THE ROLE OF RAN-BINDING PROTEIN 3 DURING INFLUENZA A VIRUS REPLICATION

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ABSTRACT

Influenza A virus (family *Orthomyxoviridae*) is one of the most important human pathogens, causing annual epidemics with significant worldwide mortality, and sporadic but potentially devastating pandemics. The influenza A viral genome encodes 14 proteins and consists of 8 segments of negative-stranded RNA. During infection, the virus exploits the host cell signaling machinery to ensure efficient replication. The PI3K/Akt and Ras/ERK are two of the signaling cascades that are induced for virus survival.

Influenza A virus replicates in the nucleus, hence the newly synthesized RNPs must be exported from the nucleus and exported to the cell membrane. Although the detailed mechanism of vRNP nuclear export is not yet fully elucidated, several studies on this process have begun to emerge. Influenza A virus nucleoprotein nuclear export is CRM1-dependent. Ran-binding protein 3 (RanBP3) is a Ran-interacting protein that is best known for its role as a cofactor of CRM1-mediated cargo nuclear export. In this study, we investigated the role of RanBP3 during the influenza A virus life cycle. We found that RanBP3 was phosphorylated at Ser58 in early and late phases of infection. Knockdown of RanBP3 expression led to a vRNP nuclear retention, suggesting that RanBP3 is involved in vRNP nuclear export. Moreover, we demonstrated that RanBP3's function during vRNP nuclear export is regulated by phosphorylation at Ser58, and the RanBP3 phosphorylation is modulated by both PI3K/Akt and Ras/ERK/RSK pathways in the late phase of viral infection. In conclusion, this study has shown that RanBP3 is a host factor that has a vital role during the late stage of influenza A virus replication, specifically as a co-factor in CRM1-mediated nuclear export. Identifying this host factor will contribute to the understanding of the mechanism of vRNP transport.

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ABBREVIATIONS USED

°C	degrees Celsius
µg	microgram
µl	microliter
µM	micromolar
Akt	serine/threonine kinase Akt/ protein kinase B
ATCC	American Type Culture Collection
cm	centimeter
CO_2	carbon dioxide
CRM1	chromosome region maintenance 1
cRNA	complementary RNA
DAPI	4',6-diamidino-2-phenylindole
ERK	extracellular-signal-regulated kinase
FBS	fetal bovine serum
h	hours
h.p.i.	hours post-infection
HA	hemagglutinin
IgG	immunoglobulin G
M	matrix
M1	matrix 1
M2	matrix 2
MAPK	mitogen activated protein kinase
MDCK	Madin-Darby canine kidney cells
MEK	MAPK/ERK-activated kinase
min	minutes
ml	milliliter
mm	millimeter
MOI	multiplicity of infection
NA	neuraminidase
NaCl	sodium chloride
NCR	non-coding region
NEP	nuclear export protein
NES	nuclear export signal
nM	nanomolar
NP	nucleoprotein

NPC	nuclear pore complex
NS1	nonstructural 1
NS2	nonstructural 2
p.f.u.	plaque-forming units
p.i.	post-infection
PA	polymerase acid
PB1	polymerase basic 1
PB2	polymerase basic 2
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3-kinases
PIP_2	phosphatidylinositol-4,5-diphosphate
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PTEN	phosphatase and tensin homolog
RanBP3	Ran-binding protein 3
RanGAP	Ran GTPase activating protein
RCC1	regulator of chromosome condensation
Raf	mitogen Raf
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RSK	ribosomal S4 kinases
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siOT	off-target siRNA
siRanBP3	RanBP3 siRNA
siRNA	small interfering RNA
TBS	tris-buffered saline
UV	ultraviolet light
v/v	volume/volume
vmRNA	viral mRNA
vRNA	viral RNA
vRNP	viral ribonucleoprotein

1.0 LITERATURE REVIEW

1.1 Influenza A Virus

1.1.1 Influenza A Virus Taxonomy and Nomenclature

Influenza A is a genus classified under *Orthomyxoviridae*, a family comprised of viruses that have a negative-sense, single-stranded, and segmented ribonucleic acid genome. *Orthomyxoviridae* contains five different genera: the influenza A, B, and C viruses, Thogotovirus and Isavirus (Kawaoka *et al.*, 2005). Each influenza genus contains only one species. Influenza viruses type A, B and C are distinguished according to antigenic differences between their nucleoprotein (NP) and matrix (M) proteins (Palese & Shaw, 2007). Further, all influenza A viruses are classified into subtypes based on the antigenic composition of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins on the viral envelope. There are 18 different HA subtypes and 11 different NA subtypes of influenza A (Tong *et al.*, 2013; Webster *et al.*, 1992; Zhu *et al.*, 2013) which implies that several virus strains can possibly emerge from any combination of these HA and NA subtypes.

Influenza A, B and C viruses could be also distinguished according to the species they infect and numbers of RNA segments. Influenza A and C infect multiple species, while influenza B almost exclusively infects humans (Hay *et al.*, 2001). Migratory birds and waterfowl usually serve as the reservoir for influenza A viruses but influenza A viruses also infect, humans, swine, horses and rarely some other mammalian species. Only 3 HA subtypes (subtypes H1, H2, and H3) and 2 NA subtypes (subtypes N1 and N2) have established stable lineages in the human population since the last century (Webster *et al.*, 1992). Influenza C viruses have been isolated mainly from humans, dogs and swine (Manuguerra & Hannoun, 1992; Yuanji & Desselberger, 1984). Influenza viruses types A and B each contain eight distinct RNA segments (Palese &

Shaw, 2007; Ritchey *et al.*, 1976), whereas the influenza C virus genome is composed of only seven segments (Herrler & Klenk, 1991).

Influenza A subtypes and B viruses are further classified into strains. An internationally accepted naming convention that was accepted by the World Health Organization (WHO) was published in 1980 in the Bulletin of the WHO. This nomenclature for influenza virus isolates includes the influenza virus type (A or B), host species (omitted if human in origin), geographical site, serial number, year of isolation and, lastly the HA and NA subtypes (for influenza A viruses only). For example, the nomenclature: A/Halifax/210/2009 (H1N1) represents the type A influenza virus isolated from humans in Halifax, Nova Scotia, Canada as a virus strain number 210 in 2009, and according to the HA and NA composition this virus is designated as H1N1 subtype.

1.1.2 Virion structure of influenza A virus

Influenza A virions (as the infectious particles are called) are pleomorphic, consisting of roughly spherical objects or long cylindrical filaments. Filamentous morphology is typical of clinical isolates of influenza virus, whereas a spherical morphology is observed in many laboratory-passaged influenza strains (Choppin *et al.*, 1960; Kilbourne & Murphy, 1960). Influenza A is an enveloped virus, with an outer lipid membrane that is derived from the host cell in which it multiplied. Its genome consists of eight separate RNA segments that encode 14 proteins (Figure 1.1). The virions are covered with projections of 3 proteins: HA, NA and matrix 2 (M2). HA is a glycosylated type I integral membrane protein with functions both as the viral receptor-binding protein and fusion protein. Because HA recognizes sialic acid (N-acetyl neuraminic acid) bound to underlying sugars on the tips of host cell glycoproteins, it has an important role in determining host tropism (Horimoto & Kawaoka, 2005; Medina & Garcia-

Sastre, 2011; Steinhauer, 1999). It also has a cleavage site that must be cleaved by host cell proteases in order to expose the HA peptide that is responsible for the fusion between the viral envelope and the endosomal membrane (Lazarowitz & Choppin, 1975). The amino acid sequence of this cleavage site modulates tissue tropism and systemic spread, affecting disease severity.

NA is a type II integral membrane glycoprotein with sialidase enzymatic activity required for release of newly produced virions and to prevent aggregation of nascent viral particles. The small protein M2 is a proton channel necessary for viral replication (Taubenberger & Kash, 2010). The matrix 1 (M1) protein, the most abundant viral protein, is found under the membrane and interacts with cytoplasmic domains of the surface glycoproteins and also with the viral ribonucleoprotein (RNP) complexes(Nayak *et al.*, 2004).The RNP complex is the core of the virion and it consists of the eight negative-stranded RNA segments and nucleoprotein (NP), and the polymerase complex heterotrimer [polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA)](Compans *et al.*, 1974; Palese & Shaw, 2007). The nonstructural 1 (NS1) protein has multiple functional domains and pleiotropic functions, including dsRNA binding, enchancement of viral mRNA translation, and type 1 interferon antagonism (Hale *et al.*, 2008; Palese & Shaw, 2007).The NS2 protein (also referred to as nuclear export protein, NEP) is found in virions and facilitates nuclear export of viral RNP complexes (Richardson & Akkina, 1991). Another small viral protein, PB1-F2, is invariably encoded within the PB1 gene by an alternative reading frame (Chen *et al.*, 2001), targets the mitochondrial inner membrane, and may play a role in apoptosis during virus infection. It modulates the host response to influenza A virus infection and is a virulence factor of influenza A virus (Conenello *et al.*, 2007; Zamarin *et al.*, 2006). It may function *in vivo* to disable virus-infected monocytes or other host innate immune

cells that impede viral transmission. The PB1 gene has also been reported to encode a third polypeptide expressed via differential AUG codon usage, called N40 (Wise *et al.*, 2009). Although virus that lacks PB1-N40 shows slower replication kinetics, the functions of the protein remain unclear. More recently, the novel influenza A virus protein PA-X was discovered. PA-X is a ribosomal frame-shifting product encoded by an alternative reading frame of PA (Jagger *et al.*, 2012). PA-X functions to repress cellular gene expression, and modulates the host response and viral virulence. Another novel protein recently reported is M42, which is an M2-related viral protein with an antigenically distinct ectodomain that can functionally replace M2 (Wise *et al.*, 2012).

In general, the influenza A virion is composed of about 1% RNA, 5-8% carbohydrates, 20% lipid and 70% protein (Frommhagen *et al.*, 1959). The internal structure of influenza A viruses is less defined (Calder *et al.*, 2010). Filamentous morphology is typical of clinical isolates of influenza virus, whereas a spherical morphology is observed in many laboratory-passaged influenza strains (Choppin *et al.*, 1960). Electron crytomography can be used to directly examine the structure of pleomorphic virions, although at a limited resolution Because of its pleomorphic nature, an understanding of the structural organization of influenza virus has remained difficult. Recent reports suggest that individual influenza A virus particles each package eight RNA segments into fully functional virion (Hutchinson *et al.*, 2010; Noda *et al.*, 2006).

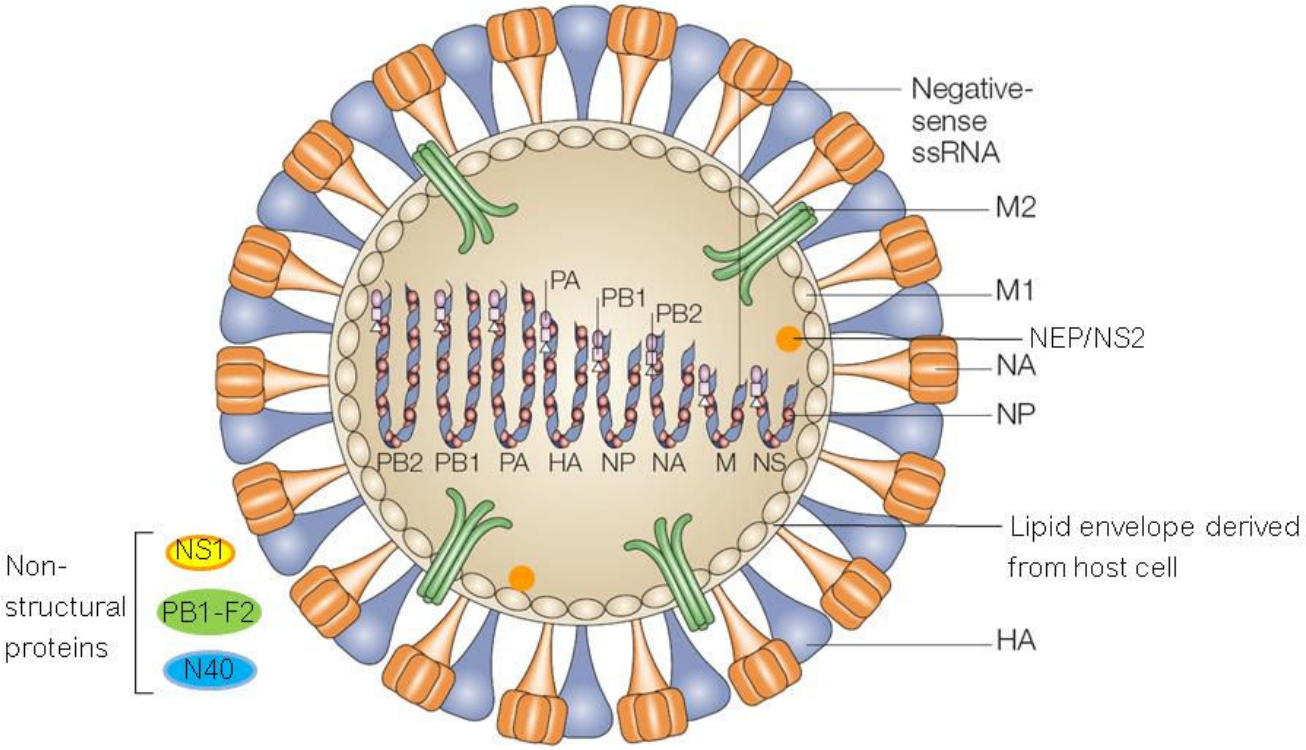

Figure 1.1 Influenza A Virus Structure
Two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) along with small numbers of M2 ion channel protein, are embedded in host-cell derived lipid bilayer. Matrix 1 (M1) protein underlies the envelope and interacts with the surface proteins and with ribonucleoproteins (RNPs). Eight negative-strand RNAs that are encapsidated with NP and associated with polymerase complex heterotrimer (PB2, PB1 and PA) comprise the viral genome. The nonstructural protein 2 (NS2, or nuclear export protein, NEP) is contained within the virion, but the nonstructural protein 1 (NS1) is not. Image adapted from: Horimoto, T. and Y. Kawaoka, 2005. Nat Rev Microbiol 3:591.

1.1.3 Structure and Organization of Influenza A Virus Genome

The influenza A virus genome contains eight separate negative-stranded RNA segments coding for up to 14 proteins that are functionally important proteins required for virus entry, replication or virus structure (Figure 1.2). These gene products are: PB1, PB1-F2, PB1-N40, PB2, PA and PA-X, HA, NP, NA, M1, M2 and M42, NS1 and NEP/NS2 proteins. Each subgenomic segment is coated by viral nucleoprotein NP and bound to a single viral RNA-dependent RNA polymerase heterotrimer (RdRp), composed of PA, PB1 and PB2 subunits. The total genome length is 12000 to 15000 nt. The largest segment is 2300 – 2500 nt long (RNA segment 1) and codes for polymerase PB2, while the smallest is 800 – 900 nt long (RNA segment 8) and codes for NS1 and NEP(Garfinkel & Katze, 1993). Most of the segments of the virus genome code for a single protein except for segments 1, 7 and 8 which are polycistronic (Clifford *et al.*, 2009). Each genome segment is transcribed to produce a single capped mRNA species, which in the case of segments 7 and 8 also undergoes splicing so that each encodes 2 proteins, M1/M2 and NS1/NEP respectively (Lamb & Horvath, 1991).The segment encoding PB1, a polymerase subunit, also generates an additional protein, PB1-F2, that is not translated from the first AUG of the mRNA, rather the PB1-F2 peptide is produced as a result of translation initiating at an alternate start codon in a different reading-frame to that used for PB1 (Chen *et al.*, 2001).

Each of the eight influenza A virus RNA segments contain terminal repeated sequences at both 3’ and 5’ ends that flank the coding sequence (Hoffmann *et al.*, 2001). The 5’ terminus of each vRNA segment has 13 conserved nucleotides, and the 3’ terminus has 12 conserved nucleotides. These non-coding regions (NCR) are conserved among all influenza A RNA segments, and they are usually followed by a segment-specific non-coding region (Desselberger *et al.*, 1980; Robertson, 1979) (Figure 1.2B). These conserved regions at the 3’ and 5’ terminal

ends are considered as the core vRNA promoter (Flick *et al.*, 1996). These conserved sequences at the 3' and 5' terminal ends exhibit partial and inverted complementarity to each other and may form secondary structures, which is critical for the influenza vRNA promoter activity, endonuclease activity of PB1 and polyadenylation (Leahy *et al.*, 2001a; Leahy *et al.*, 2001b; Pritlove *et al.*, 1999).

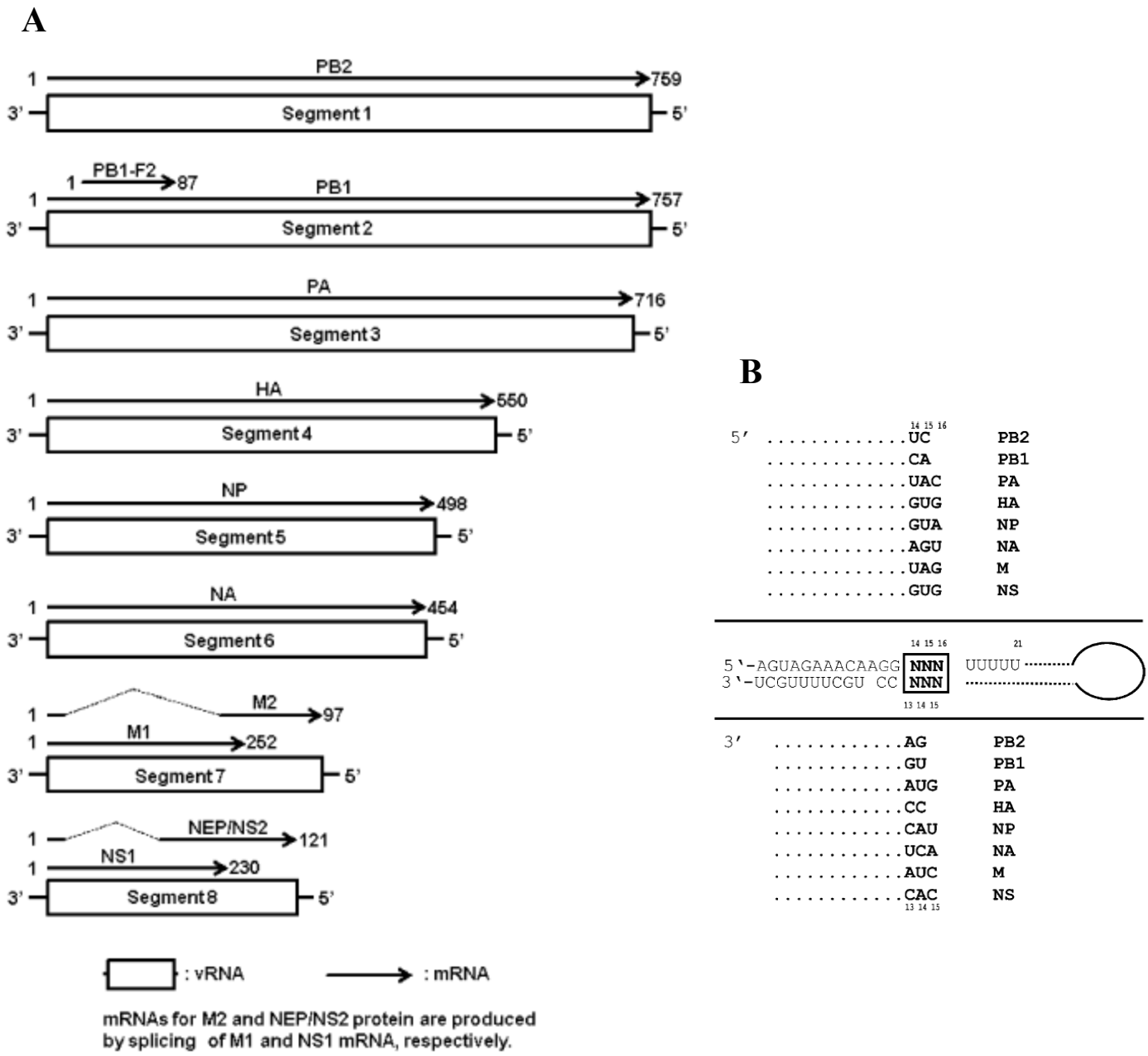

Figure 1.2 Influenza A Virus genome organization.

(A) The influenza A viral genome encodes 14 proteins and consists of 8 segments of negative single-stranded RNA. Each subgenomic segment is coated with viral nucleoprotein (NP) and bound to a single polymerase complex heterotrimer (PB1, PB2, and PA). Most of the segments of the virus genome code for a single protein except for segments 1, 7 and 8 which are polycistronic. Source: Goto and Kawaoka (2011). Influenzaviruses A, *In* Springer Index of Viruses.

(B) Schematic representation of the conserved non-coding terminal regions of the eight segments of influenza A vRNA. The 5' terminus of each influenzaA vRNA segment has 13 conserved nucleotides, and the 3' terminus has 12. Source:Hoffmann, E. et al, 2001. Arch Virol. 146:2275

1.1.4 Influenza A Virus Life Cycle

The life cycle of influenza A virus differs from that of many other negative-strand RNA viruses because it involves a nuclear phase of replication (Figure 1.3). Infection begins with the binding of the viral HA protein to sialyated host cell-surface glycoproteins (Skehel & Wiley, 1995), then the virus enters the cell by receptor-mediated endocytosis thereby forming an endosome. Cellular trypsin-like protease cleaves HA into HA1 and HA2, while the acid environment triggers a conformational change in the cleaved HA, and exposes a fusion peptide in the HA2 subunit, thereby resulting in the fusion of the viral and endosomal membranes (Stegmann, 2000). The virus envelope protein M2's ion channel activity makes the inside of the virion more acidic. As a result, viral ribonucleoprotein (vRNP) complexes of viral genomic RNA, nucleoprotein (NP), and three polymerase subunit proteins (PA, PB1, and PB2) dissociate from the M1 polypeptide and are released into the cytoplasm (Stegmann *et al.*, 1990). Influenza virus transcription and viral RNA replication occur in the nucleus. Accordingly, after release of the vRNPs in the cytoplasm, they are transported into the nucleus, in an active process that is mediated by the cellular importin α/β pathway (O'Neill *et al.*, 1995). Once in the nucleus, RdRP transcribes and replicates the negative-sense viral RNA [(−)vRNA] giving rise to three types of RNA molecules: the complementary positive-sense RNA [(+)cRNA], which is used as a template to generate more vRNA; negative-sense small viral RNAs (svRNAs), which are thought to regulate the switch from transcription to replication (Perez *et al.*, 2010; Umbach *et al.*, 2010); and the viral mRNAs, which are exported to the cytoplasm for translation (Bouloy *et al.*, 1978; Mikulasova *et al.*, 2000).

In the early stage of infection, the primary mRNA transcripts are predominantly used for translation of NP and NS1 proteins (Hay *et al.*, 1977). Newly synthesized NP and NS1 migrate

to the nucleus where increased concentration of free NP triggers the shift from the synthesis of mRNA to synthesis of cRNA and vRNA. Early synthesized NS1 protein in the nucleus interferes with the host innate defense mechanisms by blocking synthesis of antiviral cytokines and proteins in order to provide continuous virus replication (Hale *et al.*, 2008; Hay *et al.*, 1982; Shapiro & Krug, 1988). Newly synthesized vRNAs are coated by NP and serve as templates for secondary transcription of viral mRNAs and synthesis of the remaining proteins. In the late stages of the virus life cycle, newly synthesized vRNPs are exported from the nucleus through the nuclear pore complex (NPC) by the viral factors M1 and NEP/NS2 (nuclear export protein) working in accord with the host's nuclear export machinery (O'Neill *et al.*, 1998; Whittaker & Helenius, 1998). The viral envelope proteins HA, M2, and NA are translated on the rough endoplasmic reticulum (ER) and traffic to the cell surface where they integrate into the cell membrane. Interactions between M1 coupled with RNPs and the cytoplasmic domains of HA, NA or M2 activate the signals for budding which is the last stage in influenza A virus replication cycle (Compans *et al.*, 1974). Most progeny virus particles contain only one copy of each of the eight RNP complexes, which is accomplished by selective packaging. Recent reports show that each of the eight gene segments possess a unique packaging signal for efficient virion incorporation (Zheng & Tao, 2013). The final release of virions from the cell surface requires the neuraminidase activity of viral NA protein (Palese & Compans, 1976; Palese *et al.*, 1974), which destroys the sialic acid of the cellular and viral glycoproteins that would otherwise retain the new virions at the cell surface.

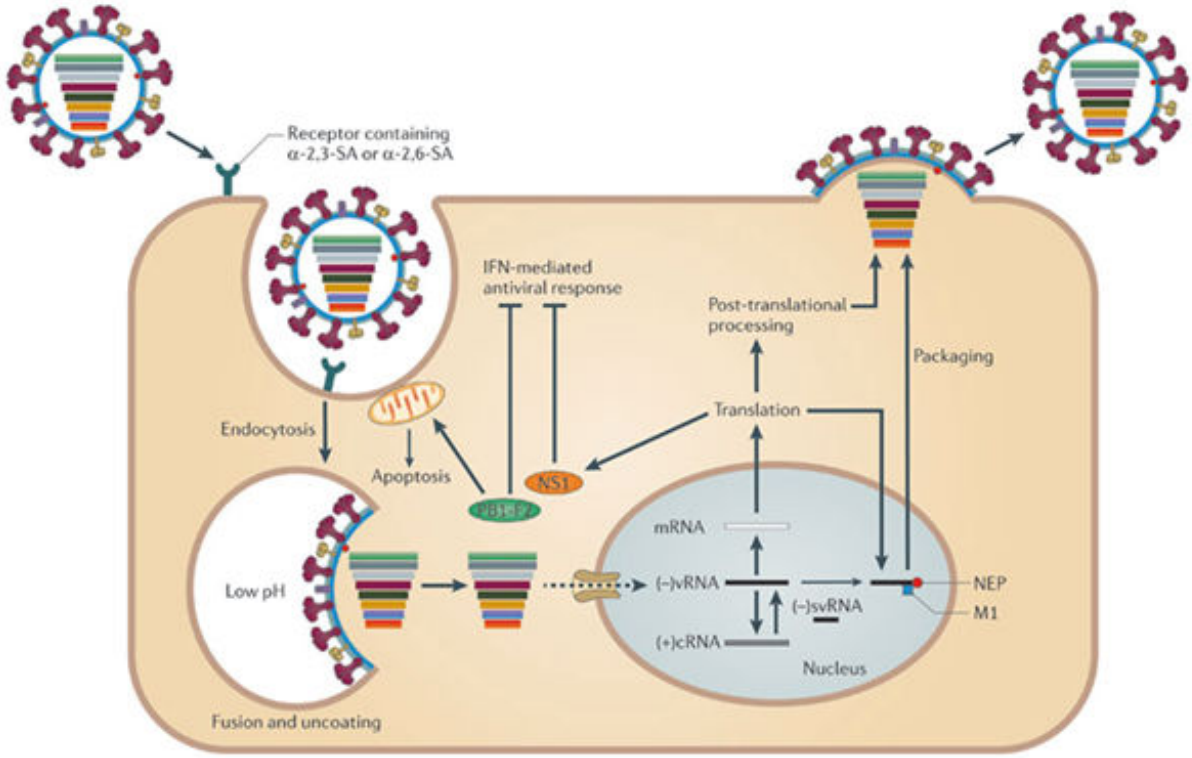

Figure 1.3 Influenza A virus life cycle.
The virion attaches to the cell surface receptor, then the receptor-bound particle enters the cell via endocytosis. After fusion of the viral and endosomal membrane, the viral genome is released into the cytoplasm. The vRNPs are transported into the nucleus where replication and transcription of vRNA segments take place. RdRp snatches 5' caps from host mRNAs to prime transcription of viral mRNA, and to create positive sense complementary RNA (cRNA) from which new viral genomes (vRNAs) are made. The vRNAs are coated with NP and exported through the nuclear pore complex by the viral factors M1 and NEP, working in junction with host nuclear export machinery. The viral envelope proteins HA, M2 and neuraminidase (NA) are translated in on the endoplasmic reticulum and transported to the cell surface where they, along with M1, RdRp and 8 distinct vRNPs are packaged into budding virions. Image source: Medina, R. and AG Sastre, 2011. Nat Rev Microbiol 9:590.

1.2 Influenza A virus induced signaling pathways

1.2.1 PI3K/Akt pathway

Successful virus replication and survival relies upon the evolution of strategies that modulate host cell signaling pathways, in particular those governing apoptosis and cell survival. The phosphatidylinositol-3-kinase (PI3K)-Akt pathway (Figure 1.4) plays a central role in modulating diverse downstream signaling pathways associated with cell survival, proliferation, migration, and differentiation (Cantley, 2002; Cooray, 2004). Identification of this pathway began in the early 1980s through determined attempts to characterize insulin receptor signaling (reviewed by Alessi, 2001; Brazil & Hemmings, 2001), which led to the identification of the components and the mechanism of insulin receptor signaling via insulin receptor substrate proteins to PI3K and consequent Akt-mediated activation by 3-phosphatidylinositol-dependent protein kinase 1 (PDK1). The enzyme phosphatidylinositol 3-kinase (PI3K) is central to many cell signal transduction pathways and acts on several downstream effectors to regulate a diverse range of cellular events (Cantrell, 2001). The serine-threonine kinase, Akt, is one such effector, and the PI3K-Akt pathway has proven to be vitally important in cell survival (Chan *et al.*, 1999).

The PI3Ks are a family of enzymes (Classes I, II and III) that produce lipid second messengers by phosphorylation of plasma membrane phosphoinositides at the 3'OH of the inositol ring (for review, see Vanhaesebroeck *et al.*, 2001). Class I PI3Ks are heterodimeric proteins consisting of a 110 kDa catalytic subunit (p110) and an adaptor/regulatory subunit, and have been subgrouped into Class I_A and Class I_B PI3Ks. Mammalian cells has Class I_A PI3Ks; the catalytic subunit has three isoforms (p110 α, β and γ), each encoded by a separate gene, while the regulatory subunit has five isoforms (p85α, p85β, p55γ, p55α and p50α) which are generated

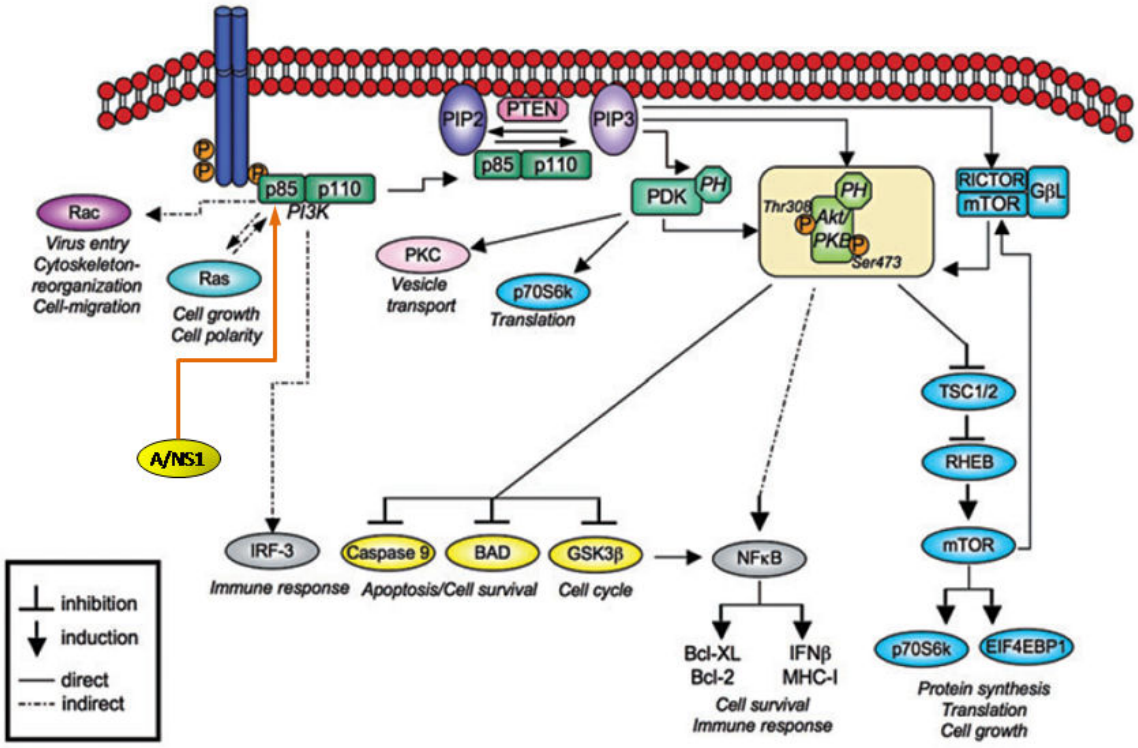

Figure 1.4 PI3K/Akt signaling pathway.
The class IA PI3Ks are heterodimeric enzymes consisting of a regulatory (p85) and and enzymatic subunit (p110). These kinases exhibit both protein and lipid kinase activity. The p85 regulatory subunit can function either as stabilizer or inhibitor of the p110 subunits. PI3K is activated by binding of p85 to autophosphorylated tyrosine kinase receptors (RTKs). Upon PI3K activation, PIP3 is generated which in turn functions as a second messenger through interaction with Akt, which is a major effector in the pathway. PI3K exhibits virus supportive functions during influenza A infection. At the late phase of influenza A replication, the viral protein NS1 induces sustained PI3K signaling by interacting with the p85β regulatory subunit of PI3K.
Source: Ehrhard C and Ludwig S. Cell Microbiol. 2009. 11:963-71

by expression and alternative splicing of three different genes (p85α, p85β and p55γ). Any of the three isoforms of catalytic subunit can bind any of the p85-related regulatory subunits (Miled *et al.*, 2007). Class I_B PI3Ks consist of a p110γ catalytic subunit, associated with a 101 kDa (p101) adaptor subunit (Cantrell, 2001; Vanhaesebroeck & Alessi, 2000).

Class I_A PI3Ks can be activated through binding of the Src homology (SH2) domain in the adaptor subunit to autophosphorylated tyrosine kinase receptors or alternatively to non-receptor tyrosine kinases in the cytoplasm, such as the Src family kinases or JAK kinases . Class I_B kinases are activated by binding of the catalytic subunit to heterotrimeric guanosine 5'-triphosphate-binding proteins or 'G proteins'. Class I_A PI3Ks can also be activated by G proteins. Activated PI3Ks preferentially phosphorylate phosphatidylinositol-4,5-diphosphate (PIP_2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Cantrell, 2001). PIP_3 then serves as a lipid second messenger and isable to interact with a wide variety of signal transduction proteins including Akt. In turn, the production of PIP_3 is negatively regulated by the protein phosphatase and tensin homolog (PTEN), which catalyses the dephosphorylation of PIP_3 to PIP_2 (Leslie & Downes, 2002; Maehama & Dixon, 1999).

The serine-threonine kinase Akt was discovered as a cellular homologue (*c-Akt*) of the viral oncogene (*v-Akt*) from an acutely transforming murine leukemia retrovirus AKT8, isolated from mice with a high incidence of spontaneous lymphoma (Bellacosa *et al.*, 1991; Jones *et al.*, 1991; Staal *et al.*, 1977). It was found to be a novel kinase similar in many respects to protein kinase A (PKA) and protein kinase C (PKC) and hence was referred to as protein kinase B (PKB)(Coffer & Woodgett, 1991). In mammals, there are three isoforms of Akt (Akt1, -2 and -3, or PKBα, -β and -γ), which have a broad tissue distribution. All three isoforms are composed of an N-terminal PH domain, a central catalytic domain, and a C-terminal hydrophobic domain.

Binding of the PH domain of Akt to the PI3K-generated PIP_3 results in its recruitment to the plasma membrane (Bottomley *et al.*, 1998). Once there, Akt is partially activated by phosphorylation at Thr^{308} of its catalytic domain by PDK-1(Alessi *et al.*, 1997). Full activation of Akt requires phosphorylation at Thr^{308} and Ser^{473}. Akt is phosphorylated at Ser^{473} in the C-terminal hydrophobic domain either by the mammalian target of rapamycin (mTOR) when in complex with the mTOR complex 2 (mTORC2)(Sarbassov *et al.*, 2005), or by the DNA-dependent protein kinase (DNA-PK)(Feng *et al.*, 2004). Although Akt is not phosphorylated directly by PI3K, these post-translational modifications occur strictly dependent on PI3K activity. Therefore, detection of phosphorylation at Ser^{473} of Akt is commonly used to monitor the activation state of PI3K. Dephosphorylation of T^{308} by the protein phosphatase 2 (PP2A)(Andjelkovic *et al.*, 1996), and Ser^{473} by the PH domain leucine-rich-repeat-containing protein phosphatases (PHLPP1/2)(Brognard *et al.*, 2007), and the conversion of PIP_3 to PIP_2 by PTEN(Stambolic *et al.*, 1998) negatively regulates Akt signaling.

Several studies have addressed the role of PI3K and its downstream effector Akt as signaling mediators during influenza A virus replication (Ehrhardt *et al.*, 2006; Hale *et al.*, 2006; Shin *et al.*, 2007b; Zhirnov & Klenk, 2007). PI3K was initially described to act in an antiviral fashion (Ehrhardt *et al.*, 2006; Sarkar *et al.*, 2004). However, later it became apparent that PI3K/Akt signaling is also actively induced by the viral A/NS1 protein to support efficient replication. At the late phase of influenza A virus infection, the PI3K/Akt pathway is activated when the viral NS1 protein is complexed with the p85β regulatory subunit of PI3K (Hale *et al.*, 2006; Shin *et al.*, 2007c). The polyproline motif in NS1 (designated SH3 binding motif 1), is essential for mediating this NS1-p85β interaction (Shin *et al.*, 2007a). Studies using mutant viruses and wildtype virus plus PI3K inhibitors also showed that PI3K/Akt functions to suppress

virus induced apoptosis (Ehrhardt *et al.*, 2007; Shin *et al.*, 2007a), whereby it inhibits Bax-mediated apoptosis by negatively regulating the c-jun N-terminal kinase (JNK) pathway (Lu *et al.*, 2010). Blockage of PI3K/Akt activation by a drug inhibitor led to reduction in progeny virus yield, suppression of viral RNA synthesis and viral protein expression, and suppression of vRNP export (Shin *et al.*, 2007b).

1.2.2 Raf/MEK/ERK pathway

The Raf/MEK/ERK pathway is another influenza A virus-induced intracellular signaling pathway that supports viral replication. This signal transduction cascade belongs to the mitogen-activated protein kinase (MAPK) cascades. MAPK cascades are important signaling pathways that convert extracellular signals into cellular responses. All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate diverse cell activities which include gene expression, mitosis, metabolism and motility, survival and apoptosis, and differentiation. MAPKs can be activated by a wide variety of stimuli, but in general, the extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2) are preferentially activated in response to growth factors and phorbol esters (Pearson *et al.*, 2001; Roux & Blenis, 2004).

Raf/MEK/ERK signaling leads to stimulus-specific changes in gene expression, alterations in cell metabolism, or the induction of apoptosis. It is induced by extracellular agents such as mitogens, hormones, and pathogens such as RNA viruses. Typically, cell surface receptors such as tyrosine kinase (RTK) and G protein-coupled receptors transmit activating signals to the Raf/MEK/ERK cascade through the small GTP-binding protein Ras (Campbell *et al.*, 1998) (Figure 1.5). Ras transmits the signal further by recruiting Raf kinases to the plasma membrane where they are activated (Wellbrock *et al.*, 2004). Activated Raf binds to and phosphorylates the MAPK/ERK-activated kinases (MEK1/2) which in turn directly

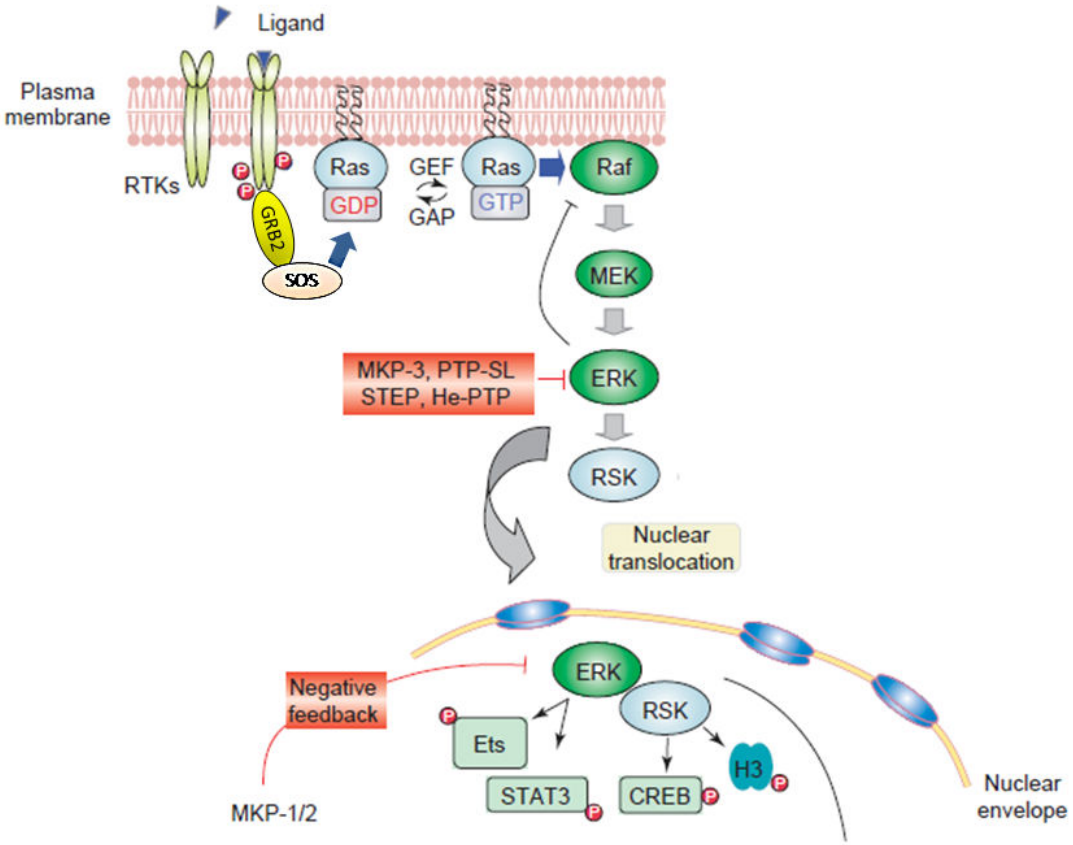

Figure 1.5 Ras/MEK/ERK signaling pathway.
After ligands such as growth factors or virions bind to the receptor tyrosine kinase (RTK) and Ras is activated, Raf kinases are recruited to the plasma membrane. Raf then activates MAPK-ERK activated kinases (MEK 1/2), which in turn phosphorylate and dissociate from ERK 1/2.Activated ERKs translocate to the nucleus and phosphorylate and activate downstream targets such as the family of ~90 kDa ribosomal S6 kinases (RSKs), RSKs are involved in multiple processes including transcriptional regulation, cell-cycle control, and protein synthesis.
Adapted from: Murphy L & Blenis J. 2006. Trends Biochem Sci. 31:265

phosphorylate and activate ERK1/2 within a conserved Thr-Glu-Tyr motif in their activation loop (Murphy & Blenis, 2006). Activated ERKs translocate to the nucleus and phosphorylate and activate downstream targets such as the family of ~90 kDa ribosomal S6 kinases (RSKs), whose human isoforms consist of RSK1–RSK4 (Roux & Blenis, 2004). RSKs are involved in multiple processes including transcriptional regulation, cell-cycle control, and protein synthesis. RSK1, RSK2, RSK3, and ERK/2 are usually present in the cytoplasm of quiescent cells, but upon stimulation, a significant portion of these proteins translocate to the nucleus.

Pleschka *et al.* (2001) reported that activation of the Raf/MEK/ERK cascade is an essential prerequisite for effective nuclear RNP export. Inhibition of Raf signaling results in nuclear retention of vRNPs, impaired function of the nuclear export protein NEP and reduction of progeny virions formed. However, Raf/MEK/ER inhibition does not impair synthesis of vRNA and proteins, or nuclear RNP import, or the normal mRNA export pathway. The investigators proposed that there must be a cellular factor that is involved in NEP export function which is regulated by ERK.

At the late phase of influenza virus infection, vRNPs are exported from the nucleus to the plasma membrane to be packaged into budding progeny virions. Accumulation of hemagglutinin in lipid rafts in the plasma membrane triggers activation of the Raf/MEK/ERK cascade via protein kinase Cα and induces vRNP export (Marjuki *et al.*, 2006)

1.3 Nucleocytoplasmic transport

1.3.1 CRM1-mediated nucleocytoplasmic transport

CRM1/Exportin1 belongs to the importin-β family of nuclear transport receptors, also referred to as karyopherins, and is responsible for exporting ribonucleoproteins and many proteins that carry the leucine-rich type of nuclear export signal (NES), from the nucleus to the cytoplasm (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Stade *et al.*, 1997). CRM1-mediated nuclear export across the nuclear membrane is regulated by the GDP/GTP cycle of a small GTPase, Ran. Most Ran in the nuclear compartment is in GTP-bound form and is catalyzed by regulator of chromosome condensation 1 (RCC1) (Bischoff & Ponstingl, 1991), while in the cytoplasm Ran is in GDP bound form that is regulated by Ran GTPase activating protein (RanGAP) (Bischoff *et al.*, 1994) – an asymmetric distribution that gives directionality of nuclear transport. In the nucleus, CRM1 associates in a cooperative manner with RanGTP and with an NES-bearing cargo to form a ternary CRM1/Ran/cargo complex that translocates through the nuclear pore complex (NPC) and is disassembled in the cytosol after GTP hydrolysis by Ran (Bischoff & Gorlich, 1997; Kutay *et al.*, 1997). In the absence of Ran, CRM1 has low binding affinity for most NES-bearing cargos. In addition to exporting NES-bearing proteins, CRM1 also mediates export of Snurportin1 which lacks a leucine-rich NES (Paraskeva *et al.*, 1999), and certain U snRNAs (Ohno *et al.*, 2000). It was later found that CRM1's binding to this diversity of export substrates requires cofactors.

1.3.2 Influenza A virus vRNP nuclear export

At the late stage of virus infection, the vRNPs exit the nucleus to assemble and bud from the host cell's plasma membrane. The trafficking of the vRNPs into and out of the nucleus is a

tightly regulated process (Cros & Palese, 2003). Although the mechanism of vRNP nuclear export is not yet fully elucidated, several studies on this process have begun to emerge. At late stages of infection, influenza virus proteins and vRNPs are transported across the nuclear envelope through the nuclear pore complex (NPC) in an active, energy-dependent process. Nuclear export is reported to be controlled by the export receptor CRM1, a member of the importin β family of the nuclear transport receptors, which is inhibited by leptomycin B (Elton *et al.*, 2001; Neumann *et al.*, 2000; Watanabe *et al.*, 2001). vRNP export proceeds through the coordinated interactions between the M1 protein (which associates with vRNP), the nuclear export receptor CRM1, and the viral nuclear export protein (NEP) (Bui *et al.*, 2000; Martin & Helenius, 1991; O'Neill *et al.*, 1998; Sakaguchi *et al.*, 2003). NEP encodes a nuclear export signal, binds CRM1 and M1, and is thought to bridge the complex between M1-vRNP and the cellular nuclear transport complexes (reviewed in Boulo *et al*, 2007). Large molecules such as RNPs may need more than one CRM1 molecule for transport.

Studies have shown that leptomycin B, which specifically inactivates the CRM1, resulted in the nuclear retention of vRNP in virus-infected cells, indicating a role for the CRM1 nuclear export pathway in influenza vRNP nuclear export (Elton *et al.*, 2001; Watanabe *et al.*, 2001). The viral M1 protein and NEP are also involved in this process. Even though no ternary complex containing vRNP, CRM1, M1 and NEP has been isolated from the infected cells, a study by Akarsu et al supported a “daisy-chain”model where CRM1 binds to the nuclear export signal located in the NEP protein, and thus bridges the complex between M1-vRNP (Akarsu *et al.*, 2003; Boulo *et al.*, 2007). Despite binary interactions between each of these components, a fully formed vRNP export complex with all four components has not been isolated from infected cells.

1.4 Ran-binding protein 3

Ran-binding protein 3 (RanBP3) is a Ran-interacting protein that is best known for its role as a cofactor of CRM1-mediated export. While located primarily in the cell nucleus, RanBP3 binds to the CRM1 transport receptor and inhibits the binding of unloaded CRM1 to the NPC (Macara, 2001). In addition, RanBP3 increases the affinity of CRM1 for both Ran-GTP and nuclear export sequences (Englmeier *et al.*, 2001; Lindsay *et al.*, 2001), thereby stabilizing the ternary CRM1/Ran/cargo complex in the nucleus. In this manner, RanBP3 coordinates efficient cargo loading, RanGTP binding, and nuclear export (Macara, 2001). RanBP3 further promotes export complex assembly by increasing the nucleoplasmic pool of CRM1 (Sabri *et al.*, 2007) and by recruiting CRM1 to RCC1, where it facilitates the association of CRM1 with RanGTP (Nemergut *et al.*, 2002). In the absence of Ran and cargo, RanBP3 inhibits CRM1 from interacting with the NPC, thereby reducing futile cycles of transport (Lindsay *et al.*, 2001).

Further, RanBP3 plays an important role in the mechanism by which extracellular signals transduced through the MEK/ERK/RSK and PI3K/Akt signaling pathways can modulate nuclear transport (Yoon *et al.*, 2008). In human cells, RanBP3 is phosphorylated at Ser58 when it interacts with either RSK or Akt. Inhibitors of the MEK/ERK/RSK and PI3K/Akt signaling pathways indicated that both pathways are involved in regulating RanBP3 phosphorylation.

Although RanBP3 is best known as a cofactor of CRM1, it has other diverse roles in nuclear transport. RanBP3 associates with RCC1 and enhances its catalytic activity towards Ran (Mueller *et al.*, 1998; Nemergut *et al.*, 2002) and stimulates the CRM1-independent nuclear export of β-catenin, Smad2 and Smad3 (Dai *et al.*, 2009; Hendriksen *et al.*, 2005).

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The host factor Ran-binding protein 3 is utilized during influenza A virus replication, where it could play a role in nuclear export of viral components to the cytoplasm.

2.2 Specific Objectives

2.2.1 To determine if RanBP3 is activated during influenza A virus replication

2.2.2 To determine the effect of RanBP3 knockdown on influenza A virus replication

2.2.3 To investigate if phosphorylation at Ser-58 of RanBP3 is essential for vRNP export

2.2.4 To examine the regulatory pathways controlling RanBP3 activation during influenza A virus replication

3.0 THE ROLE OF RAN-BINDING PROTEIN 3 DURING INFLUENZA A VIRUS REPLICATION

(As published in Journal of General Virology, May 2013 vol. 94 no. Pt 5 977-984)

3.1 INTRODUCTION

Influenza A viruses are important pathogens that are contagious and cause acute respiratory disease in humans and different animal species. Influenza A viruses are enveloped particles belonging to the RNA virus family Orthomyxoviridae (Palese & Shaw, 2007). The genome consists of eight segmented RNA molecules of negative polarity. The majority of influenza A virus strains encode 12–14 viral proteins (Muramoto *et al.*, 2013). Of those, NP and RNA polymerase subunits PB2, PB1 and PA are associated with the RNA genome, forming a viral nucleoprotein (vRNP) complex. A unique feature of the influenza virus life cycle is that

virus replication and transcription take place in the nucleus of infected cells. Thus, newly synthesized vRNP complex must be exported from the nucleus to the cytoplasm before assembling into viral particles. Although the detailed mechanism of vRNP nuclear export is not yet fully elucidated, several studies on this process have begun to emerge. At the late-stage of infection, influenza vRNPs are transported across the nuclear envelope through the nuclear pore complex (NPC) in an active, energy-dependent process. Studies have shown that leptomycin B, which specifically inactivates the chromosome region maintenance 1 protein (CRM1), resulted in the nuclear retention of vRNP in virus infected cells, indicating a role for the CRM1 nuclear export pathway in influenza vRNP nuclear export (Elton *et al.*, 2001; Watanabe *et al.*, 2001). The viral M1 protein and nuclear export protein (NEP) are also involved in this process. Even though no quadruple complex containing vRNP, CRM1, M1 and NEP has been isolated from the infected cells, a study by Akarsu et al. (2003) supported a 'daisy-chain' model where CRM1 binds to the nuclear export signal located in NEP, and thus bridges the complex between M1 and vRNP (Boulo *et al.*, 2007).

CRM1 is a member of the importin β family of nuclear transport factors, which facilitate the nuclear export of many proteins and RNPs that bear a leucine-rich nuclear export signal (Petosa et al., 2004). In the nucleus, CRM1 associates in a cooperative manner with RanGTP and with a nuclear export signal (NES)-bearing cargo to form a ternary CRM1/Ran/cargo complex that translocates through the NPC and is disassembled in the cytosol after GTP hydrolysis by Ran (Bischoff & Gorlich, 1997; Kutay *et al.*, 1997). In the absence of Ran, CRM1 has low binding affinity for most NES-bearing cargos. It was later found that the binding of CRM1 to this diversity of export substrates requires cofactors.

Ran-binding protein 3 (RanBP3) is a Ran-interacting protein that is best known for its role as a cofactor of CRM1-mediated export. It enhances the rate of nuclear export by increasing the affinity of CRM1 for RanGTP, thereby stabilizing the ternary CRM1/Ran/cargo complex in the nucleus (Englmeier *et al.*, 2001; Lindsay *et al.*, 2001). Recently, Yoon et al. (2008) showed that the function of RanBP3 is regulated by phosphorylation via the Ras/ERK and PI3K pathways. In human cells, RanBP3 is phosphorylated at Ser58 when it interacts with either RSK or Akt, which are activated downstream of Ras/ERK and PI3K, respectively. The Raf/MEK/ERK and PI3K/Aktsignalling pathways were previously shown to be activated upon influenza A virus infection (Ehrhardt *et al.*, 2006; Hale *et al.*, 2006; Pleschka *et al.*, 2001; Shin *et al.*, 2007c), and their inhibition resulted in nuclear retention of vRNPs. These findings prompted us to investigate the role of RanBP3 in regulating influenza vRNP nuclear export.

3.2 MATERIALS AND METHODS

3.2.1 Cell, viruses, and infections. A549 (human lung carcinoma cells) were maintained in Kaighn's modification of Ham's F-12 medium (ATCC) containing 10% fetal bovine serum (FBS) (Invitrogen). Madin-Darby canine kidney cells (MDCK) were cultivated in minimum essential medium (MEM) (Sigma) supplemented with 10% FBS. All media were supplemented with 50 μg/ml of gentamicin (Invitrogen) and maintained at 37 °C in an atmosphere of 5% CO2.

Influenza A/PR/8/34 (H1N1) (referred to as PR8) was propagated at 37 °C in 11-day old embryonated chicken eggs. A/Halifax/210/2009 (H1N1) (referred to as Halifax210) was cultivated in MDCK cells. Virus titres were determined in MDCK cells by plaque assay as described previously (Shin *et al.*, 2007a). UV irradiation- inactivated virus was prepared by exposing virus solution (0.5 ml in 35 mm tissue- culture dish) to a 30 W UV light at a distance of 20 cm for 20 min.

3.2.2 Antibodies and inhibitors. Rabbit polyclonal NP and M1-specific antibodies were generated in our laboratory as previously described (Shin *et al.*, 2007b). Monoclonal NP-specific antibody (Serotec) and monoclonal anti-Flag antibody (Sigma) were used in immunofluoresence staining analysis. Rabbit polyclonal anti-phospho-RanBP3(Ser58) and rabbit monoclonal anti-RanBP3 antibodies were purchased from Invitrogen. Mouse monoclonal anti-β-actin, rabbit monoclonal anti-phospho-Akt (Ser473) antibody and rabbit polyclonal anti-phospho-ERK1/2 (Thr202/Tyr204) antibody were purchased from Cell Signaling Technology. IRDye 680-conjugated polyclonal donkey anti-rabbit IgG and IRDye 800-conjugated donkey polyclonal anti-mouse IgG were purchased from LI- COR Biosciences. Secondary antibodies used for immunofluoresence staining were Alexa-Fluor (AF)594 conjugated goat anti-rabbit IgG, AF488 conjugated goat anti- mouse IgG and AF594 conjugated goat anti-mouse IgG, which were purchased from Invitrogen. Cy2-conjugated goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories.

PI3K-specific inhibitor LY294002 was purchased from Sigma. Ras/ERK/RSK pathway inhibitor U0126 was purchased from Calbiochem. Treatment of cells with LY294002 or DMSO were performed at concentrations of 20 μM and 0.4% (v/v) respectively, while treatment with U0126 was performed at a concentration of 10 μM. Chemicals were added to the cells 1 hour prior to infection, removed during virus adsorption, and added again 1 hour post infection (h.p.i.).

3.2.3 siRNAs and transfections. An siRNA set targeting human RanBP3 (catalogue numbers SI04149418, SI04223443, SI04241398, SI04261236) was purchased from Qiagen. An off-targeting siRNA (Negative Control Stealth RNAi duplex catalogue number 12935400) was

obtained from Invitrogen. A549 cells seeded in 24-well plates or 4well chamber slides (4×10^4 cells/well) were transfected with siRNAs at a concentration of 40 nM (10 nM each siRNA) using X-tremeGene siRNA transfection reagent (Roche) according to the manufacturer's protocol with minor modifications.Briefly, 40 nM of siRNA and 10 µl of X-tremeGene siRNA transfection reagent were diluted in OptiMEM in separate vials. The diluents were mixed immediately, and the mixture was further incubated at room temperature for 20 min before addition to cells at 30 to 40% confluence. At 48 hours posttransfection, cells were superinfected with influenza A virus.

3.2.4 Plasmids and transfections. Plasmids encoding HA-tagged wild-type RanBP3(pKH3-HA-RanBP3), dominant negative RanBP3 (pKH3-HA-S58A-RanBP3) and constitutively active RanBP3 (pKH3-HA-S58D-RanBP3) proteins were a gift from Dr. John Blenis (Yoon *et al.*, 2008). Flag-tagged versions of these RanBP3 proteins were created by PCR amplifying the RanBP3 genes with primers (Fw [5'- ATAGCGAATTCAATGGCGGACCTGGCGAAC-3'] and Bw [5'- AACTAGGTACCCTATGTGCTCCCGGTCGTCTG-3']) carrying EcoRI and BamHI sites. The respective genes were then cloned into the pCMV-3xFlag (Li *et al.*, 2008) at the same sites.

MDCK cells were transfected with the Flag-tagged plasmids by electroporation with Amaxa Nucleofector Kit L (Lonza). Five µg of each plasmid was used per 5×10^5 cells, and electroporations were performed according to the appropriate Nucleofector program. Prewarmed antibiotic-free medium was immediately added to electroporated cells, and about 1×10^5 cells/well were transferred to a four-well chamber slide and incubated for 10 hours. Transfected cells were then superinfected by PR8 virus at a multiplicity of infection (MOI) of 3. At 10 h.p.i. cells were fixed and subjected to immunofluorescence staining as described below.

3.2.5 Immunofluoresence and Microscopy. Influenza virus-infected A549 or MDCK cells grown on a four-well chamber slide were fixed in a mixture of acetone- methanol (1:1) for 15 min at -20°C. Cells were rehydrated with PBS, and non-specific protein binding was blocked by incubation with PBS containing 10% normal horse serum (blocking solution) for 1 hour at room temperature. Primary and secondary antibody dilutions were prepared in blocking solution and incubated with cells for 1 hour at room temperature. Between staining steps, cells were rinsed three times with PBS. For A549 cells, the primary antibodies used were mouse monoclonal anti-NP antibody (1:250) and rabbit anti-RanBP3 antibody (1:100). For MDCK cells, the primary antibodies used were rabbit polyclonal anti-NP antibody (1:400) and mouse anti-Flag antibody (20 μg/ml). Secondary antibodies included AF594 goat anti-rabbit (1:400), AF 488 goat anti-mouse (1:400), AF594 goat anti-mouse (1:400) and Cy2 goat anti-rabbit (1:400) IgG. Stained slides were mounted with glass coverslips using Invitrogen's Prolong Gold antifade reagent with DAPI, which stained cell nuclei. All fluorescence images were captured with a Leica TCS SP5 laser confocal microscope.

3.2.6 Immunoblotting. About 5×10^5A549 cells were grown on 35 mm dishesovernight and then incubated in serum-free media for 20 hours. During the last 1 hour, cells were left untreated or treated with inhibitors, then mock-infected or infected with influenza virus at a MOI of 1. At the indicated times, cell monolayers were washed with cold PBS and lysed with cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM phenylmethanesulfonyl fluoride (Sigma). The lysates were collected, incubated on ice for 10 min, sonicated, and further homogenized by passing several times through a 0.5 ml syringe with a 28 gauge needle. The lysates were

analyzed for total protein content by using a Bradford assay (Bio-Rad). Western blotting was performed as previously described (Shin *et al.*, 2007b)with minor modifications. Briefly, 20 μg of total protein was resolved by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for non-specific binding with Tris-buffered saline (TBS) [0.1 M Tris (pH 7.6), 0.9% NaCl] containing 5% bovine serum albumin (BSA) for 1 hour at room temperature, and then incubated with primary antibody diluted in TBS overnight at 4°C. Infrared dye-linked secondary antibody (1:5000) was then added, and membranes were incubated at room temperature for 1 hour. The immunoblots were visualized using an Odyssey infrared imaging system (LI-COR Biosciences).

3.3 RESULTS

3.3.1 RanBP3 is activated during the early and late phase of influenza A virus infection.

Given that RanBP3 is phosphorylated by RSK and Akt (Yoon *et al.*, 2008), and influenza virus infection activates Ras/ERK pathways and PI3K/Akt pathways, we sought to determine whether RanBP3 is activated during virus infection.

Serum-starved A549 cells were mock-infected or infected with wt PR8 virus at a MOI of 1, cell lysates prepared at the predetermined times were subjected to immunoblotting using antibodies against phospho-RanBP3 (Ser58), total RanBP3 and viral M1 protein. As shown in Fig. 3.1A, infection of cells leads to a biphasic activation of RanBP3, as manifested by a brief period of RanBP3-S58 phosphorylation during the early phase (15 to 30 min p.i.) of infection and again at the late phase which started at 6 h p.i. and was sustained for the remainder of infection. Immunoblotting with total RanBP3 showed equal amounts of RanBP3 throughout the

course of infection, indicating that the changes in phosphorylation were not due to altered cellular protein levels. When the amount of phospho-RanBP3 was normalized against total RanBP3, and the fold-change of phosphorylation relative to the 0 hr was calculated, the biphasic mode of RanBP3 activation was more apparent (Fig 3.1A lower panel). A similar pattern of RanBP3 phosphorylation was observed during the course of Halifax210 infection (Fig. 3.1B), demonstrating that this phenomenon was not limited to a specific virus strain.

As we observed biphasic RanBP3 phosphorylation, we postulated that the early phase activation might be associated with the initial virus-host cell interactions, whereas the late phase activation might be virus replication dependent. To test this hypothesis, we inactivated the virus by UV irradiation, since UV irradiation blocks the viral RNA transcription, and mRNA and protein syntheses, but has no effect on virus receptor binding and subsequent entry into host cells (Shin *et al.*, 2007b). A549 cells were infected with UV-inactivated PR8 at MOI of 1. Cells were harvested at early (15min p.i.) and late (9h.p.i.) phases of infection and the phosphorylation of RanBP3, and total RanBP3 were determined by Western blotting. As seen in Fig. 3.1C, while infection by UV-inactivated viruses did not alter total RanBP3, replication deficient virus could activate the early phase of RanBP-3 phosphorylation, but failed to trigger phosphorylation of RanBP3 at late phase. No M1 protein could be detected in UV-inactivated virus-infected cells (data not shown).

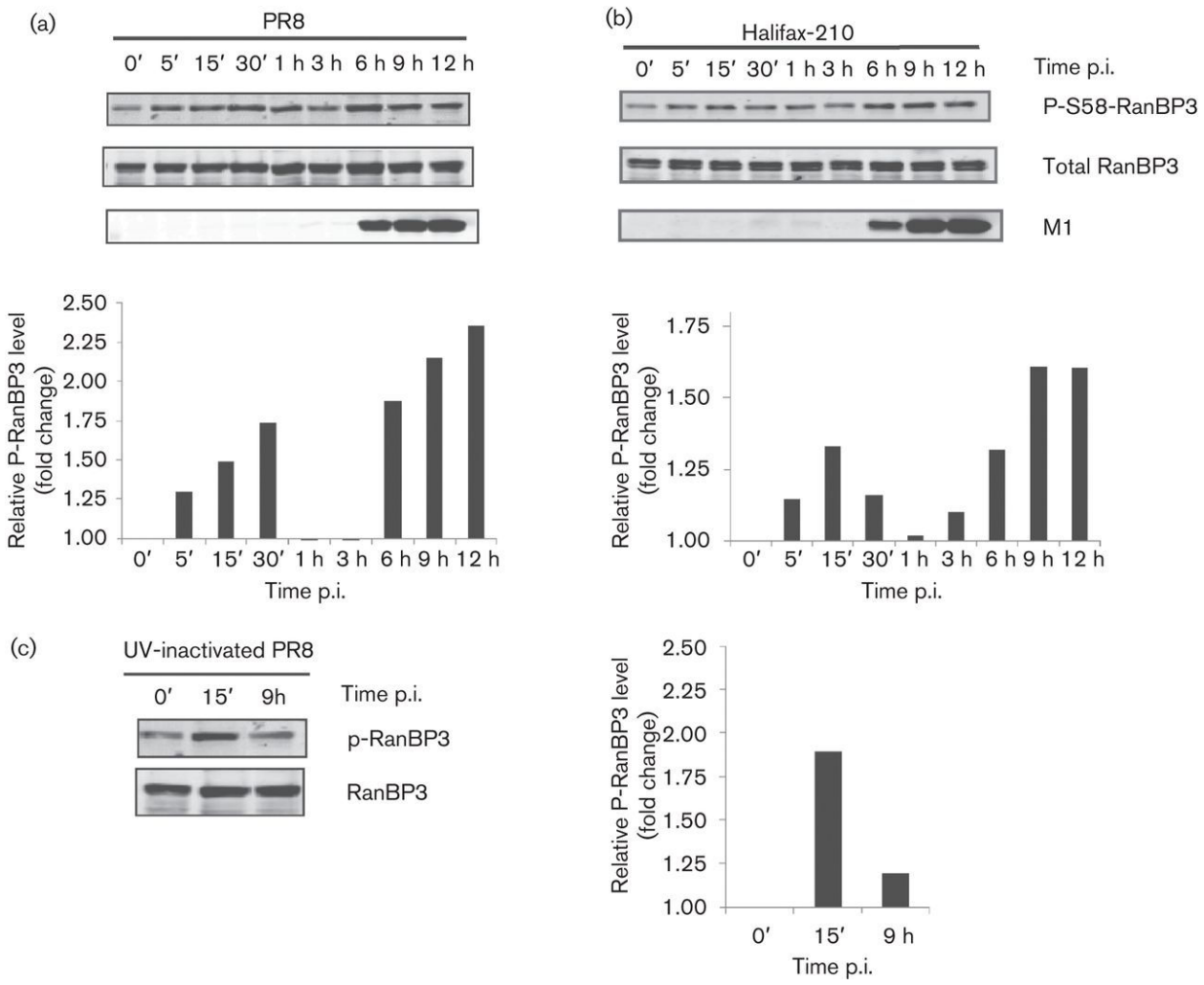

Fig. 3.1. Influenza virus infection activates RanBP3 phosphorylation. (A) Serum-starved A549 cells were infected by wt PR8 virus (A); Halifax-210 virus (B) or UV- inactivated PR8 virus (C) at an MOI of 1 for the time indicated. Phosphorylation of RanBP3 at Ser58, total RanBP3 and ongoing viral replication demonstrated by viral M1 protein synthesis were detected by the respective antibodies. Quantification of band density was performed by Odyssey software. The level of phospho-RanBP3 was normalized against total RanBP3, and the fold-change of phosphorylation relative to the mock infected cells was graphed.

3.3.2 Knockdown of endogenous RanBP3 results in reduction of progeny virus titer.

To examine the biological function of RanBP3 during influenza A virus replication, we assessed the gene knockdown effect on virus yield and NP expression. A549 cells were transfected with an siRNA mixture that targeted four different regions of RanBP3. Forty-eight hours posttransfection, A549 cells were harvested, and the knockdown effect was determined by immunoblotting with RanBP3 antibody. As shown in Fig. 3.2(a), transfection with off-target siRNA (siOT) did not alter the expression level of RanBP3 as compared to that in the non-transfected cells [si(–)]. However, transfection with RanBP3 siRNA led to a significant suppression in RanBP3 expression. siRNA treatment did not result in concomitant reduction in the level of non-targeted cellular proteins, as indicated by consistent β-actin levels.

After confirming that siRanBP3 suppressed endogenous RanBP3 expression in A549 cells, the siRNA-treated cells were then infected with PR8 virus at an m.o.i. of 1. At 8 and 16 h p.i., supernatant was harvested and virus titres were determined by plaque assay. The cells were harvested at the end-point and viral protein expression was determined by immunoblotting with NP antibody. As shown in Fig. 3.2(b), at 8 h p.i., the virus titre did not decrease significantly in RanBP3 siRNA-transfected cells compared with off-target siRNA-transfected or non-siRNA-transfected cells. However, at 16 h p.i., siRanBP3-treated cells exhibited 1 log reduction in progeny virus titre relative to off-target siRNA-treated control cells. Concomitantly, NP protein levels in siRanBP3-treated cells were lower than that in control cells.

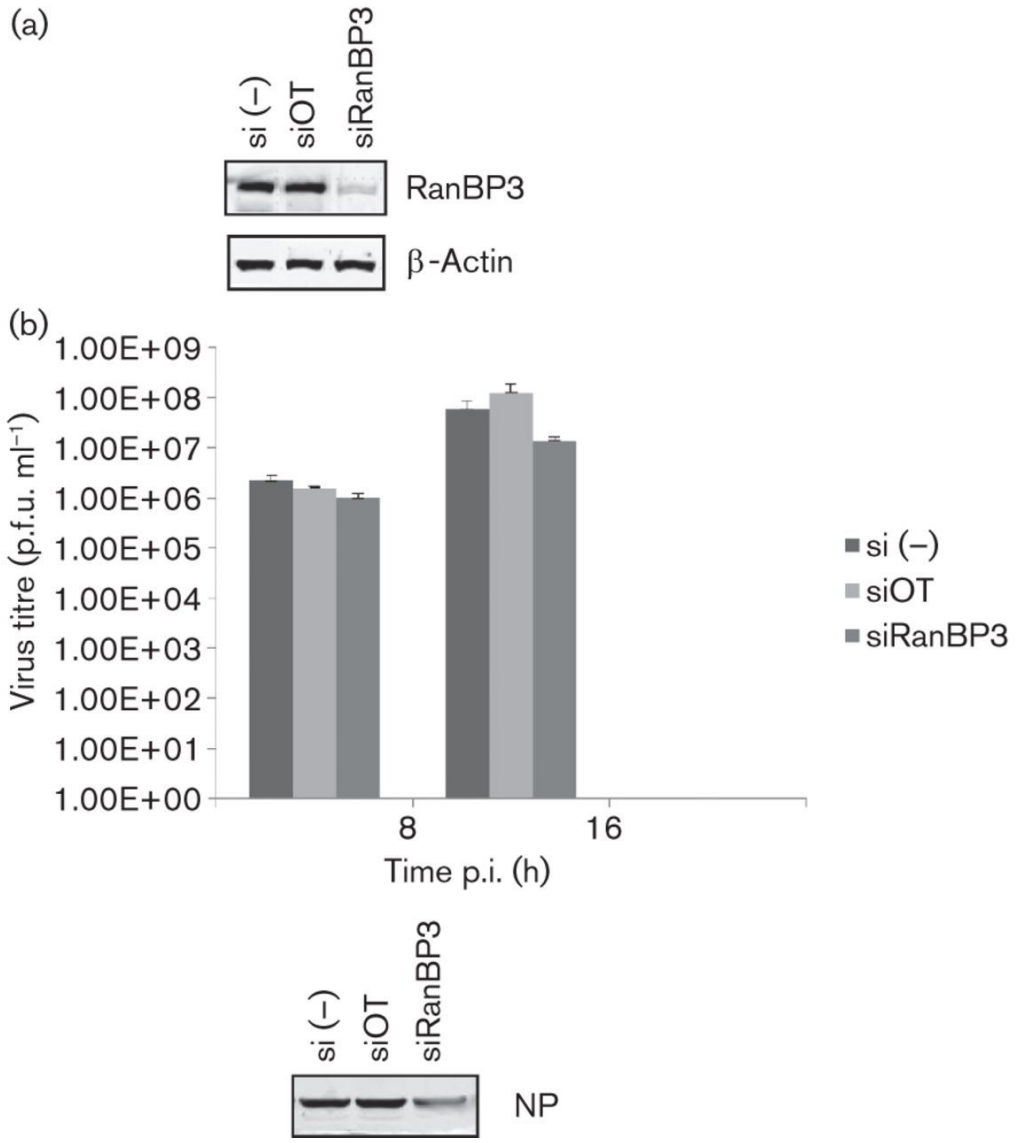

Fig. 3.2. Knockdown of RanBP3 resulted in reduction of progeny virus titer. A549cells were mock transfected or transfected with off-target siRNA (siOT) or siRNAs targeting RanBP3 (siRanBP3). At 48 h post transfection, cells were subjected to analysis. (A) Western blotting with RanBP3 or β-actin antibody to determine the knockdown effect. (B) siRNA-transfected cells were infected by WT PR8 virus at an MOI of 1; at 8 and10 h.p.i., virus titers were determined by plaque assay and at 10 h.p.i. NP synthesis was determined by Western blotting.

3.3.3 Knockdown of endogenous RanBP3 impairs influenza virus RNP nuclear export.

Having observed that RanBP3 knockdown reduced progeny virus titre, we next assessed its effect on virus replication at the single-cell level. Given that RanBP3 is a cofactor of CRM1 and influenza vRNP nuclear export is CRM1 dependent, we thus examined whether RanBP3 is involved in regulating vRNP nuclear export. Using the same transfection conditions, A549 cells were treated with siRNA and then infected with PR8 virus. At 11 h p.i., the cells were fixed and stained with antibodies specific for NP and RanBP3. As seen in Fig. 3.3(a), endogenous RanBP3 was predominantly localized to the nucleus, which is consistent with the previous report of Yoon et al. (2008). In virus-infected cells, NP was found predominantly in the cytoplasm of non-siRNA, or off-target siRNA-transfected cells, indicating that the majority of NP had been exported from the nucleus. In contrast, the majority of NP accumulated in the nucleus of RanBP3-knockdown cells, indicating that NP nuclear export is disturbed (Fig.3.3b). Cells were counterstained with DAPI. Of about 50 cells examined, 83% of siRNA non-transfected cells and 90.3% of off-target siRNA-transfected cells exhibited cytoplasmic NP staining, whereas 71.2% of siRanBP3-transfected cells showed NP nuclear staining.

3.3.4 Phosphorylation of RanBP3 at Ser58 is essential for influenza virus RNP transport.

As we observed that RanBP3 is phosphorylated at Ser58 during virus infection and downregulation of RanBP3 expression impairs vRNP export, we next assessed the possibility that RanBP3 phosphorylation at Ser58 would be responsible for vRNP nuclear export.

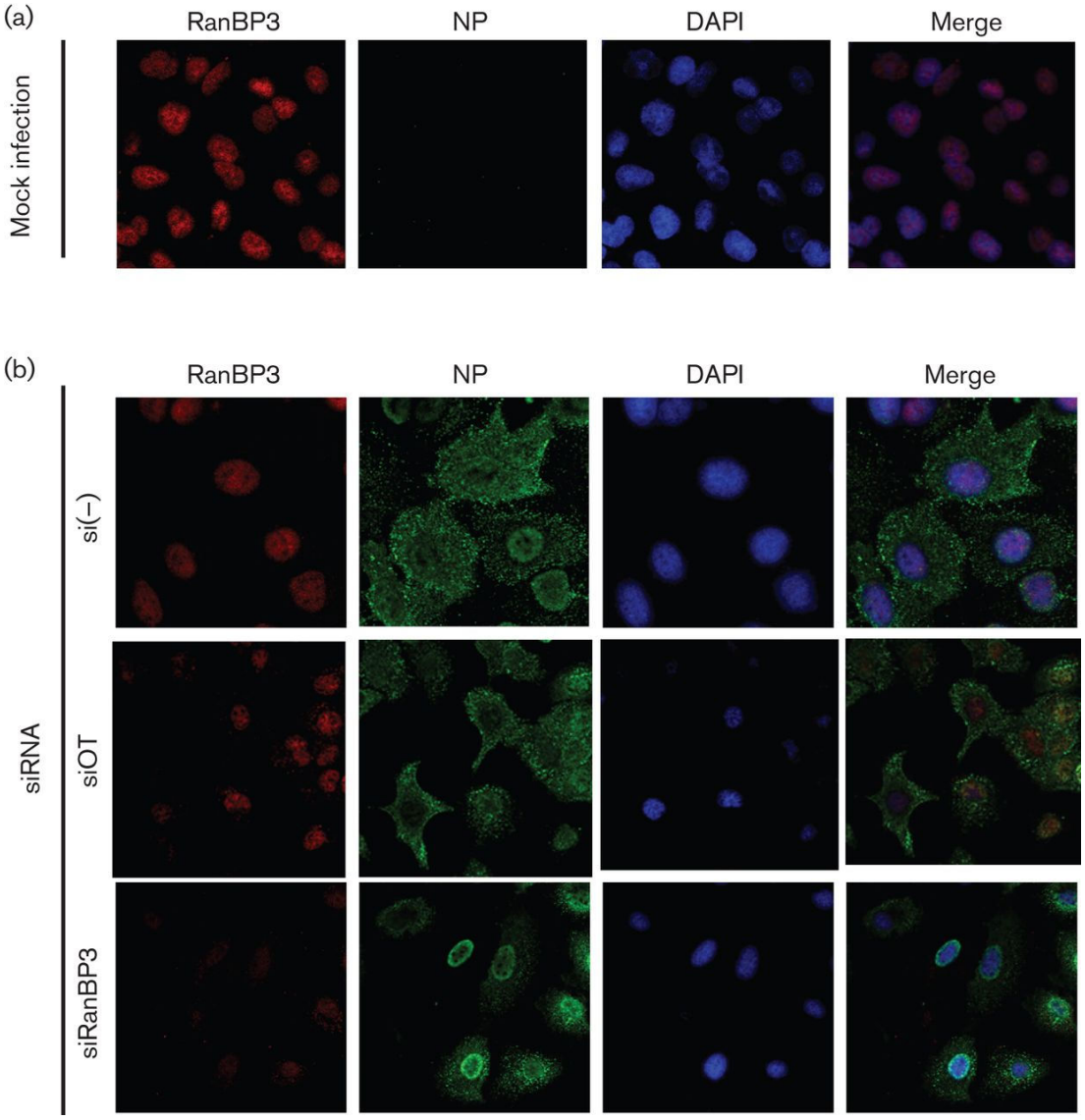

Fig. 3.3. Knockdown of RanBP3 impaired influenza virus vRNP nuclear export. A549 cells were left un-transfected, or were transfected with off target siRNA, or siRNA targeting RanBP3. Forty-eight hours later, cells were mock infected (A), or infected with PR8 at MOI of 1. At 11 h. p.i., the intracellular localization of RanBP3 and NP was determined by staining with specific antibodies. Cells were counter stained with DAPI.

Using electroporation, MDCK cells were transfected with plasmids expressing Flag-tagged wild-type RanBP3, dominant negative RanBP3-S58A, or constitutively active RanBP3-S58D (Yoon *et al.*, 2008) and allowed to express the exogenous proteins for 8 hours. The transfected cells were then super-infected with PR8 virus and at 10 h.p.i., vRNP trafficking was assessed by immunofluoresence microscopy. For each type of plasmid transfection, about 100 transfected and superinfected cells were examined, where Flag-specific antibody was used to distinguish the transfected cells from non- transfected cells; and NP antibody was used to identify the superinfected cells.

As seen in Fig. 3.4, in non-transfected infected cells, almost all vRNP was transported into the cytoplasm; 38.2% of the cells that expressed the dominant-negative RanBP3-S58A exhibited nearly complete retention of vRNPs in the nucleus. This was in stark contrast to vRNP distribution and trafficking in cells expressing wt RanBP3, in 75.5% of which the majority of vRNPs were exported to the cytoplasm. The vRNP distribution exhibited in cells expressing constitutively active RanBP3- S58D was similar to that in cells expressing wt RanBP3 (58.2% of RanBP3-S58D expressing cells showed vRNP cytoplasmic localization). It was interesting to note that the vRNP distribution in dominant-negative RanBP3-expressing cells closely resembled the vRNP distribution in RanBP3-knockdown cells (Fig. 3.3b), where vRNPs were retained in the nucleus.

3.3.5 Phosphorylation of RanBP3 at Ser58 is regulated by both PI3K/Akt and Ras/ERK pathways in the late phase of infection.

Since we observed that RanBP3 is activated during influenza virus infection, we sought to examine whether the upstream signals, specifically PI3K/Akt and Ras/ERK pathways, would

regulate RanBP3 phosphorylation. We focused on the late phase of virus life cycle, since we could not detect PI3K/Akt pathway activation until at 6 h.p.i.(Shin *et al.*, 2007b).

Serum-starved A549 cells were infected with PR8 virus, followed by treatment with Ras/ERK/RSK inhibitor U0126 and PI3K inhibitor LY294002 individually or in combination. At 9 h. p. i., cell lysates were prepared and subjected to Western blotting analysis to assess the phosphorylation of RanBP3 on Ser58. As seen in Fig. 3.5, neither U0126 alone nor LY294002 alone could completely inhibited phosphorylation of RanBP3 (lane 3 and 4). However, combination treatment of the cells with both inhibitors resulted in the inhibition of RanBP3 phosphorylation (lane 5). The levels of total RanBP3 and β-actin remained constant in all samples, indicating that the changes in RanBP3 phosphorylation were not due to changes in cellular protein levels.

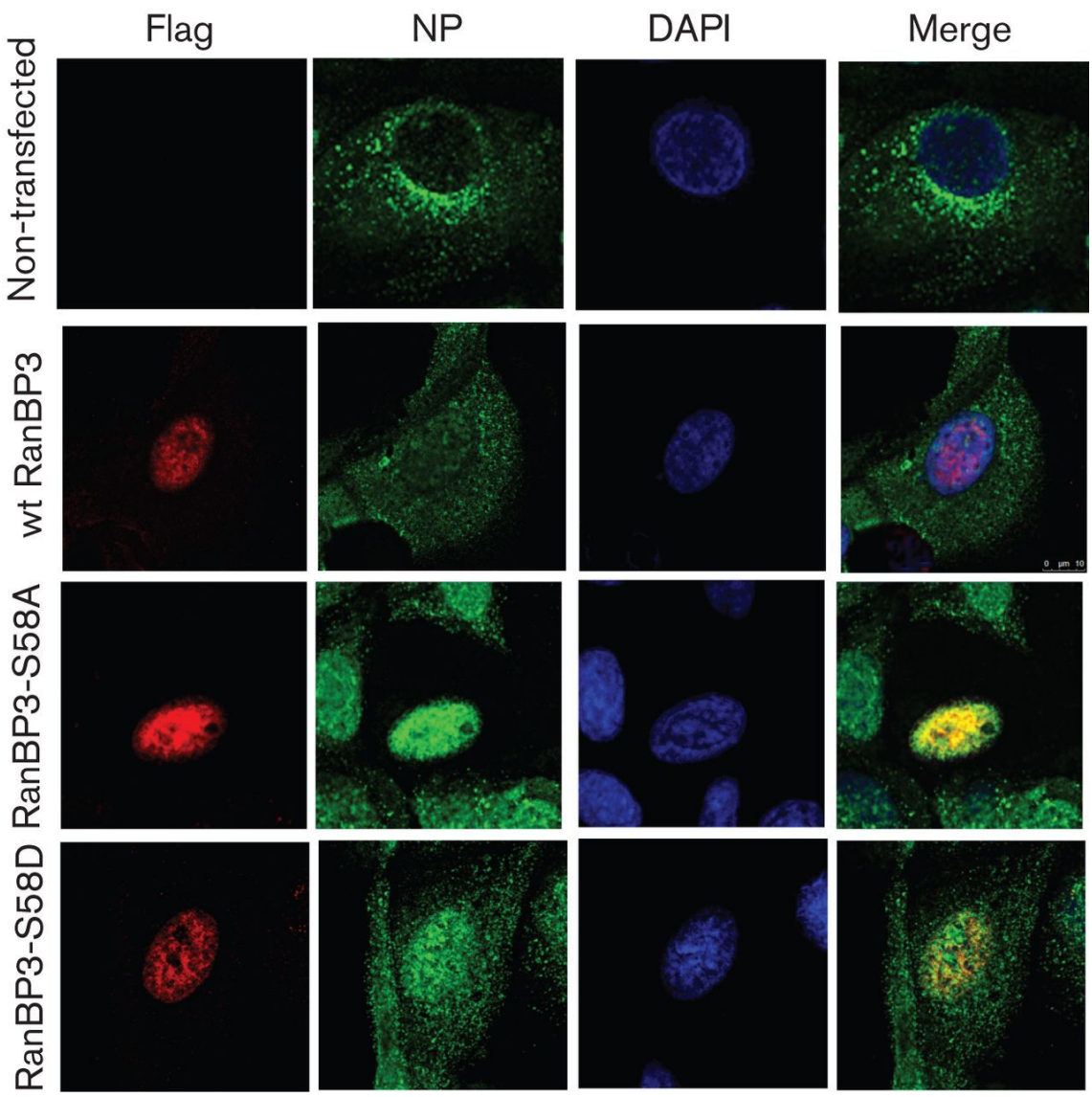

Fig. 3.4. Phosphorylation of RanBP3 at Ser58 is essential for influenza vRNP nuclear export. MDCK cells were non-transfected or transfected with plasmids expressing Flag tagged wt RanBP3, dominant negative construct RanBP3-Ser58A, or constitutive active construct RanBP3-S58D. Cells were then infected with PR8 virus at an MOI of 1 and were fixed at 10 h.p.i. The expression of extrogenous RanBP3 and the subcellular localization of NP were determined by staining with Flag antibody and NP antibody. Cells were counter stained with DAPI.

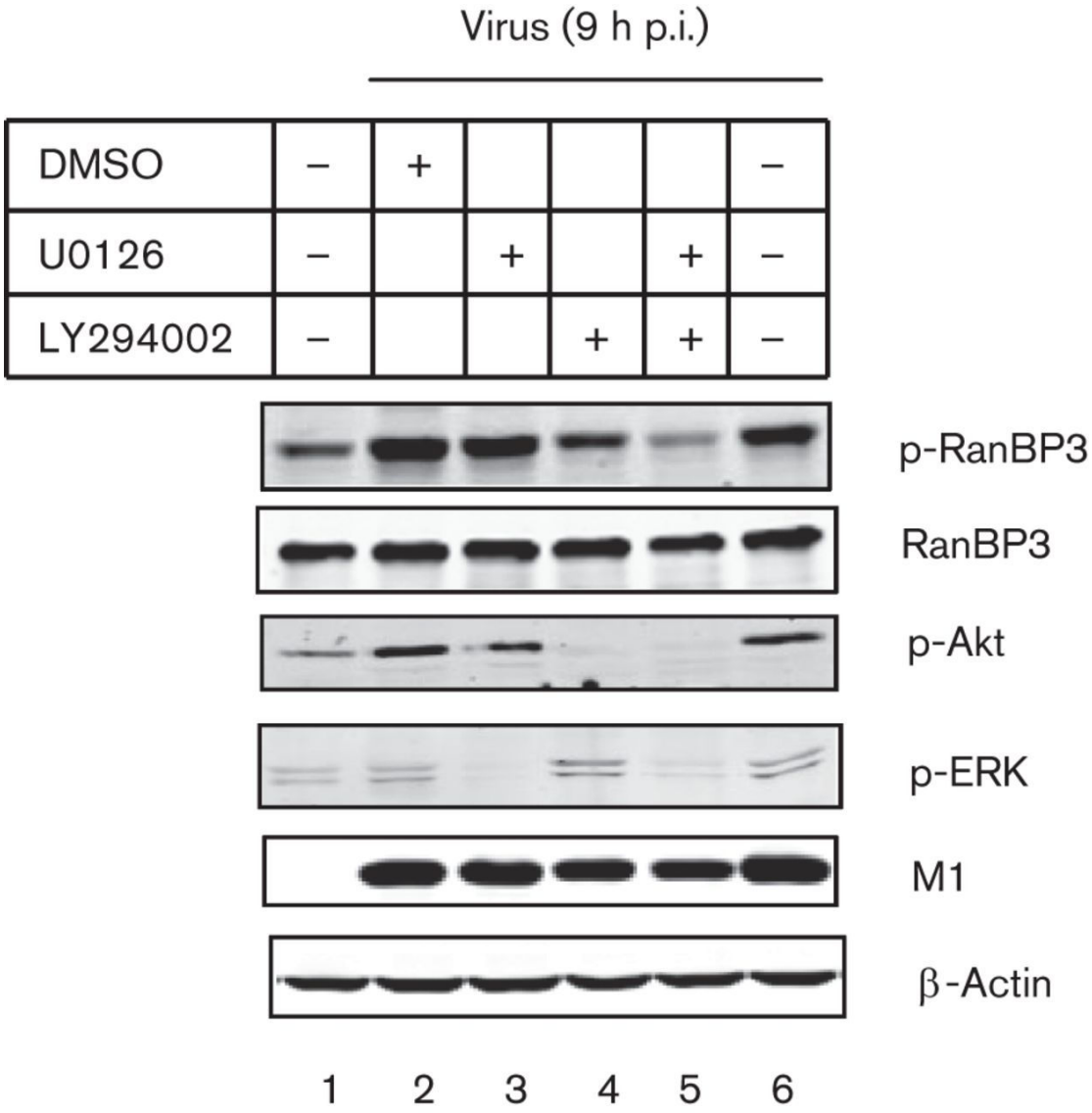

Fig. 3.5. Phosphorylation of RanBP3 at Ser58 is regulated by both PI3K/Akt pathway and Ras/ERK/RSK pathway in the late phase of virus infection. A549 cells were infected with PR8 at an MOI of 1. After 1 hour virus absorption, cells were treated with inhibitors (10μM U0126 and/or 20μM LY294002). At 9 h.p.i., cells were harvested and subjected to Western blotting analysis with specific antibodies.

3.4 DISCUSSION

Influenza A virus transcription and replication occur in the host nucleus, because the virus is dependent on the RNA processing machinery of the host cell. During the processes of importing viral genomic segments into the nucleus, exporting back out the cytoplasm, and then preventing them from re-entering the nucleus, the virus utilizes the cellular transport machinery. Active transport through the host cell nucleus via the NPC requires several host factors including RanBP3. In this study, we investigated the role of RanBP3 in influenza virus replication.

We initially examined if influenza virus infection activates RanBP3. We found that infection of cells with influenza A led to a biphasic activation of RanBP3 during the early phase of infection and again at the late phase of infection starting at 6 h p.i. (Fig 3.1). The early phase RanBP3 phosphorylation could not be eliminated in UV-inactivated cells, suggesting that is corresponds to initial virus-host cell interactions such as virus attachment and adsorption. UV-inactivated virus could not activate RanBP3 phosphorylation in the late phase of infection, suggesting that the late-phase RanBP3 phosphorylation is viral replication dependent. This late phase activation suggests that RanBP3 might be utilized by the virus.

We next examined whether RanBP3 has a biological function in virus replication by assessing the virus yield in RanBP3-knocked down cells. Knockdown of RanBP3 expression did not significantly alter expression level of non-targeted proteins, but it reduced progeny virus yield by 57% (Fig 3.2B). However, there was no difference between the expression of the viral protein NP in the RanBP3-knocked down cells and in the off-target siRNA treated cells. Upon examining the effect of RanBP3 knockdown at the individual cell level by immunofluoresence assay (Fig 3.3), we found that vRNPs were retained in the nucleus of RanBP3-knocked down cells, while vRNPs were predominantly in the cytoplasm of off-target siRNA treated cells. Since

the NP immunoblots in Fig 3.2A were from whole cell lysates, and not from separate cytoplasmic and nuclear fractions, no considerable difference in NP levels were seen between the RanBP3 knockdown and the off-target siRNA-treated cells. The total vRNPs in both treatments may be similar, but were differently partitioned between the cytoplasm and nucleus, this was in agreement with the previous report that inhibition of CRM1- mediated nuclear export did not inhibit viral protein synthesis (Elton *et al.*, 2001). However, our results from microscopic examination of vRNP transport in RanBP3 knocked down cells provided more evidence and clearly demonstrated that RanBP3 has a role in vRNP export (Fig 3.3). Recently, Chase *et al.* (2011) reported a unique chromatin- targeting strategy to facilitate vRNP export, where the chromatin association of vRNPs leads to a retention of CRM1 and Ran, which confers the advantage of vRNPs' nuclear export over other CRM1 substrates. This study further confirmed the role of CRM1 and Ran in facilitating vRNP nuclear export. Since RanBP3 could increase the affinity and association of CRM1 for RanGTP, therefore stabilizing the ternary CRM1/Ran/cargo complex in the nucleus (Langer *et al.*, 2011; Nemergut *et al.*, 2002), it is possible that in the RanBP3 knocked down cells, CRM1/Ran/vRNP complex was unstable, leading to a vRNP nuclear retention.

Upon observing that RanBP3-Ser58 is phosphorylated during virus infection and that RanBP3 knockdown abrogates vRNP export, we further investigated if Ser58 is a regulatory site for RanBP3's function in vRNP export. We found that overexpression of dominant-negative RanBP3-S58A in MDCK cells results in a nearly complete retention of vRNPs in the nucleus, which is similar to the result shown of vRNP distribution in RanBP3 knocked down cells (Fig 3.4 and 3.3B, respectively). In contrast, the cells overexpressing wild-type RanBP3, constitutively-active RanBP3-S58D, and endogenous RanBP3 all had vRNPs exported to the

cytoplasm (Fig 3.4). These results demonstrated that the function of RanBP3 in vRNP export is regulated by phosphorylation at Ser58. Yoon *et al.* (2008) reported that Ser58 is the only phosphorylation site in RanBP3 by the kinases RSK and Akt. Phosphorylation at this site regulates nuclear export by modulating the Ran gradient across the cytoplasm and nucleus in part by controlling RCC 1 activity. Whether this is the mechanism by which RanBP3 phosphorylation regulates influenza vRNP nuclear export, more detailed study is required in the future.

To identify the upstream signals that regulate RanBP3 phosphorylation upon influenza virus infection, we were particularly interested in the PI3K/Akt and Ras/ERK pathways, since previous studies showed that blockage of these pathways by the specific inhibitors led to a vRNP nuclear retention (Pleschka *et al.*, 2001; Shin *et al.*, 2007b).

We found that at the late phase of infection, single treatment of either PI3K/Akt or Ras/ERK inhibitor only partially decreased RanBP3 activation. In contrast, combination treatment of the cells resulted in a complete inhibition of RanBP3 activation (Fig. 3.5). RSK and Akt can recognize and phosphorylate serine or threonine residues in the consensus sequence RXRXXS/T, and RanBP3 has such a consensus sequence: ^{53}RERTSS^{58}. Our data suggested that for a full phosphorylation of RanBP3, both PI3K/Akt and Ras/ERK pathways are required. Pleschka et al reported that inhibition of the Raf/MEK/ERK signaling pathway by U0126 impairs vRNP nuclear export, and proposed it was through some cellular factors. Shin et al (2007b) proposed that inhibition of PI3K/Akt pathway by LY294002 led to vRNP retention in the nucleus might be due to reduced M1 protein synthesis. In the present study, our results may contribute to a novel mechanism by which inhibition of vRNP export when the aforementioned pathways are blocked is due to the inactivation of RanBP3's function. We also detected RanBP phosphorylation at early phase of infection. This early activation might be triggered by virus

attachment, since UV inactivated virus was still able to activate RanBP3 phosphorylation (Fig. 3.1). However the early activation of RanBP3 seems to be independent on both Ras/ERK and PI3K/Akt pathways, as U0126 and LY294002 did not inhibit RanBP3 phosphorylation either individually or synergistically (data not shown). While the late phase activation of RanBP3 is regulated by both PR3K/Akt and Ras/ERK/RSK pathway and contributed to regulation on vRNP nuclear export, the mechanism and function of early phase RanBP3 phosphorylation remains to be elucidated.

In conclusion, we have identified RanBP3 as a host factor that has a vital role during influenza A virus replication. Phosphorylation of RanBP3 is regulated by both Ras/ERK/RSK and PI3K/Akt pathways, and is involved in CRM1-mediated shuttling of viral RNPs from the nucleus to the cytoplasm. Identifying this host factor will contribute to the understanding of the mechanism of vRNP transport.

4.0 GENERAL CONCLUSION

Influenza A virus transcription and replication occur in the host nucleus, because the virus is dependent on the host cell's RNA processing machinery. During the processes of importing viral genomic segments into the nucleus, and exporting newly synthesized vRNA genomes in the form of vRNPs back to the cytoplasm, the virus exploits the cellular transport machinery. At the late phases of infection, the active transport of vRNPs from the host cell nucleus to the cytoplasm via the nuclear pore complex requires several host factors including the chaperone protein RanBP3.

Our study has demonstrated that RanBP3 is a host factor that is utilized during influenza A virus replication, specifically in vRNP nuclear export. RanBP3 was activated at the early and late stages of virus replication. Ablation of RanBP3 expression led to retention of vRNP in the nucleus, suggesting that RanBP3 has a role in vRNP nuclear export. Moreover, the function of RanBP3 during vRNP nuclear export is regulated by phosphorylation at Ser58, as shown by nuclear retention of vRNPs in cells expressing dominant-negative RanBP3, as compared to vRNP nuclear export in cells expressing constitutively-active and wild-type RanBP3. Since RanBP3-Ser58 phosphorylation is involved in regulating the Ran gradient between the cytoplasm and nucleus, which facilitates nuclear export, more study is needed to determine if this is the mechanism by which influenza vRNP export is regulated.

We further demonstrated that at the late phase of influenza A replication, RanBP3's function in vRNP export is regulated by both PI3K/Akt and Ras/ERK/RSK pathways (Figure 4.1). Inhibition of RanBP3 phosphorylation was only observed when both protein kinase signaling pathways were blocked, suggesting that both PI3K/Akt and Ras/ERK/RSK are required for full phosphorylation of RanBP3. Activated RanBP3 assists in vRNP export by interacting

with nuclear CRM1, and increasing the affinity of CRM1 complex with its cargo, resulting to an efficient nuclear export. These results pose a novel mechanism for the inhibition of vRNP export when the PI3K/Akt and Ras/ERK/RSK pathways are blocked. Identifying this function of RanBP3 during influenza A replication will contribute to the elucidation of the detailed mechanism of vRNP export, and further confirmed the role of CRM1 and Ran in facilitating vRNP export.

While the late phase activation of RanBP3 is modulated by both PI3K/Akt and Ras/ERK pathways, the early phase RanBP3 phosphorylation appears to be independent of these two pathways. The early phase RanBP3 activation might be the result of virus attachment, since UV-inactivated virus was still able to trigger RanBP3 phosphorylation. The mechanism and function of early phase RanBP3 phosphorylation remain to be elucidated in future studies.

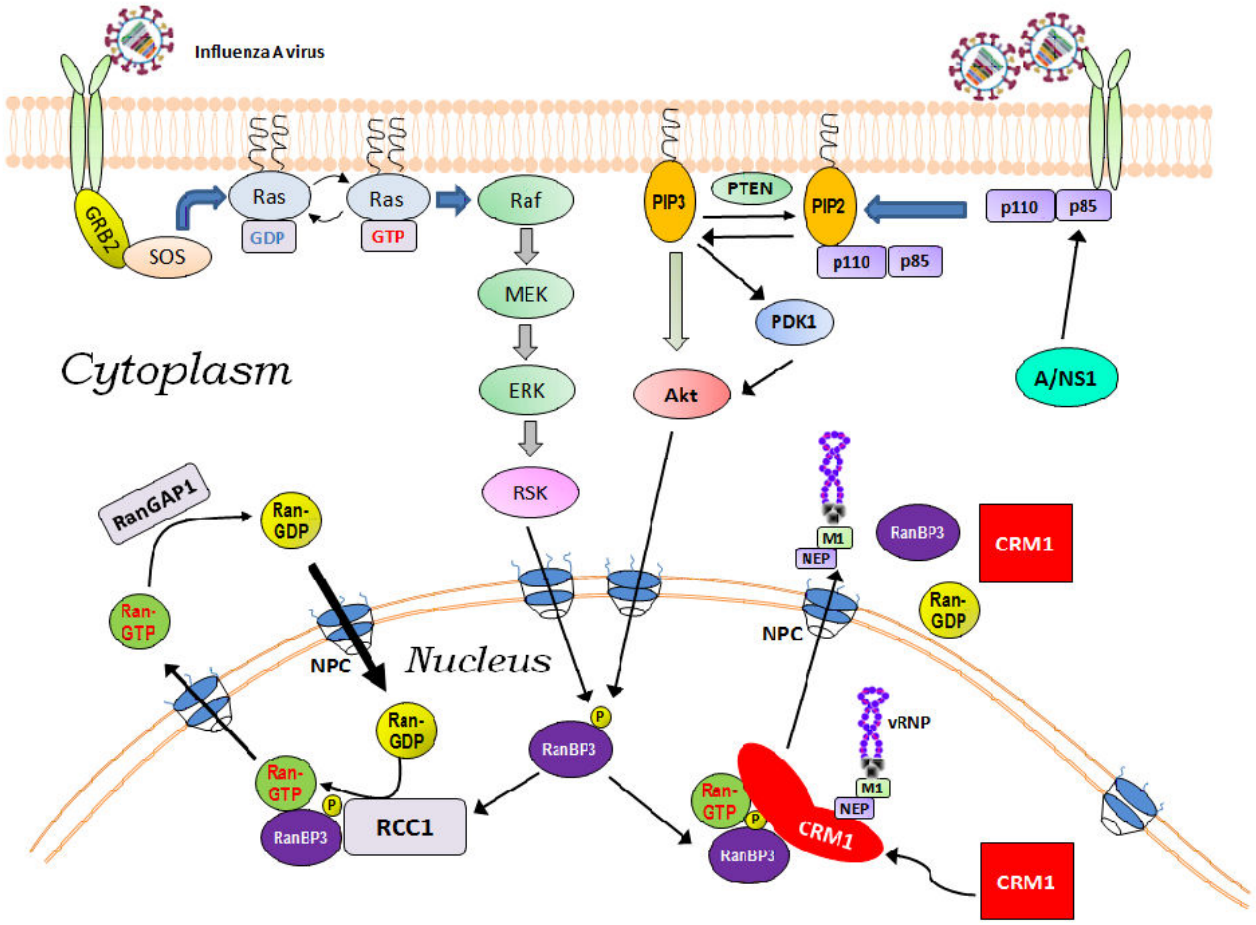

Figure 4.1 Proposed model for the regulation of RanBP3 activation during influenza A virus replication. Influenza A virus replication induces the PI3K/Akt pathway by NS1 protein during the late phase of infection. Akt is activated via PIP3 and the kinase PDK1. The activated Akt in turn phosphorylates RanBP3. Influenza virus A replication also activates the Ras/ERK pathway when virions bind to receptor tyrosine kinase and Ras is activated. Ras in turn recruits Raf to the plasma membrane and triggers downstream signals which leads to phosphorylation of RanBP3 via RSK. Both RSK and Akt/PKB phosphorylate RanBP3 at Ser58, probably in the nucleus. Phosphorylated RanBP3 has a higher affinity for Ran-GTP and stimulates the generation of Ran-GTP by RCC1 to create a Ran gradient between the cytoplasm and nucleus. Further, activated RanBP3 assists vRNP export by interacting with nuclear CRM1. It stabilizes the interaction of CRM1-RanGTP and the cargo (NEP-M1-vRNP) by increasing the affinity of the CRM1 complex for the cargo. RanBP3 induces a conformational change in CRM1 to stabilize the complex which is eventually translocated through the nuclear pore.

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