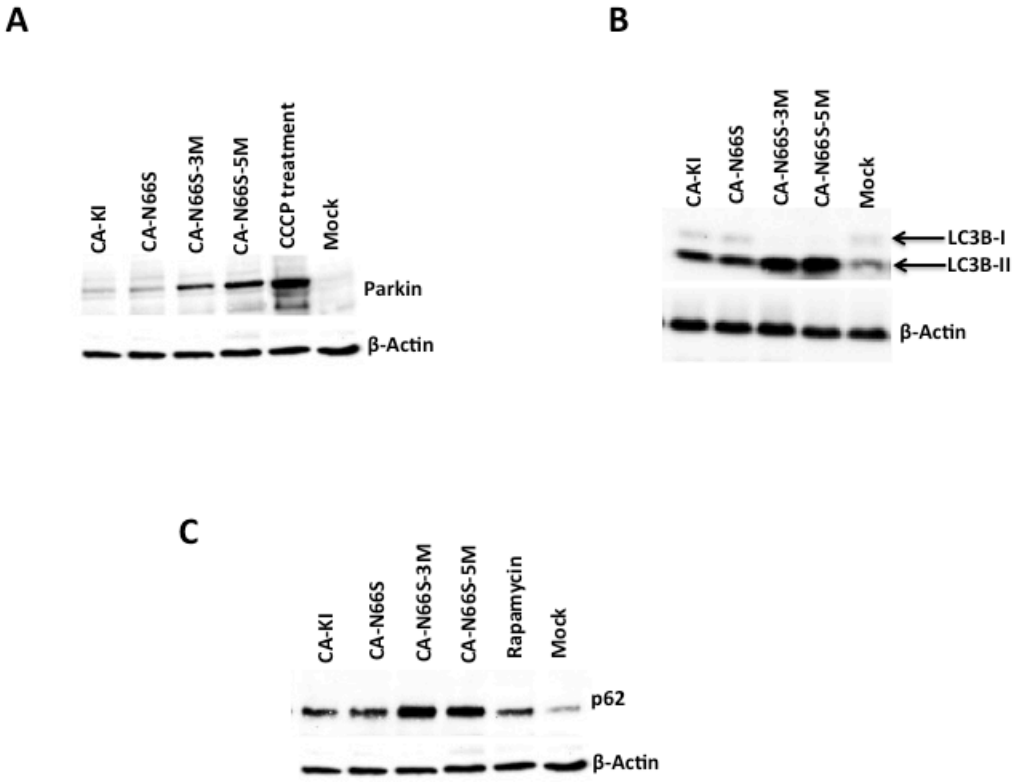

Figure 4.4: PB1-F2 C-terminal mutant viruses promote parkin-mediated type-II mitophagy but blocks autophagic degradation of damaged mitochondria. A549 cells were mock-infected or infected with PB1-F2 recombinant viruses, At 24 hpi, whole cell lysates were collected and western blot analysis was performed using antibodies specific for the indicated proteins: (A) Protein expression levels of parkin. CCCP treatment at 10 uM concentration was used as positive control. (B) Protein expression levels of LCB. At 12 hpi, 3-MA inhibitor at 10 uM concentration was added. (C) Protein expression levels of p62. Rapamycin treatment at 5 uM concentration was used as positive control. β-Actin was used as an internal loading control in all the above immunoblot analysis.

5. General Conclusions and Future Directions

5.1 General conclusions:

With increasing number of IAV outbreaks around the world, it is imperative to understand the role that specific proteins play in imparting pathogenicity. In the first phase of this study, we explored the functional role of PB1-F2 in the pathogenesis of triple-reassortant H3N2 (TR H3N2) swine influenza virus (SIV) in mammalian (swine) and avian (turkey) host. Ablation of PB1-F2 in TR H3N2 SIV modulated early stage apoptosis in monocytes but did not affect the viral replication and production of cytokines in respiratory epithelial cells. PB1-F2 expression did not affect nasal shedding, lung viral load, immunophenotypes and lung pathology in pigs. On the other hand, in turkeys, deletion of PB1-F2 resulted in early induction of clinical disease and effective transmission among the turkey poults. Interestingly, the virulence associated 66S mutation in PB1-F2 abolished the ability of the TR H3N2 virus to successfully infect turkeys and transmit to in-contacts. The relative transmission advantage and higher immunogenicity observed in turkeys infected with recombinant virus knocked out of PB1-F2 expression needs to be further explored. One plausible reason could be that deletion of a virulence marker enhances the ability of the virus to adapt and transmit in a new host species. Taken together, our results show that the PB1-F2 protein in TR H3N2 SIV did not impact pathogenesis and virulence in swine but influences clinical disease and virus transmission among turkey poults. A single asparagine to serine mutation at 66 completely abrogated pathogenicity and transmission of TR H3N2 SIV to turkeys. These results highlight the strain- and species-specific role of PB1-F2 protein.

In the second phase of this study, we demonstrated that specific C-terminal amino acid residues together with a serine at 66 highly conserved in most lethal IAV determine

the pathogenicity of 2009 pandemic H1N1 (pdm09 H1N1) virus in a mouse model. We found that C-terminal residues 73K, 75R, and 79R together with 66S increased virus replication, decreased type I interferon response and induced fulminant acute respiratory distress syndrome (ARDS) in mice with characteristic clinical and pathological features of acute lung injury (ALI). The lethal phenotypic mutants increased infiltration of neutrophils, inflammatory monocytes with the production of myeloperoxidase in the lungs consistent with ALI. Additional mutations at 74T, and 76V in PB1-F2 protein compensated the effects and alleviated ARDS. Our study suggests that these additional C-terminal residues together with 66S play a role in pathogenicity and may serve as markers for predicting the virulence of IAV. It is possible for the pdm09 H1N1 virus to acquire lethal PB1-F2 phenotype by mutations or natural ressortment with circulating influenza viruses. Screening for highly virulent strains with specific mutations identified in this study, may help in influenza virus surveillance and pandemic preparedness. Further studies are needed to know whether these C-terminal residues enhance transmission and post-influenza secondary penumonia in mammalian host.

Further, we found that PB1-F2 induces mitochondrial superoxide production and mitochondrial damage in a sequence dependent manner in IAV-infected cells. We also found that PB1-F2-mediated mitochondrial damage promotes Parkin-mediated mitophagy but suppresses the autophagic degradation of damaged mitochondria in the infected epithelial cells. Accumulated dysfunctional mitochondria can aggravate host cell death and inflammatory responses. In conclusion, the present findings enhance our understanding of PB1-F2 protein as a virulence determinant in IAV infection in a

species- and strain specific manner and provide new insights into the impact of genetic changes in PB1-F2 on the host pathogenesis of virulent influenza A virus strains.

5.2 Future directions:

Functions of PB1-F2 are influenced by multitude of factors such as its length, amino acid sequence and host species (Kosik et al, 2013). The role of PB1-F2 protein is very complex and it is necessary to understand its precise contribution to the pathogenesis of influenza virus in avian and mammalian hosts. Most of the lethal IAV strains are associated with severe lung inflammation (Tumpey et al, 2005). They possess unique and conserved C-terminal amino acid residues that may serve as virulence determinants. Our study points to the fact that the key residues in the C-terminus of lethal IAV when transferred to 2009 pandemic H1N1 enhance its pathogenicity in mice and results in acute lung injury as seen in lethal IAV. This is intriguing as the structure of PB1-F2 changes very minimally when these amino acid residues are introduced. Detailed protein-protein interaction studies and global siRNA screen may provide insights into this. Future studies should be directed at determining which amino acids are critical virulence determinants either alone or in combination with 66S?; whether 66S is critical for the other amino acid residues to exert the virulence phenotype?; and how these amino acid residues bring about the virulence phenotype?

We found that there is excessive neutrophilic infiltration in the lungs of mice infected with these PB1-F2 mutant viruses and demonstrated that they degranulate and release myeloperoxidase leading to lung damage. Neutrophil myeloperoxidase can initiate neutrophil extracellular trap (NET), also known as “Netosis” (Remijsen et al,

2011). It would be interesting to determine whether "Netosis" occurs after infection with these mutants and the pathways associated with it.

The complex immunopathology evoked in the lung of mice infected with these mutants is unprecedented and need to be teased out for its individual components starting at the cell death pathways in immune vs epithelial cells to identify common targets for antiviral therapeutic and vaccine strategies. However, these studies should await the lifting of voluntary pause on gain of function studies. Alternatively, loss of function approaches can be initiated.

Damaged mitochondria have detrimental consequences for cell viability and functioning of the immune system. Several pathways have been put forth. Dysfunctional mitochondrion releases excess reactive oxygen species (ROS) and mtDNA within the cell (Kurihara et al, 2011). ROS acts as damage associated molecular patterns that can activate both NLRP3 inflammasome (Zhang et al, 2013). NLRP3 inflammasome is an important player of innate immune response through its signaling to induce pyrogenic cytokine IL-1β production. NLRP3 inflammasomes senses dysfunctional mitochondria, which may explain the frequent association of mitochondrial damage with inflammatory diseases (Ding and Yin, 2012). mtDNA released in response to damaged mitochondria is recognized by the cytosolic DNA sensor cyclic-GMP-AMP synthase (cGAS) sensor pathway leading to robust induction of type I IFNs and IFN-stimulated genes (West et al, 2015). It would be interesting to study the pathway involved in the activation of inflammation.

Programmed necrotic cell death, necroptosis, activates mitophagy and acts as negative regulator of dysfunctional mitochondria. Receptor interacting protein kinase 2

(RIPK2)-mediated mitophagy negatively regulates ROS production, NLRP3 inflammasome and inflammation (Lupfer et al, 2013). With the help of protein homology recognition engine (Phyre) algorithm (Kelly et al, 2015), we identified that introduction of specific lethality associated amino acid residues in the PB1-F2 protein of 2009 pandemic H1N1 virus creates a F-Box domain in the protein. F-box domains are linked to the Skp1 protein and the core of Skp1-cullin-F-box protein ligase (SCF) complexes. SCF complexes constitute a new class of E3 ligases (Jia et al, 2011). It is tempting to speculate that through F-box domain interactions with SCF complexes, PB1-F2 may promote the degradation of key proteins involved in the effective sensing of damaged mitochondria. Future studies may provide additional insights into these mechanisms. Similarly the association of PB1-F2 with VDAC-1 and how it affects Parkin-mediated mitophagy should be studied in detail.

In summary, our studies only exposed a very minute tip of the iceberg that is PB1-F2 and its multivarious functions. To quote Conenello and Palese (2007), "Influenza A virus PB1-F2; A small protein with a big punch", the repercussions of the punch needs to be unraveled in layers over the future.

5.3 References:

1. Conenello GM, Palese P (2007) Influenza A virus PB1-F2: a small protein with a big punch. Cell Host Microbe 2: 207-209.
2. Ding WX, Yin XM (2012) Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem 393: 547-564.
3. Jia L, Sun Y (2011) SCF E3 ubiquitin ligases as anticancer targets. Curr Cancer Drug Targets 11: 347-356.

4. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015) The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protoc 10: 845-858.
5. Kosik I, Holly J, Russ G (2013) PB1-F2 expedition from the whole protein through the domain to aa residue function. Acta Virol 57: 138-148.
6. Kurihara Y, Kanki T, Aoki Y, Hirota Y, Saigusa T, et al. (2012) Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. J Biol Chem 287: 3265-3272.
7. Lupfer C, Thomas PG, Anand PK, Vogel P, Milasta S, et al. (2013) Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. Nat Immunol 14: 480-488.
8. Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, et al. (2005) Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 79: 14933-14944.
9. West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, et al. (2015) Mitochondrial DNA stress primes the antiviral innate immune response. Nature 520: 553-557.
10. Zhang Q, Kang R, Zeh HJ, 3rd, Lotze MT, Tang D (2013) DAMPs and autophagy: cellular adaptation to injury and unscheduled cell death. Autophagy 9: 451-458.