The Pennsylvania State University
The Graduate School
Department of Veterinary and Biomedical Sciences

THE ROLES OF POST-TRANSLATIONAL MODIFICATION OF PIV5 P PROTEIN IN VIRAL GENE EXPRESSION

A Dissertation in
Cell and Developmental Biology
by
Dengyun Sun

© 2011 Dengyun Sun

Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

May 2011
The dissertation of Dengyun Sun was reviewed and approved* by the following:

Biao He  
Associate Professor of Infectious Diseases  
University of Georgia  
Dissertation Co-Advisor  
Co-Chair of Committee  

Anthony P. Schmitt  
Assistant Professor of Molecular Immunology and Infectious Diseases  
Dissertation Co-Advisor  
Co-Chair of Committee  

Na Xiong  
Assistant Professor of Immunology  

Avery August  
Professor of Immunology  
Cornell University  

Andrea Mastro  
Professor of Microbiology and Cell Biology  

Zhi-Chun Lai  
Professor of Biology, Biochemistry and Molecular Biology  
Cell and Developmental Biology Program Chair  

*Signatures are on file in the Graduate School
ABSTRACT

Paramyxovirus RNA synthesis requires the large (L) protein and the phosphoprotein (P). It was initially thought that phosphorylation of the P protein was important for viral gene expression, however there is no direct proof supporting the assumption. Recently, our group reported that phosphorylation at S157 of the P protein of parainfluenza virus 5 (PIV5), a prototypical paramyxovirus, correlated with decreased viral gene expression. In this study, I have shown that: (1) Polo-like kinase 1 (PLK1) bound the P protein through the S(pS157)P motif; (2) PLK1 inhibitor increased PIV5 gene expression; (3) PLK1 over-expression inhibited PIV5 gene expression; (4) PLK1 inhibitor reduced phosphorylation level of the P protein in PIV5 infected cells; and (5) PLK1 directly phosphorylated the P protein in vitro, indicating that PLK1 down-regulated PIV5 gene expression by phosphorylating the P protein. Furthermore, I have determined the PLK1 phosphorylation site, S308, within the P protein and found that mutation at S308 to alanine increased the minigenome activity, which was not affected by either PLK1 inhibitor or PLK1 over-expression. Mutation at either the binding site or the phosphorylation site of PLK1 in PIV5 increased virus gene expression, which correlated with increased induction of cell death and cytokine induction. The results indicate that PIV5 limits its viral gene expression to avoid induction of innate immune responses.

Since the P protein is heavily phosphorylated, we speculate that phosphorylation of the P protein at other sites may play a positive role in PIV5 gene expression. I have identified a phosphorylation site of PIV5 P protein by mass spectrometry, T286, and found that (1)
mutation of T286 to alanine reduced PIV5 minigenome activity; (2) P-T286D showed very low activity and P-T286E showed no activity in the minigenome system; (3) rPIV5-P-T286A virus grew slower than PIV5; (4) viral RNA synthesis and protein expression in rPIV5-P-T286A infected cells were delayed. The defects of T286A/D/E were not due to NP-P-L complex formation. I also found that P-T286A/D/E affected viral transcription. It is the first time that the P protein phosphorylation is found to be important for paramyxovirus gene expression in virus infection.

Sumoylation is a protein post-translational modification and plays an important role in regulating protein function and signal transduction. I have discovered that the P protein of PIV5 can be sumoylated by SUMO1 in both transfected cells and infected cells. I have identified one sumoylation site within the P protein, K254. Mutation at K254 to arginine (P-K254R) reduced PIV5 minigenome activity. Incorporation of K254R into the genome of the virus (rPIV5-P-K254R) resulted in reduction of viral growth, viral RNA synthesis and viral protein expression, indicating that PIV5 uses host sumoylation system to regulate its growth.
TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. xi
LIST OF TABLES ...................................................................................................................... xiv
ACKNOWLEDGEMENTS .......................................................................................................... xv

CHAPTER 1: LITERATURE AND BACKGROUND ................................................................ 1

1.1 Paramyxovirus and classification .................................................................................... 2
1.2 PIV5 virion, genome and viral proteins ........................................................................... 3
1.3 PIV5 entry ......................................................................................................................... 6
1.4 PIV5 transcription ............................................................................................................. 7
1.5 PIV5 replication ................................................................................................................ 9
1.6 PIV5 assembly and release ............................................................................................. 10
1.7 PIV5 and host immune response .................................................................................... 11
1.8 PIV5 reverse genetics and minigenome system .............................................................. 12
1.9 The P protein and post-translational modification ......................................................... 13

CHAPTER 2: PLK1 DOWN-REGULATES PIV5 GENE EXPRESSION ............................... 22

2.1 Abstract .......................................................................................................................... 23
2.2 Introduction .................................................................................................................... 24
2.3 Materials and methods ................................................................................................. 27
2.3.1 Plasmids, viruses and cells

2.3.2 Immunoprecipitation

2.3.3 PLK1 inhibitors and renilla luciferase assay

2.3.4 $^{33}$P labeling

2.3.5 PIV5 minigenome system and dual luciferase assay

2.3.6 PLK1 in vitro kinase assay

2.3.7 Virus rescue and full-genome sequencing

2.3.8 Flow cytometry

2.3.9 Apoptosis assays

2.3.10 Enzyme-linked immunosorbent assay (ELISA)

2.3.11 Protein purification and CDK1/cyclin B in vitro kinase assay

2.4 Results

2.4.1 PLK1 interacts with P via the SSP motif

2.4.2 PLK1 inhibitor increases rPIV5-RL gene expression

2.4.3 PLK1 inhibitor increases PIV5, but not CPI+ gene expression

2.4.4 PLK1 over-expression reduces PIV5 gene expression

2.4.5 Kinase activity of PLK1 is required for its inhibitory effect

2.4.6 PLK1 phosphorylates the P protein in infected cells

2.4.7 PLK1 phosphorylates S308 of the P protein

2.4.8 PLK1 targets S308 of the P protein in minigenome system

2.4.9 PLK1 targets S308 of the P protein in infected cells

2.4.10 S157A and S308 viruses elevate viral protein expression and growth

2.4.11 S157A and S308A viruses induce apoptosis
2.4.12 S157A and S308A viruses induce IFN-β and IL-6 production………………..………..……….45
2.4.13 CDK1 phosphorylates S157 of the P protein……………………………………………46
2.5 Summary…………………………………………………………………………………………47

CHAPTER 3: IDENTIFICATION OF A PHOSPHORYLATION SITE OF THE P
PROTEIN IMPORTANT FOR PIV5 GROWTH …………………………………………..72

3.1 Abstract…………………………………………………………………………………..………..73
3.2 Introduction…………………………………………………………………………………..74
3.3 Materials and Methods………………………………………………………………….76
3.3.1 Plasmids, viruses, and cells …………………………………………………………….76
3.3.2 Mass spectrometry analysis……………………………………………………………..76
3.3.3 PIV5 minigenome system and dual luciferase assay…………………………………77
3.3.4 Protein purification and circular dichroism………………………………………………77
3.3.5 Virus rescue and viral genome sequencing……………………………………………78
3.3.6 Growth curve and plaque assay…………………………………………………………79
3.3.7 Western blotting…………………………………………………………………………79
3.3.8 Flow cytometry………………………………………………………………………………80
3.3.9 Real time PCR……………………………………………………………………………..80
3.3.10 Immunoprecipitation-immunoblotting (IP-IB)………………………………………..81
3.3.11 Immunoprecipitation and DSP crosslinking……………………………………………82
3.4 Results…………………………………………………………………………………………83
3.4.1 T286 of the P protein is phosphorylated in PIV5 infected cells………………………83
3.4.2 P-T286A reduces PIV5 minigenome activity.................................83
3.4.3 Effect of P mutations at T286.................................................84
3.4.4 rPIV5-P-T286A shows slower growth...................................84
3.4.5 rPIV5-P-T286A shows delayed protein expression................85
3.4.6 rPIV5-P-T286A affects viral RNA synthesis............................86
3.4.7 Mutations at T286 affect viral transcription............................86
3.4.8 P mutants at T286 do not affect the formation of NP-P-L complex...87
3.4.9 Analysis of revertant viruses.................................................87
3.5 Summary.................................................................................89

CHAPTER 4: SUMOYLATION OF THE P PROTEIN AT K254 IS IMPORTANT
FOR PIV5 GROWTH ........................................................................111

4.1 Abstract.................................................................................112
4.2 Introduction ............................................................................113
4.3 Materials and methods.............................................................116
4.3.1 Plasmids, viruses and cells....................................................116
4.3.2 Western blotting.................................................................116
4.3.3 Immunoprecipitation-Immunobloting (IP-IB)........................117
4.3.4 PIV5 minigenome system and dual luciferase assay..............118
4.3.5 Protein purification and circular dichroism............................118
4.3.6 Growth curve and plaque assay............................................119
4.3.7 Flow cytometry.................................................................119
4.3.8 Reverse transcription and real time PCR .......................................................... 120
4.3.9 Immunoprecipitation and DSP crosslinking .................................................. 121

4.4 Results .............................................................................................................. 122

4.4.1 The P protein is sumoylated by SUMO1 but not SUMO2 or SUMO3 .......... 122
4.4.2 Identification of one consensus sumoylation site within P protein ............ 123
4.4.3 K254R mutation reduces PIV5 minigenome activity ................................. 123
4.4.4 K254 of the P protein is a sumoylation site in PIV5 infected cells ............ 124
4.4.5 rPIV5-P-K254R grows slower and to a lower titer than PIV5 ..................... 124
4.4.6 K254R mutation reduces viral protein expression ................................. 125
4.4.7 rPIV5-P-K254R has lower levels of viral mRNA and genome RNA .......... 125
4.4.8 K254R mutation affects viral transcription ............................................ 126
4.4.9 P-K254R does not affect the NP-P, P-L interaction and tetramer formation 126

4.5 Summary ........................................................................................................ 127

CHAPTER 5: CONCLUSIONS AND DISCUSSION ............................................ 145

5.1 Conclusions ................................................................................................... 146

5.1.1 PLK1 down-regulates PIV5 gene expression ............................................ 146
5.1.2 Phosphorylation of the P protein at T286 is important for PIV5 growth ........ 146
5.1.3 Sumoylation of K254 of the P protein is important for PIV5 growth ........ 147

5.2 Discussion .................................................................................................... 149

5.2.1 Phosphorylation in viral gene expression: negative role ....................... 149
5.2.2 Phosphorylation in viral gene expression: positive role ...................... 153
5.2.3 Sumoylation in viral gene expression.........................................................157

REFERENCES ......................................................................................................161
LIST OF FIGURES

Chapter 1

Figure 1-1: PIV5 virion and genome structure ......................................................15
Figure 1-2: Paramyxovirus life cycle ................................................................. 16
Figure 1-3 Paramyxovirus RNA synthesis (transcription and replication) ............17
Figure 1-4: RNA editing of representative paramyxoviruses ..............................18
Figure 1-5: Rescue of PIV5 from cloned cDNA ..................................................19
Figure 1-6: PIV5 minigenome system ...............................................................20

Chapter 2

Figure 2-1: Interaction between P and PLK1 .....................................................48
Figure 2-2: Effect of PLK1 inhibitor on rPIV5-RL gene expression ..................50
Figure 2-3: PLK1 inhibitor increases PIV5, but not CPI+ gene expression ..........52
Figure 2-4: Effect of PLK1 over-expression on the minigenome system ..........54
Figure 2-5: Effect of kinase-deficient PLK1 on PIV5 minigenome activity .......56
Figure 2-6: Effect of PLK1 inhibitor on phosphorylation of the P protein ........58
Figure 2-7: PLK1 phosphorylates S308 of the P protein ................................ 59
Figure 2-8: PLK1 targets S308 of the P protein in minigenome system ............60
Figure 2-9: PLK1 phosphorylates S308 of the P protein in infected cells .........63
Figure 2-10: Gene expression and growth of -S157A and S308A viruses .........65
Figure 2-11: Induction of apoptosis by S157A and S308A viruses.................................67
Figure 2-12: Induction of cytokines by S157A and S308A viruses.............................70
Figure 2-13: CDK1/Cyclin B phosphorylates S157 of the P protein .........................71

Chapter 3

Figure 3-1: Effect of P mutants on PIV5 minigenome system.................................90
Figure 3-2: Effect of P mutations on T286...........................................................93
Figure 3-3: Effect of P-T286A on viral growth....................................................95
Figure 3-4: Viral protein expression in PIV5-P-T286A infected cells........................97
Figure 3-5: Viral RNA synthesis in PIV5-P-T286A infected cells............................100
Figure 3-6: Viral transcription activity of P mutations at T286.................................103
Figure 3-7: The NP-P-L complex formation.......................................................105
Figure 3-8: Effect of revertant mutations..........................................................107

Chapter 4

Figure 4-1: Sumoylation of PIV5 P protein by SUMO1........................................128
Figure 4-2: K254 of the P protein was one sumoylation site.................................130
Figure 4-3: P-K254R reduces PIV5 minigenome activity.....................................132
Figure 4-4: Sumoylation of K254 in PIV5 infected cells.....................................133
Figure 4-5: Growth curve of rPIV5-P-K254R......................................................135
Figure 4-6: Viral protein levels in rPIV5-P-K254R infected cells............................136
Figure 4-7: Viral RNA levels in rPIV5-P-K254R infected cells.................................139
Figure 4-8: P-K254R affected viral RNA transcription........................................142
Figure 4-9: Interaction of P with NP, L and itself.................................................143

Chapter 5

Figure 5-1: A working model for regulation of PIV5 gene expression by PLK1.............160
LIST OF TABLES

Chapter 1

Table 1-1 Paramyxovidae classification and examples.................................................21

Chapter 3

Table 3-1 Summary of mass spectrometry results.........................................................109
Table 3-2: Sequencing results of 26 plaques from rescuing rPIV5-P-T286E.....................110
ACKNOWLEDGMENTS

I would like to give my sincere thanks to people who directly or indirectly contribute to my thesis and graduate study.

Firstly, I am particularly grateful to my advisor Dr. Biao He for mentoring me to pursue my Ph.D degree. He has created an excellent environment for serious science and free ideas. He has taught me the importance of passion, hard work and independence. I thank him as an excellent mentor and scientist.

I would also like to thank other committee members: Dr. Anthony Schmitt, Dr. Avery August, Dr. Na Xiong, and Dr. Andrea Mastro. Special thanks to Dr. Anthony Schmitt for improving the PIV5 minigenome system, which enable my experiments to progress smoothly. Special thanks for Dr. Avery August, Dr. Na Xiong and Dr. Andrea Mastro for their input of time and helpful suggestions.

I am also grateful to my labmates: Yuan Lin, Jie Xu, Matthew Wolfgang, Jui Patel, Dr. Zhuo Li, Dr. Minghao Sun, Dr. Khalid Timani, Dr. Sandra Fuentes, Priya Luthra, Pei Xu, and Haeyoung Yoon for their kindly help; and my graduate fellows: Qian Wang, Jiehuan Huang, Jian Li, Xiaofan Zhou and Suting Zheng for their unforgettable friendships.

Finally, I will give my dear husband Shuai Shi my special thanks for his great love and spiritual supports. I always know that I can rely on him whenever I need help.
CHAPTER 1: LITERATURE AND BACKGROUND
1.1 Paramyxovirus and classification

*Paramyxovidae*, a family of enveloped, non-segmented, negative-stranded RNA viruses, belongs to *mononegavirales*, and shares special similarity with *Orthomyxoviridae* (in properties of envelope glycoproteins) and *Rhabdoviridae* (in genome organization and gene expression) (57, 58). *Paramyxovidae* contains *Paramyxovirinae* and *Pneumovirinae* subfamilies. *Paramyxovirinae* is further classified into five genera: *Rubulavirus*, *Avulavirus*, *Respirovirus*, *Henipavirus* and *Morbillivirus*. *Pneumovirinae* contains two genera: *Pneumovirus* and *metapneumovirus*. Some recently identified viruses such as Tupaia paramyxovirus (TPMV), Tioman virus (TiV), Beilong virus and J virus are grouped into unclassified paramyxoviruses (Table 1-1). *Paramyxoviridae* contains many important human and animal pathogens such as human parainfluenza virus type 2 (HPIV2) and mumps virus (MuV), which belong to genus *Rubulavirus*; Sendai virus (SeV), human parainfluenza virus type 1 (HPIV1) and type 3 (HPIV3), which belong to genus *Respirovirus*; measles virus (MeV), Rinderpest virus, and canine distemper virus (CDV), which belong to genus *Morbillivirus*; Hendra virus (HeV) and Nipah virus (NiV), which belong to genus *Henipavirus* and are newly emerging viruses; human respiratory syncytial virus (hRSV) and bovine respiratory syncytial virus (RSV), which belong to *Pneumovirinae* (58).

Parainfluenza virus 5 (PIV5) belongs to genus *Rubulavirus* and is a prototypical paramyxovirus. It was originally isolated from cultured monkey cells and was formerly known as simian virus 5 (SV5) (58). However, its natural host is dog in which it may cause kennel cough. PIV5 can infect human and other animals, but no known disease is associated
with PIV5 infection (4, 109). PIV5 minigenome and reverse genetics system have been successfully established and have been very useful in studying viral RNA synthesis of paramyxovirus (47). Foreign genes can be inserted into PIV5 genome and stably expressed (48, 116), indicating that PIV5 is a potential viral vector for vaccine development (116).

1.2 PIV5 virion, genome and viral proteins

PIV5 virions are generally spherical, 150 to 350 nm in diameter, and contain lipid bilayer envelopes derived from the plasma membrane of infected cells (58). Glycoproteins fusion (F) protein and hemagglutinin-neuraminidase (HN) protein in the envelope form spikes which extend 8 to 12 nm from the membrane surface (Figure 1-1A) (58, 101). Small hydrophobic (SH) protein is incorporated into the envelope. The nucleocapsid core or ribonucleoprotein core is located inside the membrane and is separated by a matrix (M) protein layer from the membrane. Nucleocapsid protein (NP) encapsidates the RNA genome and with the phosphoprotein (P) and the large protein (L) forms the RNP complex (58). The PIV5 genome contains 15,246 nucleotides including 55 nucleotides as the 3’ leader sequence and 31 nucleotides known as the 5’ trailer sequence (48). The rest of the RNA genome contains seven genes in the order of NP-V/P-M-F-SH-HN-L, and encodes eight proteins, with the V/P gene encoding both the V and the P proteins (48, 58). Each gene has conserved transcriptional control sequences: gene start (GS) and gene end (GE). Between the gene boundaries are intergenic region (IG). The V/P gene transcribes both the V and the P mRNA through a process called RNA editing. While the V mRNA is transcribed faithfully from the V/P gene, the P mRNA is produced when two non-template guanines are added at a precise
site during transcription (58). As a result, the V and the P proteins share the N-terminal 164 amino acid residues and differ in their C-terminal sequences.

The RNP complex, which is the machinery for viral RNA synthesis, consists of the RNA genome, the NP, the P, and the L proteins (58). Nucleocapsid protein (NP, or N) is the first transcribed gene and is expressed at the highest level among all the viral genes. Studies of Sendai virus N protein indicate that the N protein binds six nucleotides and 13 N subunits constitute a nucleocapsid helix turn (58). The binding of the N protein to RNA can protect the RNA from nuclease digestion, align RNA to serve as a functional promoter, and provide interaction sites for virus assembly and budding. Mutational and biochemical studies have divided the N protein into two structural regions: (1) The N-terminal core, which contains about 400 conserved residues. This region is essential for RNA binding, the N protein self-assembly, and viral replication; (2) The C-terminal tail region, which is less conserved. This region may serve as the region interacting with the P and the L protein (94). A recent study on PIV5 has shown that the C-terminal end of the NP protein is important for the NP-M interaction and virus-like particle (VLP) formation (102). In infected cells, the N protein exists in at least two forms. N⁰ is an unassembled soluble form; another form is stably associated with RNA (58, 94). The P protein is an essential component of viral RNA dependent RNA polymerase (vRdRP) and associates with N⁰ to form nascent chain assembly complex. Studies from Sendai virus have shown that the P protein binds nascent N⁰ through its N-terminus and binds to the NP: RNA complex and the L protein through its C-terminus. The P protein itself can form homo-oligomer though a region within the C-terminus (20, 25, 112), which is important for the function of the P protein in viral RNA synthesis. The large
(L) protein, encoded by the last gene in PIV5 genome contains 2255 amino acid residues and is about 250 kDa in size (58, 88). Although at very low amount in virions, the L protein is an essential component of RNP complex. The L protein is the RNA dependent RNA polymerase (RdRP) and is thought to contain all the enzymatic activities required for viral RNA synthesis, including nucleotide polymerization, methylation, 5’ capping, and 3’ poly (A) addition (58, 78).

The M protein, the most abundant protein in the virion, is critical for virus assembly and budding. The M protein can associate with the plasma membrane, the NP protein as well as the F and the HN glycoproteins (58, 101). PIV5 HN protein, a type II integral membrane protein, has hemagglutinase and neuraminidase activities, and plays an important role in viral attachment. The Fusion (F) protein, a type I integral membrane protein, mediates the fusion between virion and host membrane at neutral pH (58). The F protein can form a homotrimer that is synthesized as a precursor (F0). The F0 protein contains a signal peptide at the N-terminus, fusion peptide, one hydrophobic haptad repeat (HRA) following fusion peptide, and another haptad repeat (HRB) close to the transmembrane (TM) domain at the C-terminus. After cleavage by host protease, it forms a biologically active protein consisting of F1 and F2 linked by a disulfide bond. Small hydrophobic protein (SH) is the third membrane protein of PIV5. SH is not required for virus life cycle in vitro, since knocking out the SH gene does not affect PIV5 growth in MDBK cells (46). However, SH protein plays an important in blocking TNF-α induced apoptosis in virus infected cells (71).

The V protein is an accessory protein, which contains 222 amino acid residues, and shares
the N-terminus with the P protein. PIV5 V protein has multiple functions including counteracting host antiviral responses, slowing down cell cycle, and regulating viral RNA synthesis (69, 72, 73). The V protein, through its Cys-rich C-terminus, binds to several cellular proteins including Damage-specific DNA-Binding protein 1 (DDB1) and Melanoma Differentiation-Associated protein 5 (MDA-5) (24, 66). Disruption of Cys-rich C-terminus of the V protein in PIV5 (rPIV5VΔC) results in severe cytopathic effect (CPE) and apoptosis in infected cells (111).

1.3 PIV5 entry

The general life cycle of paramyxovirus is summarized in Figure 1-2. The first step is entry, which is mediated by the HN and the F proteins. The HN tetramer binds to its receptor (sialic acid) on the cell surface. Receptors or coreceptors have been identified for measles virus (CD46 or SLAM), Nipah and Hendra virus (ephrine B2) (17, 58, 86, 114). The receptor of PIV5 is not clear. The fusion between virion envelope and cell membrane is mediated by the F protein, a class I fusion protein. Fusion peptide within the F protein causes the membrane to merge. Before activation, the hydrophobic fusion peptide is buried inside. The fusion peptide is exposed due to conformational change and inserts into the plasma membrane of the target cell. After insertion of the fusion peptide, the F protein folds irreversibly into a more stable form, which brings the two haptad repeats (HRA and HRB) together to form a six-helix bundle (9). This six-helix bundle forces the membranes of the virion and the host cell to fuse together, resulting in the release of the contents of virion into the cytoplasm of the cell (9, 58).
The triggering mechanism that regulates the F protein conformational changes is unknown. However, the binding between the HN and its receptor is required (60). Association of the F and the HN protein has been found by coimmunoprecipitation. Coexpression of the HN protein and the F protein either is necessary or makes fusion more efficient (29, 64). A model which may explain the involvement of the HN in fusion promotion has been proposed: after binding to its receptor, the HN undergoes a conformational change which triggers the structural change of the F protein and causes the exposure of fusion peptide within the F protein (60). For PIV5, an alternative model explaining involvement of the HN protein in fusion is that binding of the cell surface receptor results in partial disassembly of the HN tetramer and association of HN-F, which activates the F protein for membrane fusion (58, 60).

1.4 PIV5 transcription

After PIV5 enters a cell, the viral RNP accesses viral genes through a single entry site near the 3’ leader sequence of the genome and produces the leader RNA and viral mRNA from the viral genome, which is called primary transcription (Figure 1-3). At a later time, when progeny genomes have been produced through viral replication, they serve as additional templates to produce more viral mRNA, which is called secondary transcription (58). The viral RNA polymerase, the L protein, transcribes mRNA from the 3’ leader sequence of PIV5 and the NP gene is the first gene transcribed. When the polymerase reaches the gene end (GE) region which contains a stretch of uridine residues (four to seven), it stutters and adds
poly (A) tail to the nascent mRNA. The cap structure is added to the 5’ end of viral mRNA during transcription by the polymerase. The viral RNA polymerase passes the intergenic region (IG) and reinitiates transcription at the next gene start (GS) site. This sequential transcription is called stop-start mechanism which continues across the whole genome from the 3’ end to the 5’ end (58). However, the frequency of reinitiation is not 100%. Therefore, not every RNP that terminates at a GE region reinitiates at the next GS region, which leads to a gradient of mRNA abundance, with the NP mRNA being the highest and the L mRNA being the lowest (58). Sometimes, RNP ignores the GE sequence and synthesizes a readthrough product containing both upstream and downstream mRNA.

For the V/P gene of PIV5, a pseudotemplated addition of nucleotides known as RNA editing leads to production of the V mRNA and the P mRNA (Figure 1-4) (45). The V mRNA is the unedited transcript of the V/P gene, and the P mRNA is produced by insertion of two guanines at a precise site. The frequency of RNA editing is about 50%. Therefore, the V mRNA and the P mRNA show similar level in infected cells. In morbilliviruses, and respiroviruses, the P mRNA is the unedited copy (58). When a single residue is added, the P gene produces the V Open Reading Frame (ORF). The number of guanine insertion and the number of different mRNA produced differ for different virus groups (57, 58). This RNA editing, a mechanism for virus to obtain more coding potential, was first identified in PIV5 and has now been found in most paramyxoviruses (58).
1.5 PIV5 replication

After primary transcription and accumulation of viral proteins, the negative-sense genome is used as the template to produce a full-length antigenome, which is further used as the template to produce more genome copies (Figure 1-3) (58). Those progeny genomes can serve as the templates for mRNA synthesis (secondary transcription), the templates to produce more antigenomes (replication), and content for progeny virions (assembly) during virus budding. The newly synthesized genome and antigenome are quickly encapsidated by the NP protein to prevent nuclease degradation.

The sequences for replication initiation are located at the 3’ end (leader) of the genome and the 3’ end (trailer) of the antigenome. Antigenomes are typically found at a lower level than genomes in infected cells, suggesting that there is a bias for vRdRP to produce more genomes and the function of the antigenome is likely an intermediate in virus replication (58). This could be because the 3’ end promoter of the antigenome is stronger than that of the genome. Interestingly, the number of nucleic acids in genome and antigenome affects the replication efficiency. In PIV5, Sendai virus, NDV, hPIV3, and Nipah virus, the number of nucleotides needs to be a multiple of six, called the rule of six, to have the highest efficiency (44, 45, 58). This requisite may be that the NP protein contacts six nucleotides to form a subunit. A genome length with a multiple of six nucleotides can be precisely encapsidated by the NP protein, with no free nucleotide protruding from the end of the nucleocapsid (58). Genome and antigenome replication can also be regulated by viral accessory protein. The V
proteins from PIV5 and Sendai virus inhibit virus replication (58, 72). NS1 protein of RSV is also found to be able to inhibit RNA replication in RSV minigenome system (8).

The switch from transcription to replication is regulated by the amount of unassembled N⁰ and other accessory proteins. The leader region contains the N encapsidation site, therefore, the newly synthesized leader RNA must be separated from the NP mRNA by termination and reinitiation (58). After infection, unassembled NP protein is limited. vRdRP prefers mRNA synthesis, which results in an increased level of viral proteins, including unassembled NP protein. When unassembled NP protein reaches a certain amount, some vRdRP will switch to the replication model to produce more antigenome and genome, and reduce the relative level of unassembled NP protein. The phosphorylation status of the P protein and viral accessory protein such as M2-2 protein of RSV may play a role in the switch from transcription to replication (5, 14, 58, 98).

1.6 PIV5 assembly and release

Like other enveloped viruses, PIV5 particles are released by a process called budding, in which viral components assemble underneath plasma membrane and pinch off to release virus particles (58). The assembly of the nucleocapsid includes the association of the NP subunits with the genome to form the helical RNP structure and the association of the P-L complex. The assembly of the envelope proteins and the RNP occurs at plasma membrane, in which the M protein plays important roles (58, 101). The M protein can bind both RNP core and envelope glycoprotein cytoplasmic tail, self assembles into ordered structure, and induce
viral components to concentrate at certain sites underneath the plasma membrane (58). Viral like particles (VLP) has been used to study virus assembly and budding (101). In PIV5, expression of the M protein alone by transfection does not induce efficient VLP formation. When the M protein is coexpressed with the NP protein and a viral glycoprotein F or HN, the VLP formation becomes very efficient, similar to that of infected cells. In addition to the M protein, RNP, cytoplasmic tail of glycoproteins, host machineries such as multivesicular body formation machinery are also required for virus budding. The M protein may recruit host factors to assembly sites though the late domain. In PIV5, the M protein recruits angiomotin-like 1 (AmotL1), a component of tight junctions, for PIV5 budding (90). The binding between the M protein and 14-3-3 negatively regulates the VLP formation (91). Depletion of free ubiquitin by the Proteasome inhibitor MG-132 in the cell reduces PIV5 or PIV5 VLP budding, suggesting that ubiquitination machinery is utilized by the virus to regulate its budding (58).

1.7 PIV5 and host immune responses

A virus infection can be recognized by the host, resulting in clearance of the virus and infected cells (87). For viruses to produce progenies, it is very important for them to develop strategies to antagonize host antiviral response (87). Paramyxoviruses encode accessory proteins such as the V protein of PIV5, the C protein of respiroviruses, and the V and the W proteins of Nipah virus that limit interferon (IFN) synthesis, a major antiviral cytokine (73). The conserved Cys-rich domain of the V protein blocks dsRNA-induced activation of IFN promoter though a direct interaction with MDA-5, an IFN-inducible DExD/H box helicase
The NS1 and NS2 proteins of hRSV and bRSV alone or cooperatively block IFN production, likely through inhibiting interferon regulatory factors (IRFs) (18, 41, 85). In addition, the V protein can also antagonize IFN signaling pathways through affecting STAT proteins (signal transducers and activators of transcription) (93). The V proteins of PIV5 and SV41 target STAT1 for degradation; hPIV2 V protein targets STAT2 for degradation; the V protein of mump virus directs both STAT1 (IFN signaling) and STAT3 (IL-6 signaling); The V proteins of Hendra and Nipha virus, although do not induce STAT degradation, prevent STAT transport to the nucleus (50, 58, 87). In Sendai virus and hPIV3, another accessory protein, the C protein can alter STAT phosphophorylation patterns or induce ubiquitination and degradation of STAT1 in different experimental system (42, 58). Although showing little similarity, the SH proteins of PIV5, RSV, J virus, and mumps virus, can block or reduce TNF-α mediated signaling (35, 67, 71, 122).

1.8 PIV5 reverse genetics and minigenome system

The studies of paramyxovirus and virus-host interaction have benefited greatly from the reverse genetics system, a technology that allows engineering of specific mutant virus possible. For negative stranded RNA viruses, such as paramyxovirus, the viral genome RNA needs to be assembled into an active transcriptase-replicase complex to initiate virus transcription and replication (Figure 1-5) (48, 58). PIV5 can be rescued from cells transfected with plasmids encoding the NP, the P, the L protein, and a plasmid expressing the full-length genome of PIV5.
Similarly, the minigenome system with a reporter gene replacing all the coding sequence of the virus has been successfully constructed for PIV5, J virus, and Nipah virus (44, 67, 72). Chloramphenicol acetyltransferase (CAT) and renilla luciferase (R-luc) genes are common reporter genes used in the minigenome system (Figure 1-6). A minigenome plasmid has been constructed that contains a Renilla luciferase (R-luc) reporter gene, from which a negative-sense minigenome is generated from T7 RNA polymerase transcription. In the presence of the NP, the P and the L protein, this negative-sense RNA template can be replicated to produce more negative-sense RNA and transcribed to give rise to the R-luc mRNA, resulting in luciferase activity. The plasmid encoding a firefly luciferase (FF-Luc) reporter gene was cotransfected for transfection efficiency control. Minigenome system provides a good strategy to study virus transcription and replication without interference from other steps of virus infection.

1.9 The P protein and post-translational modification

The P proteins of paramyxoviruses are heavily phosphorylated. However, the role of the P protein phosphorylation is an enigma. It was initially thought that phosphorylation of the P protein in paramyxovirus was important for viral RNA synthesis. However, most previous studies from Sendai virus and RSV seem to suggest that phosphorylation of the P protein did not play a role. While it may be true that some of the phosphorylation sites may not play any significant role in virus gene expression, it is hard to imagine that none of the phosphorylation sites within a heavily phosphorylated P protein has any role in its function. Therefore, further detailed studies need to be performed to examine the role of the P protein.
phosphorylation in viral gene expression. Sumoylation is a post-translational modification that plays important roles in regulating gene transcription in eukaryotes. Since the P protein of PIV5 is the cofactor for the viral RNA dependent RNA polymerase and plays essential roles in virus gene expression, I have examined whether the P protein of PIV5 undergoes sumoylation and whether sumoylation plays a role in regulating PIV5 gene expression.
Figure 1-1. PIV5 virion and genome structure. A. Negative stained PIV5 virion by electron microscope (EM). B. Schematic view of PIV5 virion and genome structure. Leader and Trailer: important sequences for recognition of the viral RNA polymerase. NP: nucleocapsid protein, which encapsidates PIV5 genome to form RNP. P: phosphoprotein, a component of RNP and cofactor of the viral RNA polymerase. V: an accessory protein with multiple functions. M: matrix protein, a central organizer of virus assembly. F: fusion protein, mediating virus-host cell membrane fusion. HN: hemagglutinin neuraminidase, mediating attachment of virus to host cell membrane. SH: small hydrophobic protein, blocking TNFα induced apoptosis. L: large protein, which is the RNA dependent RNA polymerase and contains all the enzymatic activities required for viral RNA synthesis (Fields Virology, 5th Edition).
Figure 1-2. Paramyxovirus life cycle. The incoming virion binds to and fuses with the plasma membrane of target cell to release the negative stranded RNA genome and associated viral proteins. Viral mRNA is then transcribed and viral proteins are translated. More genome copies are produced by viral replication. Viral proteins and genomes are packed into progeny virions, which are released outside by a process called budding (Fields Virology, 5th Edition).
Figure 1-3. Paramyxovirus RNA synthesis (transcription and replication). The RNA genome is completely encapsidated by the N protein. The viral RNA polymerase (L-P) recognizes the promoter sequence at the 3’ end and transcribes genome template to produce leader RNA and successive capped and polyadenylated mRNA by stopping and starting between each two genes. These transcripts are translated into viral protein by the host machinery. Once unassembled N (N-P) accumulates, they assemble the nascent leader chain and the polymerase ignores the junctions, switching to replication model to produce full-length antigenome, which is further used as a template to produce more genomes. (Fields Virology, 5th Edition).
Figure 1-4. RNA editing of representative paramyxoviruses. The shared region of different proteins due to RNA editing is shown as the white box. The RNA editing site is indicated by the vertical line. For rubulaviruses, the V mRNA is transcribed faithfully, and the addition of two G nucleotides produces the P mRNA. The addition of one or four G nucleotides produces the W or the I mRNA. In respiroviruses, morbilliviruses, avulaviruses, and henipaviruses, the unedited mRNA encodes the P protein (Fields Virology, 5th Edition).
Figure 1-5. Rescue of PIV5 from cloned cDNA. BSR-T7 cells provide the T7 RNA polymerase. pT7-SV5 (PIV5) contains complete sequence of PIV5 genome and a T7 polymerase promoter. PIV5 antigenome, first transcribed by T7 polymerase is encapsidated by the NP protein. The P and the L proteins further transcribe antigenome to produce other viral proteins for virus life cycle (Fields Virology, 5th Edition).
Figure 1-6. PIV5 minigenome system. BSR-T7 cells are transfected with plasmids encoding the NP, the P and the L protein, and a minigenome plasmid contains a report gene replacing the coding region of the PIV5 genome. The minigenome plasmid was transcribed to produce a negative sense RNA by T7 polymerase provided by BSR-T7 cells. The transcripts can be further replicated and transcribed into mRNA by the NP-P-L complex. The Renilla luciferase mRNA is translated by the host cells. FF-luc was transfected as a control for transfection efficiency (modified from Lin et al 2005).
Table 1-1 *Paramyxovidae* classification and examples

<table>
<thead>
<tr>
<th>Family Paramyxoviridae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfamily Paramyxovirinae</td>
</tr>
<tr>
<td>Genus Rubulavirus</td>
</tr>
<tr>
<td>Mumps virus (MuV)</td>
</tr>
<tr>
<td>Parainfluenza virus 5 (previously called simian virus 5 [SV5] or canine parainfluenza virus type 2) (PIV5)</td>
</tr>
<tr>
<td>Human parainfluenza virus type 2, type 4a and 4b (hPIV2/4a/4b)</td>
</tr>
<tr>
<td>Mapuera virus</td>
</tr>
<tr>
<td>Porcine rubulavirus (La-Piedad-Michoacan-Mexico virus)</td>
</tr>
<tr>
<td>Genus Avulavirus</td>
</tr>
<tr>
<td>Newcastle disease virus (avian paramyxovirus 1) (NDV)</td>
</tr>
<tr>
<td>Genus Respirovirus</td>
</tr>
<tr>
<td>Sendai virus (mouse parainfluenza virus type 1) (SeV)</td>
</tr>
<tr>
<td>Human parainfluenza virus type 1 and type 3 (hPIV1/3)</td>
</tr>
<tr>
<td>Bovine parainfluenza virus type 3 (bPIV3)</td>
</tr>
<tr>
<td>Genus Henipaviruses</td>
</tr>
<tr>
<td>Hendra virus (HeV)</td>
</tr>
<tr>
<td>Nipah virus (NiV)</td>
</tr>
<tr>
<td>Genus Morbillivirus</td>
</tr>
<tr>
<td>Measles virus (MeV)</td>
</tr>
<tr>
<td>Cetacean morbillivirus</td>
</tr>
<tr>
<td>Canine distemper virus (CDV)</td>
</tr>
<tr>
<td>Peste-des-petits-ruminants virus</td>
</tr>
<tr>
<td>Phocine distemper virus</td>
</tr>
<tr>
<td>Rinderpest virus</td>
</tr>
<tr>
<td>Subfamily Pneumovirinae</td>
</tr>
<tr>
<td>Genus Pneumovirus</td>
</tr>
<tr>
<td>Human respiratory syncytial virus A2, B1, B2 (hRSV)</td>
</tr>
<tr>
<td>Bovine respiratory syncytial virus (bRSV)</td>
</tr>
<tr>
<td>Pneumonia virus of mice (PVM)</td>
</tr>
<tr>
<td>Genus Metapneumovirus</td>
</tr>
<tr>
<td>Human metapneumovirus (hMPV)</td>
</tr>
<tr>
<td>Avian metapneumovirus</td>
</tr>
<tr>
<td>Unclassified paramyxoviruses</td>
</tr>
<tr>
<td>Fer-de-Lance virus (FDLV)</td>
</tr>
<tr>
<td>Tupoi paramyxovirus (TPMV)</td>
</tr>
<tr>
<td>Menangle virus (MenV)</td>
</tr>
<tr>
<td>Tioman virus (TiV)</td>
</tr>
<tr>
<td>Belono virus</td>
</tr>
<tr>
<td>J virus</td>
</tr>
<tr>
<td>Mossman virus (MoV)</td>
</tr>
<tr>
<td>Salem virus (SaV)</td>
</tr>
<tr>
<td>Nariva virus</td>
</tr>
</tbody>
</table>

*(Fields Virlogy, 5th Edition)*
CHAPTER 2: PLK1 DOWN-REGULATES PARAINFLUENZA VIRUS 5 GENE

EXPRESSION
2.1 Abstract

Paramyxovirus is a family of negative-sense RNA viruses that includes many important human and animal pathogens. Paramyxovirus RNA dependent RNA Polymerase consists of the viral phosphoprotein (P) and the large (L) protein. Phosphorylation of the P protein is thought to regulate viral gene expression, though direct proof remains elusive. Recently, we have reported that phosphorylation of a specific residue S157 of PIV5 P protein correlated with decreased viral gene expression. Here, I show: Polo-Like Kinase 1 (PLK1), a serine/theronine kinase that plays a critical role in regulating the cell cycle, interacts with PIV5 P protein through the S157 residue; PLK1 inhibition increases viral gene expression; PLK1 over-expression inhibits viral gene expression; and PLK1 directly phosphorylates the P protein, indicating that PLK1 down-regulates viral gene expression by phosphorylating the P protein. Furthermore, I have determined the PLK1 phosphorylation site within the P protein and found that mutant PIV5 whose P protein cannot either bind to or be phosphorylated by PLK1 had similar phenotypes. Increased viral gene expression in PIV5 with mutation in the PLK1 binding/phosphorylation sites correlates with increased induction of cell death and cytokine expression, suggesting that PIV5 limits its viral gene expression to avoid these host effects. It is possible that targeting PLK1 will enhance host innate immune responses, leading to a novel strategy of quickly clearing paramyxovirus infections.
2.2 Introduction

Paramyxovirus RNA dependent RNA polymerase (RdRP) consists of two proteins, the large (L) protein and the phosphoprotein (P) (52, 58, 59). While all P proteins of paramyxoviruses are heavily phosphorylated (therefore named phosphoprotein), the role of the P protein phosphorylation in the viral RNA synthesis of paramyxovirus remains an enigma. It was initially thought that P protein phosphorylation was important for viral RNA synthesis (57). However, recent studies seem to indicate that the P protein phosphorylation does not have a role in viral replication. In Sendai virus, mutations at S249, the major phosphorylation site as well as other phosphorylation site did not affect viral growth and pathogenesis in vitro and in vivo (51, 52). The P protein of RSV is also well studied. Two clusters of phosphorylation sites have been identified (7, 10). Mutations of the phosphorylation sites in recombinant viruses did not affect viral gene expression (75, 99, 118). Further studies of RSV P protein phosphorylation using mass spectrometry identified T108 as a phosphorylation site, and mutation at this residue resulted in diminished minigenome activity, suggesting that this phosphorylation site may play a role in regulating viral RNA synthesis (5). However, this study has not been confirmed in the context of virus infection.

Previously, a strain of PIV5 called canine parainfluenza virus (CPI) was isolated from dog and caused neurological disorders (33). During the study of CPI virus, a derivative of CPI was isolated and showed a different phenotype (12). Therefore the previous strain was named CPI+, and the derivative form was called CPI-. Several differences were found among the genome sequences of PIV5, CPI+ and CPI- (22, 23, 106). Compared with PIV5 W3A strain
(referred to wild type), the CPI+ virus genome has five amino acid differences in the V/P gene and three of them are in the V and P shared region. CPI- virus genome contains eight amino acid changes in the V/P gene and six of them are in the V and P shared region (22). For later studies, the recombinant CPI+ (rPIV5-CPI+) and CPI- (rPIV5-CPI-) contains three and six amino acid changes in the V and P shared region only (Figure 2-1A) (22, 120, 132). Previous studies found that the rPIV5-CPI- showed elevated viral gene expression compared with wild type PIV5, and induced expression of host anti-viral response genes such as interferon-β (IFN-β) and interleukin-6 (IL-6) (28, 120, 132). In our recent study, we identified that a single amino acid change of the P protein, S157F, was responsible for the increased viral gene expression. Based on mass spectrometry results, we also found that S157 of the P protein was phosphorylated in PIV5 infected cells, indicating that phosphorylation of the P protein at S157 plays a role in regulating viral gene expression (115). However, the mechanism for this regulation is unknown.

Intriguingly, S157 of the P protein is within a consensus binding motif of Polo-Like-Kinase 1 (PLK1), a serine/theonine kinase that plays a critical role in mitosis and cell cycle checkpoints (30, 31, 104, 108). Four PLK members have been identified in mammals, PLK1 (PLK, the best characterized member), PLK2 (also called SNK), PLK3 (also called FNK or PRK), and PLK4 (also called SAK) (108). PLK1 is associated with tumorigenesis and overexpression of PLK1 has been found in many cancer types (108). The C-terminus of PLK1 contains two polo-box domains (PBD) and its N-terminus contains the kinase activity domain (53, 108). It has been reported that the PBDs of PLK1 binds to the target protein through a S(pS/T)P motif (SSP motif or STP motif), where the second amino acid residue
serine or threonine needs to be phosphorylated for the optimal binding (32, 104, 108). The binding of PLK1 to a target protein through a SSP motif enables PLK1 to phosphorylate either the target protein itself or a protein associated with the target protein, which is mediated by the kinase domain of PLK1 (31, 104). In this chapter, I investigate the role of PLK1 in PIV5 gene expression and identify both the binding site and the phosphorylation site of PLK1 within PIV5 P protein, which provide a mechanism for the increased viral gene expression and cytokine production of rPIV5-CPI-.
2.3 Materials and methods

2.3.1 Plasmids, viruses and cells

Plasmids expressing P-S157A and P-S308A were cloned from a copy of P wide type in the pCAGGS vector as described before (115). The human PLK1 gene was obtained from Open Biosystems (AL, USA). The coding region was cloned into pCAGGS vector with a Flag tag added at the N-terminus. The kinase dead PLK1 (Flag-PLK1-K82M) was constructed by mutating nucleic acid AAG to ATG at amino acid 82 using Flag-PLK1 as the template and using pfX50TM polymerase (Invitrogen). Plasmids encoding full-length genome of rPIV5-V/P-S157A and rPIV5-P-S308A viruses were constructed similar to that of rPIV5-CPI+ as previous report (115). PIV5 (W3A), rPIV5-CPI+ and rPIV5-RL viruses used in this study were described previously (110, 115). HeLa (HeLa CCL, ATCC) and MDBK cells were grown in Dulbecco modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 100IU/ml penicillin and 100ug/ml streptomycin. Growth medium for BHK cells contains additional 10% Tryptose Phosphate Broth (TPB). BSR-T7 cells, which are originated from BHK cells and constitutively express T7 RNA polymerase, were grown in the same medium as BHK cells plus 100 µg/ml G418. All cell lines are incubated at 37°C with 5% CO2. The growth medium for infected cells contains only 2%FBS.
2.3.2 Immunoprecipitation

To detect interaction between the P protein and endogenous PLK1, HeLa cells in 10 cm plate were mock infected or infected with PIV5 or rPIV5-CPI+ at a MOI of 3. After 12 hours post infection (hpi), the cells were treated with 100 ng/ml Nocodazole to increase PLK1 expression level by arresting cells at M phase (11, 63). After 10-12 hours, the cells were lysed with Whole Cell Extraction Buffer (WCEB, 50mM Tris-HCl pH 8.0, 280mM NaCl, 0.5% NP-40, 0.2mM EDTA, 2mM EGTA and 10% glycerol) containing protease inhibitor cocktail and 1mM phenylmethylsulfonyl fluoride (PMSF) (110, 115). The same amount of cell lysates were pre-cleared with 40 µl protein G sepharose beads (Invitrogen) and then incubated with rabbit anti-P antibody and 30 µl protein G sepharose beads for 2-3 hours at 4°C on a shaker. The precipitated proteins were mixed with 2X protein loading buffer [60mM Tris-HCl pH 6.8, 40% glycerol, 4%SDS, 3% dithiothreitol (DTT), and few bromophenol blue] as described before (115) and resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto Polyvinylidene fluoride (PVDF) membrane (Milipore). Mouse anti-PLK1 (Santa Cruz Biotechnology) was incubated with the membrane to detect PLK1. Aliquots of the cell lysates were resolved in SDS-PAGE and transferred onto PVDF membrane. Mouse anti-PLK1 or mouse anti-P (Pp antibody) was incubated with the membrane to show the input amount of the proteins.

To examine whether the interaction between PLK1 and P/Pcpi+ is direct, not via other viral proteins, BSR-T7 cells in 6 cm plate were transfected with pCAGGS-P or pCAGGS-Pcpi+
alone or together with pCAGGS-Flag-PLK1. The transfected cells were incubated with DMEM deficient of Cys/Met for 30 min and then labeled with \(^{35}\text{S}-\text{Cys/Met promix} (\text{Amersham Life Science)} \) (100 \(\mu\)Ci/ml) in DMEM deficient of Cys/Met. After 3 hours, the cells were lysed in RIPA buffer (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.2% Triton-X100, 0.1% SDS, 5 mM Indoacetanide) containing protease inhibitor cocktail and 1mM PMSF (72). The lysates were incubated with protein G sepharose beads together with Pk antibody or Flag antibody at 4\(^{\circ}\)C for 2-3 hours. The precipitates were washed three times with RIPA buffer containing 0.3M NaCl and once with RIPA buffer containing 0.15 M NaCl. The precipitated proteins were resolved in 10% SDS-PAGE. After the gel was dried, the proteins were visualized by autoradiography using storm phosphoimager (Molecular Dynamics Inc).

2.3.3 PLK1 inhibitors and renilla luciferase assay

BI2536, a highly selective PLK1 inhibitor was purchased from the Axon Company (Netherlands) and dissolved in Dimethyl sulfoxide (DMSO) (61, 107). GW843682 (Sigma) is another PLK1 inhibitor used in this study. It mainly inhibits PLK1 activity with weaker effect on PLK3, which has similar function as PLK1 (61). To examine the effect of PLK1 inhibitor on rPIV5-RL (rPIV5 with a Renilla luciferase gene inserted between HN and L gene) gene expression, HeLa or BSR-T7 cells were infected with rPIV5-RL at a MOI of 1. PLK1 inhibitor with various concentrations shown in the Figure 2-2 was added into the growth medium. After 16-20 hours post infection, the supernatant was removed and the cells were lysed by the lysis buffer provided in the Renilla luciferase assay kit (Promega). Aliquots of the lysates were used for Renilla luciferase assay. Four replicates for each
sample were used for statistic analysis and three individual experiments were performed. The PLK1 inhibitor was also used in PIV5 or rPIV5-CPI+ infected cells for immunoprecipitation experiment to show viral protein expression level using Pk antibody. Briefly, HeLa cells in 6 cm plates were mock infected or infected with PIV5 or rPIV5-CPI+ with duplicate. BI 2536 (1 µM) was added to the infected cells for 16-18 hours with DMSO treatment as a control. The cells were incubated with DMEM without Met/Cys then metabolically labeled with 35S-Met/Cys for 3 hours at 37°C. The labeled cells were lysed with WCEB and the supernatant was immunoprecipitated with Pk antibody.

2.3.4 33P labeling

To examine the effect of PLK1 inhibitor on the phosphorylation level of the P protein or Pcpi+, HeLa cells in 6 cm plates were mock infected or infected with PIV5 or rPIV5-CPI+. After 16-18 hours post infection, one group of the infected cells were incubated with medium lacking Cys/Met and labeled with 35S-Met/Cys with DMSO or 1 µM BI 2536. Another group of the infected cells were incubated with DMEM lacking sodium phosphate for 30 min and then labeled with DMEM containing 33P-orthophosphate (100 µCi/ml) in the present of DMSO or 1 µM BI2536. After 4 hours, the cells were lysed with WCEB and immunoprecipitated with Pk antibody as previously described (115). The proteins were resolved in 10% SDS-PAGE and visualized by a phosphoimager (Storm scanner 860, Molecular dynamics Inc). Three individual experiments were performed and protein intensity was quantified by Imagequant™ TL (GE Healthcare). The relative phosphorylation levels were normalized as the ratio of the phosphorylated P protein from 33P labeling to total
amount of the P protein from $^{35}$S labeled. The relative phosphorylation level of the P protein in PIV5 infected cells with DMSO treatment was set to 100.

2.3.5 PIV5 minigenome system and dual luciferase assay

The PIV5 minigenome system was described previously except that the negative control (NC) contained no P plasmid, instead of no L plasmid (115). Briefly, BSR-T7 cells in 24-well plates were transfected with 0.2 µg pSMG-RL, 0.2 µg NP, 0.01-0.16 µg P, 0.3 µg L and 1 ng plasmids expressing Firefly luciferase (FF-luc). Transfection was performed using Plus and Lipofectamine reagents (Invitrogen) as the provided protocol. A plasmid expressing GFP was used to keep the same total amount of transfected plasmids. For cotransfection of Flag-PLK1 or Flag-PLK1-K82M with PIV5 minigenome system, 0.02 µg P or P mutant was used due to the highest activity. Dual luciferase assay was carried out at 18-22 hours post transfection. The cells were lysed with 100 µl passive lysis buffer and 10 µl lysates were used for dual luciferase assay (Promega). The ratio of R-luc to FF-luc was normalized as relative luciferase activity and six replicates were performed for statistic analysis. Aliquots of the cell lysates were mixed with the same volume of 2X protein loading buffer as described above and resolved in 10% SDS-PAGE. The proteins were transferred onto PVDF membrane and mouse anti-NP together with mouse anti-P (Pk) were incubated with the membrane to show the input amount of NP and P proteins. Mouse anti-Flag was used in western blotting to detect the input amount of Flag-LK1 or Flag-PLK1-K82M.
2.3.6 PLK1 *in vitro* kinase assay

For the PLK1 *in vitro* kinase assay, HeLa cells in 10 cm plates were transfected with 4 µg plasmid encoding the P protein or P-S308A. At 24 hours post transfection, 30 µl anti-V5 agarose gel (Pk antibody conjugated to the agarose beads, Sigma) was used to immunoprecipitate the P protein or P-S308A. Similar amounts of the P protein and P-S308A were used for PLK1 (Cell Signal, MA) *in vitro* kinase assay with kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl$_2$, 2 mM dithiothreitole, and 0.1 mM NaVO$_3$). The mixture was incubated with 10 µCi γ$^{-}$32P-ATP (Amersham Biosciences) and 100 ng PLK1 for 1 hour at room temperature in a total volume of 30 µl (110). The reaction was terminated by addition of the same volume of 2X protein loading buffer. Proteins were separated by 10% SDS-PAGE and the phosphorylation was detected with a Storm Phosphorimager (Molecular Dynamics Inc). The same amounts of the proteins were used for Coomassie blue staining (50% Methanol, 10% Acetic acid, 0.25% brilliant blue, in water) to show the input amounts of the P or P-S308A.

2.3.7 Virus rescue and full-genome sequencing

To rescue rPIV5-V/P-S157A and rPIV5-P-S308A viruses from cDNA, BSR-T7 cells in 6-well plate were transfected with 1 µg pCAGGS-NP, 1.5 µg pCAGGS-L, 0.2 µg pCAGGS-P-S157A or pCAGGS-P-S308A, and 3 µg large plasmid encoding full-length genome of rPIV5-V/P-S157A or rPIV5-P-S308A. After 4 days post transfection, the supernatant were used to infect BHK cells. The infected BHK cells were overlayed with DMEM-2% FBS-10%
TPB-1% agarose with antibiotics. After 3-5 days, single plaques were picked and used to infect MDBK cells for virus amplification. The supernatant were collected at 3 days post infection and stored as stock virus. To sequence virus genome, MDBK cells were infected with the virus stock and viral RNA was extracted from the supernatant using QIAmp viral RNA mini kit (Qiagen, CA) as the protocol described. The viral RNA was reverse transcribed with random primer (Superscript III first-strand synthesis system, Invitrogen). 12 pairs of oligomers were used for PCR and 30 oligomers were used for sequencing at Nucleic acid facility at Pennsylvania State University. The leader or trailer sequences were obtained by Rapid Amplification of cDNA Ends (RACE) as previous described (68). Briefly, an adaptor JX129 was ligated to viral RNA by T4 RNA ligase and the ligation products were reversed transcribed by JX130 (complementary to JX129). JX130 and JX131 were used to amplify the cDNA and sequence. To obtain 5’ trailer sequence, the viral RNA was firstly reverse transcribed using JX133. The cDNA were purified and ligated to JX129. The ligation product was amplified by oligomers JX130 and JX132. The PCR products were sequenced and the sequences were compared with wide type PIV5 genome using BLASTn.

2.3.8 Flow cytometry

HeLa cells in 6-well plates were mock infected or infected with PIV5, rPIV5-V/P-S157A, or rPIV5-P-S308A at a MOI of 5. The infected cells were collected at 16 hpi and fixed with 1 ml of phosphate buffered saline without Mg$^{2+}$ and Ca$^{2+}$ (PBS-) containing 0.5% formaldehyde for 2 h at 4°C. After centrifugation (1000 rpm) and removal of the supernatant, the cells were resuspended in 500 µl solution of FBS-DMEM (50:50), then permeabilized by
adding 1.5 ml 70% ethanol and incubated at 4°C overnight. The cells were washed with PBS- and incubated with mouse anti-HN in PBS- with 10% FBS for 30 min at room temperature. After washing with PBS-, the cells were further stained with Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibody at room temperature for 30 min in the dark and washed with PBS-. The fluorescence intensity was measured using a flow cytometer (FC-500, Benckman-Coulter). Four replicates were used for statistic analysis and three individual experiments were performed.

2.3.9 Apoptosis assays

To detect apoptotic cells, Annexin-V staining, Propidium Iodide (PI) staining, and Terminal deoxynucleotidyltransferase-mediated dUTP-FITC nick end labeling (TUNEL) assay were performed as previously described (47, 71, 111). Briefly, MDBK cells in 6-well plates were mock infected or infected with PIV5, rPIV5-P-S308A or rPIV5-V/P-S157A at a MOI of 5. At 48 hours post infection, both the floating and the trypsinized monolayed cells were harvested. For Annexin-V staining, the cells were washed with cold PBS- and incubated with FITC-labeled Annexin-V (Annexin-V-FLIOS, Roche Diagnostics Corp. Mannheim, Germany) for 15 min at room temperature. 10,000 cells were examined for fluorescence by a FC-500. For PI staining, the collected cells were fixed and permeabilized with 70% ethonal as before. The permeabilized cells were washed and incubated with 100 µl Pk antibody solution (1:100 dilution in PBS-1% BSA). After washing, the cells were incubated with 100 µl FITC-labeled anti-mouse antibody (1:5,000 in PBS-1%BSA). The cells were finally incubated with 500 µl of PI solution (50 µg/ml) (Sigma). The stained cells were examined
with a FC-500. For the TUNEL assay, the permeabilized cells were incubated with 25 µl of TUNEL reaction mixture (Roche Diagnostics Corp. Mannheim, Germany) for 3 hours in the dark at 37 °C. After washing, the cells were incubated with 100 µl Pk antibody (1:100 dilution in PBS-1% BSA) followed by 100 µl Phycoerythrin (PE)-labeled anti-mouse antibody (1:100 dilution in PBS-1% BSA) and analyzed with a flow cytometer (FC-500) (35). Four replicates of each sample were used for statistic analysis and three individual experiments were performed.

2.3.10 Enzyme-linked immunosorbent assay (ELISA)

HeLa cells were mock infected or infected with PIV5, rPIV5-P-S308A, rPIV5-V/P-S157A at a MOI of 5. The medium were collected at 48 hours post infection and centrifuged to remove cell debris. 50 µl cleared medium and an IFN-β standard in duplicates were used for IFN-β ELISA by using human IFN-β ELISA kit (PBL InterferonSource, NJ) as the provided protocol. For IL-6 production, the same medium from infected cells were sent to The General Clinical Research Center (GCRC) at Pennsylvania State University to measure IL-6 concentration as described in Lin et al (73).

2.3.11 Protein purification and CDK1/cyclin B in vitro kinase assay

P and P-S157A in pET15b vector with 8 His tag at the N-terminus were transformed into BL21(DE3)pLysS competent cells. Single colonies were picked and grown in LB medium with Ampcillin (50 ng/ml) and Chloramphenicol (34 ng/ml) at 37°C. When OD600 of the
bacteria reached 0.5-0.6, 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) of final concentration was added to induce protein expression. The proteins were purified using Ni charged resin (Novagen) and examined by SDS-PAGE and Coomassie blue staining. The same amount of His-P or His-P-S157A was used for CDK1/cyclin B (Cell signaling) in vitro kinase assay similar to PLK1 in vitro kinase assay. Half of the mixture was incubated with 10 µCi γ-32P-ATP and 100 ng CDK1/cyclin B for one hour at room temperature in a total volume of 30 µl. Half of the mixture was used for Coomassie blue staining. The reaction was terminated by addition of protein loading buffer. The proteins were resolved in SDS-PAGE and visualized by a Storm Phosphorimager (Molecular Dynamics Inc).
2.4 Results

2.4.1 PLK1 interacts with the P protein via the SSP motif

Previously, it was reported that rPIV5-CPI+, a recombinant virus containing three amino acid changes (V32I, T33I, and S157F) within the V and the P proteins showed elevated viral gene expression level, and that S157F of the P protein was responsible for the phenotype (115). The amino acid differences among PIV5, rPIV5-CPI+ and rPIV5-CPI- are shown in Figure 2-1A. To ascertain the mechanism of the increased viral gene expression, the P protein sequence was searched for known kinase binding motifs. S157 is within a PLK1 binding motif (SSP), suggesting that PLK1 may play a role in regulating viral gene expression. To investigate the possible role of PLK1 in PIV5 gene expression, the interaction between PLK1 and P was examined in PIV5 or CPI+ infected cells. PIV5 P protein interacted with endogenous PLK1, while P protein of rPIV5-CPI+ (Pcpi+) did not (Figure 2-1B). P interacts with viral proteins NP and L. To examine whether PLK1 interacts with P in the absent of NP or L, the interaction between PLK1 and P in transfected cells was examined (Figure 2-1C). PLK1 was co-immunoprecipitated with P, but not with Pcpi+ using antibody against P; the reciprocal immunoprecipitation with anti-Flag antibody confirmed the interaction of PLK1 with P but not Pcpi+, indicating that PLK1 interacts with P, likely through the SSP motif centered at the residue S157. Furthermore, similar experiment using immunoprecipitation followed by immunoblotting (IP-IB) was performed and similar result was obtained: PLK1 bound the P protein but barely bound Pcpi+ (Figure 2-1D).
2.4.2 PLK1 inhibitor increases rPIV5-RL gene expression

To study the role of PLK1 in PIV5 gene expression, the effect of a PLK1 inhibitor (BI 2536) on reporter gene expression using a recombinant PIV5 expressing Renilla luciferase (rPIV5-RL) was examined (Figure 2-2A). BI 2536 of 0.05 µM and higher concentration increased Renilla luciferase activity in HeLa cells (P<0.01). In BSR-T7 cells, BI2536 was also effective in increasing the luciferase activity, albeit at higher concentrations (from 0.5 µM). Because of concerns over potential off-target effects of this molecule, the effect of a structurally different PLK1 inhibitor, GW843682 (Sigma), was also tested in HeLa cells and the same effect on reporter gene expression was observed as BI 2536: GW843682 of 0.05 µM and higher concentration significantly increased the virus gene expression (Figure 2-2C).

2.4.3 PLK1 inhibitor increases PIV5, but not CPI+ gene expression

Furthermore, the effect of PLK1 inhibition on PIV5 gene expression was examined by immunoprecipitation, using CPI+ as a control (Figure 2-3A). BI 2536 treatment increased viral protein expression in PIV5 infected cells. In contrast, BI 2536 treatment did not greatly affect overall protein expression (right panel, Figure 2-3A), indicating that PLK1 regulation of gene expression in infected cells is specific to PIV5. Interestingly, BI 2536 did not increase viral protein expression in rPIV5-CPI+ infected cells (Figure 2-3B), suggesting that PLK1 does not play a role in regulating viral gene expression of rPIV5-CPI+. Since PLK1 fails to bind Pcpi+, these results suggest that the SSP motif plays a critical role in the inhibition of PIV5 gene expression by PLK1.
To further examine the role of PLK1 in PIV5 gene expression, we used PIV5 minigenome system, in which viral gene expression can be examined free of virus infection. PLK1 inhibitor BI 2536 increased reporter gene expression from the PIV5 minigenome using wild type P, but had little effect on reporter gene expression driven by Pcpip+ (Figure 2-3C). We also attempted to utilize siRNA technology to knock down expression of endogenous PLK1 in HeLa cells (data not shown) to examine the effect PLK1 depletion on viral gene expression. Unfortunately, the experiment was inconclusive due to the toxicity of siRNA against PLK1. Optimal knock down of PLK1 expression occurs between 48 to 72 hours after transfection of siRNA, after which we infected the siRNA-transfected cells. Since PLK1 plays a critical role in cell cycle progression, this long time of PLK1 depletion, coupled with virus infection, was cytotoxic.

2.4.4 PLK1 over-expression reduces PIV5 gene expression

Since phosphorylation of S157 binds to PLK1 and reduces the function of the P protein in PIV5 gene expression, we reasoned that over-expressing PLK1 would further reduce the function of the P protein. When ectopically expressed in the minigenome system, PLK1 reduced the minigenome activity in a dose dependent manner (Figure 2-4A): there was about 50% reduction of luciferase activity using 2 ng, and 95% reduction with 16 ng, of PLK1 plasmid in the transfection. PLK1 over-expression had a much weaker effect on the minigenome activity when Pcpip+ was used in place of wild type P: PLK1 plasmid at 4 ng, 8 ng and 16 ng moderately inhibited the Pcpip+ minigenome activity; there was no significant
effect at 2 ng though expression levels of PLK1 were consistent with the amount of plasmids transfected. To confirm that PLK1 functions through the SSP motif, we performed similar experiments using P containing a single point mutation S157A instead of Pcpi+, which has three point mutations (V32I, T33I, and S157F). As expected, P-S157A failed to bind PLK1 (Figure 2-4B) and the minigenome activity using P-S157A was weakly affected by PLK1 over-expression (Figure 2-4C). These data indicate that PLK1 binds to PIV5 P protein through the SSP motif centered at the residue S157 and reduces PIV5 gene expression. The weak inhibitory effect of PLK1 on Pcpi+ or P-S157A minigenome system is likely due to an interaction between PLK1 and a STP sequence within the P protein when PLK is over-expressed. The P protein contains an additional S(S/T)P sequence centered at T108. This STP sequence is not known to be phosphorylated and mutating this T to A had no effect on PLK1 binding or viral gene expression (data not shown). Since STP, even without T residue being phosphorylated, can weakly interact with PLK1, over-expression of PLK1 may result in interaction between PLK1 and P-S157A/Pcpi+(a weak interaction between Pcpi+ and PLK1 was indeed detected when PLK1 was over-expressed (Figure 2-1D).

2.4.5 Kinase activity of PLK1 is required for its inhibitory effect

To examine whether the binding of PLK1 is sufficient for its inhibitory effect on PIV5 gene expression, or its kinase activity is required, we tested the effect of over-expression of a kinase dead PLK1 (PLK1-K82M), which has a lethal point mutation at the ATP binding site (K82M) (127, 128), on PIV5 minigenome activity. PLK1-K82M, at amounts from 2 to 16 ng, failed to inhibit PIV5 minigenome activity, in contrast to wild type PLK1, though PLK1 and
PLK1-K82M were expressed at similar levels (Figure 2-5). These data indicate that the kinase activity is required for the inhibitory effect of PLK1 on PIV5 gene expression. Since the binding between PLK1 and target protein is through the PBD at the C-terminus of PLK1, not the kinase domain, we expected that PLK1-K82M would still bind the P protein, which was confirmed by co-immunoprecipitation (Figure 2-5B). Thus, the kinase activity of PLK1 is required for the effect of PLK1 on PIV5 gene expression, while the binding itself is not sufficient.

2.4.6 PLK1 phosphorylates the P protein in infected cells

To investigate the mechanism of how PLK1 regulates PIV5 gene expression, it is important to determine the phosphorylation target of PLK1. We hypothesize that after binding to the P protein, PLK1 phosphorylates the P protein. To test this hypothesis, we compared the level of phosphorylated P protein with or without PLK1 inhibitor BI 2536 treatment in PIV5 or rPIV5-CPI+ infected cells. Consistent with previous studies, rPIV5-CPI+ infected cells showed higher levels of viral protein expression than PIV5 infected cells and Ppni+ had lower level of phosphorylation than that of PIV5 (Figure 2-6A) (115). Because we only treated the infected cells for a short period of time during labeling (4 hours), no significant difference of PIV5 protein levels between DMSO and BI 2536 treated cells was detected by 35S-Cys/Met labeling. However, there was a significant reduction (80% reduction) of phosphorylation of the P protein due to BI 2536 treatment in PIV5 infected cells (Figure 2-6B), but not in rPIV5-CPI+ infected cells. Interestingly, while NP was also phosphorylated in infected cells, but its phosphorylation was not affected by PLK1 inhibitor, indicating that NP
phosphorylation is PLK1 independent even though it interacts with P.

2.4.7 PLK1 phosphorylates S308 of the P protein

Since the PLK1 inhibitor reduces the P protein phosphorylation, it is possible that PLK1 directly phosphorylates the P protein. Therefore, we planned to determine the PLK1 phosphorylation site within the P protein, which may provide more evidence for the role of PLK1 in PIV5 gene expression. A consensus PLK1 phosphorylation site has been previously reported (53). According to the report, the serine residue at position 308 (S308) of P protein resembles a PLK1 phosphorylation site (Figure 2-7A). To confirm that PLK1 directly phosphorylates the P protein at S308, a PLK1 in vitro kinase assay was carried out. P and P-S308A proteins were purified from HeLa cells transfected with plasmids encoding either P or P-S308A by affinity chromatography using anti-V5 conjugated agarose gel (Sigma). The purified P proteins were then incubated with PLK1 (Figure 2-7B). Wild type P was phosphorylated by PLK1, while P-S308A was not, indicating that S308 of P protein is indeed the PLK1 phosphorylation site.

2.4.8 PLK1 targets S308 of the P protein in minigenome system

If S308 of the P protein is the PLK1 phosphorylation site, mutation of S308 should thus result in a P protein with higher activity in the minigenome system that is insensitive to PLK1 inhibitor or PLK1 over-expression. Mutating S to A at 308 (P-S308A) increased the minigenome activity when compared with wild type P (Figure 2-8A). In addition, PLK1
inhibitor BI 2536 treatment did not affect P-S308A minigenome activity (Figure 2-8B). Furthermore, over-expression of PLK1 had no effect on the activity of the minigenome system using P-S308A (Figure 2-8C), indicating that S308 is indeed a PLK1 phosphorylation site. To exclude the possibility that the mutation at S308 caused a conformational change in the P protein such that it can no long be bound by PLK1, we examined the interaction between PLK1 and P-S308A. P-S308A co-immunoprecipitated with PLK1 and vice versa, suggesting that P-S308A still binds to PLK1 (Figure 2-8D).

2.4.9 PLK1 targets S308 of the P protein in infected cells

Using the PIV5 reverse genetics system, we generated two recombinant viruses encoding mutant P protein: rPIV5-V/P-S157A, which carries an amino acid change in the PLK1 binding site; and rPIV5-P-S308A, which has an amino acid change at the PLK1 phosphorylation site (Figure 2-9A). To investigate whether PLK1 phosphorylates the P protein at S308 in infected cells, $^{33}$P labeling was performed in infected cells with or without BI 2536 treatment. P-S308A in rPIV5-P-S308A infected cells had lower level of phosphorylation than that of the P protein in PIV5 infected cells (Figure 2-10CD). Phosphorylation level of wild type P was reduced by PLK1 inhibitor BI 2536, while phosphorylation of P-S308A in infected cells was not sensitive to BI 2536 treatment, indicating that S308 is a phosphorylation site for PLK1 in virus infected cells. These results mirror those presented in Figure 2-6 using rPIV5-CPI+, whose P protein encodes Ser at position 308 but no longer binds PLK1 due to the S157F mutation, underscoring the importance of both the binding and the phosphorylation of the P protein by PLK1.
2.4.10 S157A and S308 viruses elevate viral protein expression and growth

To confirm the phenotype of P-S157A and P-S308A in recombinant viruses, viral gene expression levels from cells infected with the viruses were examined by flow cytometry using HN antibody. The cells infected by rPIV5-V/P-S157A or rPIV5-P-S308A expressed higher levels (2-fold increase) of the HN protein than PIV5-infected cells, consistent with previous minigenome data and rPIV5-CPI+ infected cells (Figure 2-11). Therefore, the elevated gene expression in CPI+ virus infected cells is indeed due to the failure in binding and phosphorylation by PLK1 therefore failure of inhibition by PLK1.

To examine the growth of the recombinant viruses in tissue cultured cells, HeLa cells and Vero cells were infected with the recombinant viruses at a MOI of 0.01 and the virus titers in the medium of infected cells were determined using plaque assay. Interestingly, while rPIV5-V/P-S157A and rPIV5-P-S308A grew faster than wild type PIV5 initially as expected, the growth of the two recombinant viruses dropped at later time points in HeLa cells, which are capable of producing and responding to interferon. In Vero cells, the two recombinant viruses grew faster than PIV5. The drop of growth for the two recombinant viruses in HeLa cells was not observed in Vero cells, which are IFN signal defective (Figure 2-11C), indicating that interferon may affect the growth of the viruses.
2.4.11 S157A and S308A viruses induce apoptosis

It has been reported that rPIV5-CPI- virus, which contains mutation at S157, causes increased cell death and cytokine production besides the increased levels of viral gene expression (23, 132). To investigate whether rPIV5-V/P-S157A and rPIV5-P-S308A cause increased cell death, HeLa and MDBK cells were infected and photographed at 2 days post infection. The two mutant viruses caused cell death (Cytopathic effect, CPE), unlike PIV5, which did not cause cell death (Figure 2-12AB). To further investigate the nature of the cell death, apoptosis assays were performed using MDBK cells mock infected or infected with PIV5, rPIV5-P-S308A or rPIV5-V/P-S157A. Both mutant viruses induced increased apoptosis after infection using Propidium iodine staining, which detects the sub-G₀-G₁ population; Annexin V staining, which detects phosphatidylserine (PS) on the surface; and TUNEL assay, which detects nicked DNA (Figure 2-11C, D and E).

2.4.12 S157A and S308A viruses induce IFN-β and IL-6 production.

Previous studies found that CPI- induced production of cytokines such as IFN-β and IL-6 (119, 120, 132). To investigate induction of cytokines by rPIV5-V/P-S157A and rPIV5-P-S308A viruses we made, HeLa cells were infected with the viruses and the medium from infected cells were collected. Amounts of IFN-β, an anti-viral cytokine, and IL-6, a proinflammatory cytokine, were measured using ELISA. Expression levels of both cytokines were elevated in rPIV5-P-S308A and rPIV5-V/P-S157A infected cells compared to mock or wild type PIV5 virus infected cells (Figure 2-12A and B).
2.4.13 CDK1 phosphorylates S157 of the P protein

Cycline Dependent Kinase 1 (CDK1) is a proline directed serine/threonine kinase, which has been previously reported to phosphorylate the serine or threonine within a SSP/STP motif (32). Therefore, CDK1 is a candidate kinase phosphorylating the PIV5 P protein at S157, providing a docking site for PLK1. To demonstrate that CDK1 phosphorylates S157 of the P protein, CDK1/Cycline B \textit{in vitro} kinase assay was performed using His tagged P or P-S157A purified from \textit{E.coli}. Phosphorylation level of P-S157A by CDK1 was reduced, compared with P wild type, indicating that S157 is a phosphorylation site of CDK1 (Figure 2-13).
2.5 Summary

During studying the mechanim of CPI+ phenotype, I identified a host kinase, PLK1, that bound to PIV5 P protein at S157, the amino acid residue responsible for the different gene expression levels of PIV5 and CPI+, suggesting that PLK1 may play a role in regulating PIV5 gene expression. Further studies showed that a PLK1 inhibitor increased PIV5, but not CPI+ gene expression; PLK1 over-expression dramatically reduced PIV5 minigenome activity, but only slightly reduced CPI+ minigenome activity. These data suggest that PLK1 down-regulates PIV5 gene expression without affecting CPI+ gene expression. Metabalical $^{33}$P labeling experiments with PLK1 inhibitor showed that PLK1 phosphorylated the P protein of PIV5. Based on PLK1 in vitro kinase assay, S308 of the P protein was determined as the PLK1 phosphorylation site, and mutation of S308 of the P protein to alanine increased the minigenome activity. In addition, both PLK1 inhibitor and PLK1 over-expression had no effect on P-S308A minigenome activity. Incorporation of S157A (rPIV5-V/P-S157A) or S308A (rPIV5-P-S308A) into the virus genome enhanced the viral gene expression and viral growth. rPIV5-V/P-S157A and rPIV5-P-S308A viruses also induced apoptosis of the host cells, and production of cytokines including IFN-β and IL-6.
A

B

IP: anti-P
IB: anti-PLK1
IB: anti-PLK1
IB: anti-P
Figure 2-1: Interaction between P and PLK1. (A). Amino acid differences among wild type P, Pcep- and Pcep+. (B). Interaction between P and endogenous PLK1 in infected cells. HeLa cells, infected with mock, PIV5 or rPIV5-CPI+ were immunoprecipitated (IP) with rabbit anti-P antibody and immunoblotted (IB) with mouse anti-PLK1. (C). Interaction between P and PLK1 in transfected cells. The transfected BSR-T7 cells were metabolically labeled with 35S-Met/Cys. The cells were lysed by RIPA buffer and immunoprecipitated with Pk antibody or Flag antibody. The precipitated proteins were resolved in SDS-PAGE and visualized using a phosphoimager. (D). Interaction between P/Pcip+ and PLK1 was further confirmed in transfected cells using IP-IB. Rabbit anti-P was used for IP and mouse anti-PLK1 was used for IB.
A

![Graph A]

B

![Graph B]
Figure 2-2: Effect of PLK1 inhibitor on rPIV5-RL gene expression. (A). HeLa cells in 24-well plates were mock infected or infected with rPIV5-RL at a MOI of 1 and incubated with DMSO, 0.01, 0.05, 0.1, 0.2, 0.5 or 1 µM BI 2536. Renilla luciferase activity was measured at 18-20 hpi with 4 replicates and the average luciferase activity +/- standard derivation of mean (SEM) is shown. (B). BSR-T7 cells in 24-well plates were mock infected or infected with rPIV5-RL at a MOI of 1. Similar luciferase assay was performed as (A). (C). GW843682, another PLK1 inhibitor was tested in HeLa cells infected with rPIV5-RL.
A

IP: V/P Ab

Cell lysate

B

P<0.01

DMSO

BI 2536

PIV5

rPIV5-CPI+

Relative Protein Levels

0 50 100 150 200 250 300

PIV5

rPIV5-CPI+
Figure 2-3: PLK1 inhibitor increases PIV5, but not CPI+, gene expression. (A). Effect of PLK1 inhibitor BI 2536 on viral gene expression. HeLa cells were mock infected or infected with PIV5 or rPIV5-CPI+ and incubated with 1µM BI 2536. The cells were metabolically labeled with 35S-Met/Cys and immunoprecipitated with Pk antibody, which recognizes both the V and the P protein and co-immunoprecipitates the NP and the L protein. The right panel shows the labeled cell lysates without immunoprecipitation. (B). Quantification of (A). Three independent experiments were performed and the relative amount of the P protein was measured. The level of PIV5 P in infected cells with DMSO treatment was set at 100 and the rest was normalized to the level of PIV5 P. The average of relative expression level of P +/- SEM is shown. (C). Effects of PLK1 inhibitor on the PIV5 or CPI+ minigenome system. BSR-T7 cells were transfected with necessary plasmids and incubated with 0.25 µM BI 2536. At 18-20 hours post transfection, the cells were lysed and dual luciferase assay was performed. The relative luciferase activities were calculated as ratios of Renilla luciferase activity (indicative of minigenome activity) to FF-Luc activity (indicative of transfection efficiency). The average of relative luciferase activity +/- SEM is shown.
Figure 2-4: Effect of PLK1 over-expression on the minigenome system. (A). Effect of PLK1 over-expression on minigenome composed of P or Pcp1+. Plasmids encoding Flag-PLK1 at various concentrations were transfected along with the minigenome system. Dual luciferase assay was carried out as described above. Aliquots of the cell lysates were used for immunoblotting to detect expression levels of Flag-PLK1 (the bottom panel). (B). Interaction between P-S157A and PLK1 in transfected cells by coimmunoprecipitation, similar to Figure 2-1C. (C). Effect of PLK1 over-expression on the minigenome system composed of P or P-S157A, similar to Figure 2-4A.
Figure 2-5: Effect of kinase dead PLK1 on PIV5 minigenome activity. (A). Effect of over-expression of PLK1 kinase-dead mutant K82M on the minigenome system. BSR-T7 cells in 24-well plates were transfected with various concentrations of pCAGGS-Flag-PLK1
or pCAGGS-Flag-PLK1-K82M together with the plasmids necessary for the PIV5 minigenome system. Dual luciferase assay was carried out after 18-20 hours post transfection as above. NC: negative control, no plasmid encoding P; PC: positive control, no plasmid encoding PLK1 or PLK1 K82M. The relative luciferase activity of positive control was set as 100. The average of relative luciferase activity +/- SEM is shown. Aliquots of the cell lysates were used for immunoblotting to detect the expression level of Flag-PLK1 or Flag-PLK1-K82M. (B). Interaction between P and PLK1-K82M. A plasmid encoding P was transfected into cells with a plasmid encoding Flag-PLK1 or Flag-PLK1-K82M. The cells were metabolically labeled and immunoprecipitated with anti-P or anti-Flag.
Figure 2-6: Effect of PLK1 inhibitor on phosphorylation of the P protein. (A). Effect of BI 2536 on the P protein phosphorylation. HeLa cells were mock infected or infected with PIV5 or rPIV5-CPI+. At 18-20 hours post infection, the cells were metabolically labeled with $^{35}\text{S}$-Cys/Met or $^{33}\text{P}$-orthophosphate in the presence of 1 µM BI 2536 or DMSO. The cells were lysed and immunoprecipitated with Pk antibody. (B). Quantification of the P protein phosphorylation. Three individual experiments as described in (A) were quantified. The ratio of phosphorylated P from the $^{33}\text{P}$-labeling to total amount of P from $^{35}\text{S}$-labeled in PIV5 infected cells treated with DMSO was set as 100 and the others are normalized to the ratio. The average of relative level of the P protein phosphorylation +/- SEM is shown.
Figure 2-7: PLK1 phosphorylates S308 of the P protein. (A). Prediction of the PLK1 phosphorylation site within the P protein. The bold S/T residue is the PLK1 phosphorylation site. Ψ, amino acid residue with a hydrophobic side chain. (B). Phosphorylation of the P protein by PLK1 in vitro. HeLa cells were transfected with plasmids pCAGGS-P or pCAGGS-P-S308A. P and P-S308A were purified from transfected cells using anti-V5 agarose gel. Kinase assay was carried out in a total volume of 30 µl containing 100 ng PLK1 for 1 hour at room temperature. The bottom panel is the Coomassie blue stained gel to show input of P and P-S308A.
Figure 2-8: PLK1 targets S308 of the P protein in the minigenome system. (A). Effect of mutating S308 into A of the P protein on the minigenome activity. To ensure a validity comparison of P versus P-S308A, a range of concentrations of the P proteins in the experiments were used. Cell lysate aliquots of the transfected cells were subjected to
immunoblotting using anti-NP and anti-P antibodies. The average of relative luciferase activity +/- SEM is shown. (B). Effect of PLK1 inhibitor on the minigenome system using P-S308A. BI 2536 was added the minigenome system using P-S308A in a similar fashion as above. (C). Effect of PLK1 over-expression on the minigenome system using P-S308A. Plasmids encoding pCAGGS-Flag-PLK1 at various concentrations were transfected along with the minigenome system as above. Dual luciferase assay was carried out as described above. Aliquots of the cell lysates were used for immunoblotting to detect expression levels of Flag-PLK1. (D). Interaction between P-S308A and PLK1. A plasmid encoding Flag-PLK1 was transfected into cells alone or together with a plasmid encoding P, P-S308A, or P-S157A. The cells were metabolically labeled with $^{35}$S-Met/Cys and immunoprecipitated with anti-P or anti-Flag.
A

PIV5:  
155 156 157 158 159 ...... 306 307 308 309 310  
acc agt tcc ccc atc......aat gat agt ttc ttg  
T S S P I ...... N D S F L  

rPIV5-V/P-S157A: acc agt gec ccc atc......aat gat agt ttc ttg  
T S A P I ...... N D S F L  

rPIV5-P-S308A: acc agt tcc ccc atc......aat gat gct ttc ttg  
T S S P I ...... N D A F L  

B

35S

<table>
<thead>
<tr>
<th>BI 2536</th>
<th>Mock</th>
<th>Mock</th>
<th>PIV5</th>
<th>PIV5-P-S308A</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

33P

<table>
<thead>
<tr>
<th>BI 2536</th>
<th>Mock</th>
<th>Mock</th>
<th>PIV5</th>
<th>PIV5-P-S308A</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NP
P
Figure 2-9: PLK1 phosphorylates S308 of the P protein in infected cells. (A). Sequences of PIV5, rPIV5-V/P-S157A and rPIV5-P-S308A at regions around residue 157 and 308 of the P protein. The entire genomes of the mutant viruses were sequenced and no mutation was observed except in the designated site (S157A or S308A). (B). Effect of PLK1 inhibitor on P-S308A phosphorylation in infected cells. HeLa cells were mock infected or infected with PIV5 or rPIV5-P-S308A. At 18-20 hours post infection, the cells were metabolically labeled with $^{35}$S-Cys/Met or $^{33}$P-orthophosphate in the presence of 1 µM BI 2536 or DMSO. The cells were lysed and immunoprecipitated with Pk antibody. (C). Quantification of P-S308A phosphorylation with or without PLK1 inhibitor. Three individual experiments as described in (B) were quantified. The ratio of phosphorylated P from the $^{33}$P-labeling experiment to total amount of P from $^{35}$S-labeled P in PIV5 infected cells treated with DMSO was set to 100 and the others are normalized to the ratio. The average of relative phosphorylation level of P +/- SEM is shown.
Figure 2-10: Gene expression and growth of S157A and S308A viruses. (A). Viral gene expression from PIV5, rPIV5-V/P-S157A and rPIV5-P-S308A infected cells. HeLa cells were infected with PIV5, rPIV5-V/P-S157A, or rPIV5-P-S308A. At 16 hours post infection, the infected cells were processed for flow cytometry using anti-HN antibody. Average mean fluorescence intensity of infected cells +/- SEM is graphed. (B). Growth of the two mutant viruses in HeLa cells. HeLa cells were infected with PIV5, rPIV5-V/P-S157A or rPIV5-P-S308A viruses at a MOI of 0.01 and the supernatants from the infected cells were collected at indicated time points. Titers of the viruses were determined by plaque assay using BHK cells. (C). Growth curve of the two mutant viruses in Vero cells. Vero cells were infected with the viruses at a MOI of 0.01 and the supernatants were collected at the indicated time points. Viral titers were determined by plaque assay.
Figure 2-11: Induction of apoptosis by S157A and S308A viruses. (A). Induction of cytopathic effect by virus infection. HeLa cells were infected with PIV5, rPIV5-V/P-S157A, or rPIV5-P-S308A. At 48 hours post infection, the infected cells were photographed using microscope (Nikon ECLIPSE TE300, Japan). (B). MDBK cells were infected with PIV5, rPIV5-V/P-S157A, or rPIV5-P-S308A. Similar experiments were performed as (A). (C). Propidium iodine (PI) staining. MDBK cells were infected and collected 2 days post infection as above. The cells were stained with PI and cellular DNA profiles were examined using flow cytometry. Percentages of sub G0-G1 population, which is considered apoptotic, are graphed. S157A: rPIV5-V/P-S157A; S308A: rPIV5-P-S308A. (C). Annexin V staining. The infected cells were stained with FITC-labeled annexin V and measured using a flow cytometer. Annexin V, which binds to phosphatidylserine (PS), is an indication of cells undergoing apoptosis when it is present on cell surface. Percentages of annexin V positive cells are graphed. (D). TUNEL assay. TUNEL positive cells were measured using a flow cytometer and percentages of TUNEL positive cells are graphed.
Figure 2-12: Induction of cytokines by S157A and S308A viruses. (A). Induction of IFN-β. HeLa cells were infected and the levels of IFN-β in the supernatants were measured using ELISA at 2 days post infection. (B). Induction of IL-6. HeLa cells were infected and the levels of IL-6 in the supernatants were measured using ELISA at 2 days post infection. Error bars show the SEM of three replicates.
Figure 2-13: CDK1/Cyclin B phosphorylates S157 of the P protein. His-P or His-P-S157A was purified from E.coli as described before. Similar amounts of His-P or His-P-S157A were used for CDK1/Cyclin B in vitro kinase assay (top panel) and Coomassie blue staining (bottom panel).
CHAPTER 3: IDENTIFICATION OF A PHOSPHORYLATION SITE OF THE PROTEIN IMPORTANT FOR PIV5 GROWTH
3.1 Abstract

Paramyxovirus family includes many important human and animal pathogens. The viral RNA dependent RNA polymerase of paramyxovirus consists of the large (L) protein and the phosphoprotein (P). It has been reported that the phosphorylation of the P protein at S157 and S308 of PIV5 down-regulates viral gene expression. However, no phosphorylated amino acid residue within the P protein in paramyxovirus has been identified to play a positive role in viral RNA synthesis in virus infection. Using mass spectrometry, we have identified T286 of PIV5 P as a phosphorylation site. Mutating T286 to alanine (T286A), aspartic acid (T286D), or glutamic acid (T286E) reduced the minigenome activity. Circular dichroism analysis showed that P-T286A/D/E did not have detectable secondary structure defects. Recombinant virus containing a mutation at T286 position (rPIV5-P-T286A) grew slower than wide type virus; viral RNA synthesis and protein expression in rPIV5-P-T286A infected cells were delayed. I also found that mutations of T286A/D/E affected viral transcription. Biochemical studies showed that the binding of NP or L protein, or tetramer formation by the mutant P proteins remained the same as wide type P. While I failed in rescuing rPIV5-P-T286E viruses, several revertant viruses were obtained. All revertants had mutations at the same site T286 and showed defects in both minigenome activity and viral growth.
3.2 Introduction

The P proteins of all paramyxoviruses are heavily phosphorylated and hence the name of phosphoprotein (58). However, the role of the P protein phosphorylation in the RNA synthesis of paramyxoviruses is an enigma. It was initially suggested that phosphorylation of the P protein was essential for viral RNA synthesis. In paramyxovirus, the P proteins of Sendai virus and RSV were studied the most extensively. Up to 11 phosphorylation sites of SeV P protein were detected and S249 was identified as the major phosphorylation site. However, mutation at S249 of the P protein showed normal function in the minigenome system (36, 52, 109). In addition, recombinant SeV containing P mutation at S249 also showed similar growth characteristics in vitro and in vivo (51), indicating that this phosphorylation site of the P protein was dispensable for SeV gene expression and pathogenesis. Mutating other sites besides S249 of SeV P protein resulted in a decrease of over 90% in phosphorylation. The mutant P protein still had the same activity in the minigenome system (51). In RSV, two clusters of phosphorylation sites (amino acid residue 116, 117 and 119 cluster and residue 232 and 237 cluster) of the P protein have been identified (7). Phosphorylation of S232 of the P protein was thought to regulate RSV transcription, however, this study based only on in vitro assays (75). When mutations at major phosphorylation sites were incorporated into recombinant RSV using reverse genetics system, phosphorylation level of the P protein was reduced by 90%, yet there was no difference in terms of viral gene expression, indicating that phosphorylation of the P protein was dispensable for viral gene expression (36). Further studies of the RSV P protein by mass spectrometry identified T108 as a phosphorylation site. Mutation of T108 diminished RSV
minigenome activity, possibly through affecting its interaction with M2-1, an important factor for the viral RNA polymerase (5). Mutation at S54 of RSV P protein was found to affect viral uncoating by using LiCl treatment which affects phosphorylation of S54 (6). However, the roles of these phosphorylation sites have not been examined in the context of virus infection.

Recently, it was found that S157 of PIV5 P protein was phosphorylated in infected cells and its phosphorylation correlated with decreased viral gene expression as well as inhibition of cytokine induction, suggesting that phosphorylation of P protein at S157 plays a role in down-regulating viral gene expression (115). Further studies showed that Polo-Like-Kinase 1 (PLK1) was involved in the process by binding the P protein at S157 and phosphorylating the P protein at S308 (109). This finding was surprising because P protein phosphorylation was initially thought to be critical for viral RNA synthesis. Phosphorylation of the P protein at S157 and S308 reduced viral gene expression to prevent cytokine production, which was very important for viral growth in the long term. However, since the P protein is heavily phosphorylated (58), I speculate that the P protein phosphorylation at certain site(s) plays a positive role in regulating viral gene expression. In the present study, I have identified T286 of PIV5 P protein as a phosphorylation site that is important for PIV5 gene expression.
3.3 Materials and methods

3.3.1 Plasmids, viruses, and cells

Plasmids expressing P mutants and other plasmids used in this work were cloned into pCAGGS vector and made as previously described (109, 115). Plasmids for protein purification from *E. coli* were cloned into pET15b vector with 8 histidines at the N-terminus. Plasmids containing full-length genome for rPIV5-P-T286A and rPIV5-P-T286E viruses were made similarly to rPIV5-CPI+, rPIV5-V/P-S157A and rPIV5-P-S308A as previously described (109, 115). The genomes of the mutant viruses were confirmed by sequencing as described in chapter 2. The cell lines used in this chapter were grown in the same condition as described in chapter 2 (109).

3.3.2 Mass spectrometry analysis

To determine the phosphorylation sites within the P protein in PIV5 infected cells, liquid chromatograph-mass spectrometry/mass spectrometry (LC-MS/MS) was performed as previously described (115). HeLa cells in 10 cm plates were infected with PIV5 at a MOI of 5. At 24 hours post infection, the cells were lysed by WCEB and anti-V5 conjugated agarose (Sigma) were used to precipitate the P protein. After washed with WCEB, the agarose were mixed with one volume of 2X protein loading buffer and resolved in 10% SDS-PAGE. The gel was stained with Coomassie blue in the solution of 50% methanol and 10% acetic acid. After destaining, the P protein band was excised and digested with trypsin (Promega) (115).
The phosphopeptides were enriched using a TiO$_2$ column and analyzed by LC-MS/MS (Waters Q-Tof Ultima mass spectrometer at W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University). The MS/MS spectra were searched against the NCBI database using the automated Mascot algorithm to identify peptides with possible phosphorylation residues of tyrosine (Tyr), threonine (Thr) and serine (Ser).

### 3.3.3 PIV5 minigenome system and dual luciferase assay

The PIV5 minigenome system used in this study was previously described (109, 115). Increasing amounts (0.01-0.16 µg/well, 24-well plate) of P, P-T286A, P-S36A, P-S126A, P-T286S, P-T286D, P-T286E, P-T286V or other P mutants were transfected together with other plasmids (0.2 µg pSMG-Rluc, 0.2 µg NP, 0.3 µg L, and 1 ng FF-Luc) into BSR-T7 cells. At 20-22 hpi, one tenth of the lysate from each well was used for dual luciferase assay (Promega). The relative luciferase activity was normalized as the ratio of Renilla luciferase (R-Luc) to Firefly luciferae (FF-Luc) activity. Six replicates of each sample were used for statistical analysis and the error bars showed the SEM. Aliquots of the cell lysates from the minigenome system were used for western blotting. Mouse anti-NP and Pk antibody were used together to detect the expression of the NP protein and the P protein or P mutants.

### 3.3.4 Protein purification and circular dichroism

Circular dichroism (CD) was carried out using His-P, His-P-T286A, His-P-T286D and His-P-T286E purified from *E.coli*. Briefly, P and P mutants in pET15b vector were transformed
into BL21(DE3)pLysS competent cells. Single colonies were picked and grown in LB medium with ampicillin (50 ng/ml) and chloramphenicol (34 ng/ml) in 37°C. When OD600 of the bacteria reached 0.5-0.6, IPTG with a final concentration of 1mM was added into the medium to induce protein expression for four hours at 37°C. The P proteins were purified using Ni charged resin (Novagen) and examined by SDS-PAGE and Coomassie blue staining. The purified proteins were desalted and resuspended in the buffer containing 10 mM potassium phosphate (KH$_2$PO$_4$) and 10 mM potassium chloride (KCl) (pH 7.0). 300 µl of 10 µM of His-P and His-P mutants were measured on Jasco-J715 spectropolarimeter using 0.1 cm path-length cuvette (39). Three measurements were taken for each sample and the average was compared.

### 3.3.5 Virus rescue and viral genome sequencing

To rescue rPIV5-P-T286A and rPIV5-P-T286E viruses from plasmids, 1 µg NP, 0.2 µg P or P mutants, 1.5 µg L and 3 µg recombinant virus genome plasmid were cotransfected into BSR-T7 cells in 6-well-plate. For negative control, 0.2 µg GFP, instead of P was used. At 4 days post transfection, the supernatant was collected and used for plaque assay with serial dilution (1:1, 1:10 and 1:100). Single plaques were picked and further amplified in MDBK cells. The viral RNA was extracted from the supernatant using QIAmp viral RNA mini kit and Reverse transcription (RT) was performed by using random primer as the protocol (Invitrogen). Reverse transcription product was further amplified by PCR and the PCR products were sequenced at Nucleic acid facility at Pennsylvania State University. To get T286E virus, we collected the transfected cells after 6 days post transfection and cocultured
the transfected cells with Vero cells for another 7 days. The supernatant from the cocultured cells was collected for plaque purification. Single plaques were picked and further amplified in MDBK cells and sequenced.

3.3.6 Growth curve and plaque assay

To characterize the rPIV5-P-T286A virus, MDBK cells in 6-well plates were infected with PIV5 or rPIV5-P-T286A at a MOI of 0.01 or 5. The supernatants were collected at different time points and stored in -70°C. BHK cells in 6-well plate were infected with the viruses with serial dilution (1:10 to 1:10^7). After one to two hours, the infection medium was removed and replaced with 5 ml growth medium of 2% FBS, 10% TPB, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% low melting agarose in DMEM. The plaques were counted 4-5 days post infection. Three replicates for each time were collected for statistical analysis.

3.3.7 Western blotting

To compare the viral protein expression levels in PIV5 and rPIV5-P-T286A infected cells, HeLa or BSR-T7 cells were infected with PIV5 or rPIV5-P-T286A at a MOI of 5. The medium was replaced after 2 hours and the cells were lysed at 4, 8, 12, 16 and 20 hours post infection with WCEB and mixed with protein loading buffer (115). Aliquots of the total lysates were resolved in 10% SDS-PAGE. The proteins were transferred onto PVDF membrane and incubated with mouse anti-NP and Pk antibodies, followed by incubation with horseradish peroxidase (HRP) labeled anti-mouse secondary antibody. The PVDF membrane
was washed and incubated with ECL advance substrate (GE Healthcare) and scanned by Kodak image station 440. Rabbit anti-β-actin was used as the control for the input amount of the proteins. Three individual experiments were performed and one representative result was shown.

### 3.3.8 Flow cytometry

Flow cytometry was carried out following a previous description (71, 109). HeLa cells at 90% confluence were infected with PIV5 or rPIV5-P-T286A at a MOI of 5 or 1. The Cells were collected at the time points given in the figure legends and fixed with PBS-5% formaldehyde. After washing, the cells were resuspended in 0.5 ml FBS-DMEM (1:1) and permeabilized with 1.5 ml 70% ethanol. Mouse anti-P/V antibody and FITC-labeled anti-mouse were incubated with the cells. The fluorescence intensity was measured using a flow cytometer (LSR II, BD). Three replicates were performed for statistic analysis. Similar experiment was carried out in BSR-T7 cells.

### 3.3.9 Real time PCR

To compare the levels of viral mRNA and genome in infected cells, real time PCR was performed as previously described (115). Briefly, MDBK cells in 6-well plate were infected with PIV5 or rPIV5-P-T286A. Total RNAs from the infected cells were extracted using RNeasy Mini Kit (Qiagen). One fifth of the total RNA from each sample was used for reverse transcription (RT) using Superscripct III reverse transcriptase (Invitrogen). Oligo
(dT)$_{15}$ was used in RT to detect viral mRNA level; BH191 which anneals to a region of the genomic RNA in the M gene was used in RT to measure viral genome. 2% of the cDNA was used for real time PCR reaction on Step one Plus Real Time PCR System using Taqman Universal PCR Master Mix and custom made Taqman Gene Expression Assays with FAM dye and NFQ quencher (Applied Biosystems) (115). Relative levels of viral mRNA and viral genome at each time point were determined by calculating ΔCt, and normalized with the levels of the input genome defined as the genome levels at 2hpi. Three replicates for each sample were used for statistic analysis.

3.3.10 Immunoprecipitation-immunoblotting (IP-IB)

To detect the interaction between the P and the L protein, BSR-T7 cells were transfected with 3 µg Flag-L together with 1 µg P, P-T286A, P-T286E, or P-T286D. After 24 hours, the cells were lysed with WCEB and the lysates were immunoprecipitated with mouse anti-P together with protein G sepharose beads for 2-3 hours at 4°C. The IP products were washed and resolved in SDS-PAGE. The gel was transferred onto PVDF membrane and immunoblotted with Flag antibody (Stratagene). Aliquots of the cell lysates were resolved in SDS-PAGE and immunoblotted with antibody against P or Flag to measure the expression levels of P/P mutants and Flag-L.
3.3.11 Immunoprecipitation and DSP crosslinking

To compare interaction between the NP and the P/P mutants, BSR-T7 cells in 6 cm plates were transfected with 1µg NP together with 1 µg P, P-T286A, P-T286E, or P-T286D. After 18-20 hours, the cells were metabolically labeled with $^{35}$S-Met/Cys. The labeled cells were lysed with WCEB and half of the lysates were immunoprecipitated with Pk antibody and half with antibody against NP. The IP products were washed and resolved in SDS-PAGE. The gel was dried and the proteins were visualized with a Typhoon 9700 (GE Healthcare). To detect tetramer formation of P, P-T286A, P-T286E, or P-T286D, the transfected BSR-T7 cells were metabolically labeled with $^{35}$S-Cys/Met for 3 h at 37°C. The labeled cells were incubated with 1mM Dithiobis succinimidyl propionate (DSP) (Pierce, Rockford, IL) in PBS-0.5% NP40 to cross link the disulfide bond as previously described (115). After crosslinking, the cells were lysed with WCEB and the supernatant was immunoprecipitated with Pk antibody. After washing, half of the mixture was mixed with protein loading buffer without Dithiothreitol (DTT), and half of the mixture was mixed with protein loading buffer with DTT. The mixture was resolved in 10% SDS-PAGE and the proteins were visualized with a Typhoon 9700 (GE Healthcare).
3.4 Results

3.4.1 T286 of the P protein is phosphorylated in PIV5 infected cells

To determine phosphorylation sites within the P protein, the P protein was purified from PIV5 infected cells using affinity purification. The purified protein was then subjected to tryptic digestion, TiO$_2$ enrichment, and analyzed by LC-MS/MS. The results from two individual experiments were summarized in Table 3-1. S157 and T286 were found to be phosphorylation sites in both experiments. S36 and S126 were identified as phosphorylation sites once. For the peptide from amino acid residue 293 to 331, the mass spectrometry identified two phosphorylation serines/threonines. However, it did not pinpoint the exact sites. The total coverage of the two mass spectrometry analyses is 74.2%. Phosphorylation of T286 can be demonstrated by: (1) y7-98, a neutral loss of H$_3$PO$_4$ (a mass of 98) from y7; (2) mass difference between y7 and y6: 182.09, which is equal to the mass of phosphorylated threonine.

3.4.2 P-T286A reduces PIV5 minigenome activity

To study whether phosphorylation of T286 of the P protein affects PIV5 gene expression, T286 was mutated to alanine and the function of P-T286A was compared with wild type P using the minigenome system. P-T286A showed lower level of the minigenome activity (2 fold reduction) (Figure 3-1B), indicating that T286 is important for PIV5 gene expression in the minigenome system. S36 and S126 are two possible phosphorylation sites (Table 3-1).
However, mutating these two sites to alanine had no effect on the minigenome system (Figure 3-1CD), indicating that they are incidental phosphorylation sites or their phosphorylations are not important for PIV5 gene expression.

3.4.3 Effect of P mutations at T286

To further confirm the role of T286 phosphorylation in viral gene expression, T286 was mutated to serine (P-T286S), aspartic acid (P-T286D), and glutamic acid (P-T286E). P-T286S showed the same function as the P wild type in the minigenome system (Figure 3-2A). P-T286D showed very little activity, although there was significant difference comparing P-T286D and negative control (without P) (P<0.01). P-T286E mutant showed no activity at all, the same as negative control (P>0.05). Circular dichroism was used to examine the secondary structures of the P mutants purified from E.coli, which does not have serine/threonine phosphorylation. As shown in Figure 3-2D, His-P, His-P-T286A, His-P-T286D and His-P-T286E showed similar CD patterns under continuous wavelength from 190 to 260 nm, suggesting that the overall protein secondary structure is not affected by the mutations at T286.

3.4.4 rPIV5-P-T286A shows slower growth

To examine the role of T286 phosphorylation in virus infection, a recombinant virus containing a T to A change at 286 of the P protein (rPIV5-P-T286A) was obtained. The differences in nucleic acid residues and amino acid residue between rPIV5-P-T286A and
PIV5 were shown in Figure 3-3A. To study the role of T286 phosphorylation of the P protein in infected cells, MDBK cells were infected with PIV5 or rPIV5-P-T286A at a low MOI (MOI=0.01). The supernatant were collected for plaque assay. rPIV5-P-T286A titer was lower than that of PIV5 (Figure 3-3B) with about 10 fold reduction (P<0.01) at each time point. At high MOI infection (MOI=5, Figure 3-3C), PIV5 started to release virions at 12 hours post infection (hpi), while there was almost no virion release from rPIV5-P-T286A infected cells at 12 hours post infection. The viral titer of rPIV5-P-T286A was lower than PIV5 at 12 and 24 hours post infection (P<0.01). At 36 hours post infection and later on, rPIV5-P-T286A showed similar titer as PIV5 (Figure 3-3C). These data suggest that lack of phosphorylation at T286 of the P protein resulted in a delay in virus growth.

3.4.5 rPIV5-P-T286A shows delayed protein expression

To investigate the mechanism of the delayed growth of rPIV5-P-T286A, viral protein expression was examined in HeLa cells infected with PIV5 or rPIV5-P-T286A at a MOI of 5. At 4 hours post infection, protein levels of the NP, the P and the V proteins in PIV5-P-T286A infected cells were similar to those in PIV5 infected cells. At 8 and 12 hours post infection, the viral protein expression levels of rPIV5-P-T286A were lower than those of PIV5. At late time points 16 hpi and 20 hpi, the viral protein expression levels were similar in PIV5 and rPIV5-P-T286A infected cells (Figure 3-4A). The results were further confirmed with flow cytometry by staining both the P and the V proteins (Figure 3-4B), which indicates that rPIV5-P-T286A virus gene expression is delayed, but not completely defective. The same phenotype of rPIV5-P-T286A was observed in BSR-T7 cells and MDBK cells (Figure
3-4CD and data now shown), indicating that the phenotype is not cell type specific.

### 3.4.6 rPIV5-P-T286A affects viral RNA synthesis

To further investigate the mechanism of the delayed viral protein expression in rPIV5-P-T286A infected cells, viral mRNA synthesis and genome replication were examined using real time PCR. At a high MOI (MOI=5) infection, rPIV5-P-T286A had about half of the viral mRNA of that of PIV5 at 8 and 12 hpi. At 16 hpi, the mRNA level of rPIV5-P-T286A was similar to that of PIV5 (Figure 3-5A). rPIV5-P-T286A also showed lower level of viral genome than PIV5 (P<0.05) (Figure 3-5B). The ratio between viral mRNA to genome was lower in rPIV5-P-T286A infected cells at 8 and 12 hours post infection (Figure 3-5C), indicating that T286A mutation affects viral transcription. For infection at a MOI of 1, the difference of viral mRNA between PIV5 and rPIV5-P-T286A was seen at 12 hpi (P<0.05) and later time points (Figure 3-5D). Genome copy of rPIV5-P-T286A was lower than PIV5 at 16 hpi and 20 hpi (P<0.05) (Figure 3-5E). This result suggests that rPIV5-P-T286A reduces viral RNA synthesis, consistent with the differences observed in viral protein expression as well as growth curve.

### 3.4.7 Mutations at T286 affect viral transcription

To further confirm that T286 mutations affect viral transcription, a PIV5 deficient minignome system which only has transcription activity, no replication activity, was used to compare P mutants with P wild type. P-T286A showed less function in PIV5 replication
defective minigenome system (ANOVA, P<0.01) (Figure 3-6A). P-T286D mutant showed little activity in the mutant minigenome system compared with negative control (P<0.01) (Figure 3-6B). For P-T286E, there was no transcription activity at all (P>0.05, compared with negative control) (Figure 3-6C) which may explain the lethal effect of P-T286E mutation.

3.4.8 P mutants at T286 do not affect the formation of NP-P-L complex

The P protein interacts with the NP and the L protein (88, 92). To investigate the mechanism for the defect of P-T286A, P-T286D and P-T286E, interactions between the P and the NP, the P and the L were examined. Based on the coimmunoprecipitation and immunoprecipitation-immunoblotting (IP-IB) results in transfected cells (Figure 3-6AB), P-T286A, P-T286D and P-T286E still bound to the NP or the L protein, similar to the P wild type. The P protein of paramyxovirus is known to form homo-oligomer (112). Abilities of the P mutants to form tetramers were examined. P-T286A, P-T286D and P-T286E formed homotetramers (Figure 3-6C) similar to P wild type. Taking these data together, the interactions between the P and the NP, the L or itself were not affected by the mutations at T286.

3.4.9 Analysis of revertant viruses

I tried to rescue rPIV5-P-T286E virus but failed. Prolonged co-incubation of rPIV5-P-T286E rescue cells with Vero resulted in recombinant viruses. Twenty-six isolates from three
separate experiments were obtained. Sequencing results indicated that they all contained revertant mutations at E286 including glutamic acid (E, GAA) to alanine (A, GCA), lysine (K, AAA), valine (V, GTA), aspartic acid (D, GAC) or threonine (T, ACT) (Table 3-2). This result indicates that T286E is lethal in the function of the P protein in PIV5 growth, which is consistent with the minigenome data. We sequenced the full-length genome of one for each revertant virus of T286V, T286D, and T286K, and did not find other mutation, indicating that T286 of the P protein is a key regulatory site for PIV5 gene expression. All of those revertent viruses grew slower than PIV5 (Figure 3-8A). Both P-T286K and P-T286V showed much less activity than the P wide type in the minigenome system (Figure 3-8BC).
3.5 Summary

Using mass spectrometry, I have identified S36, S126 and T286 of the P protein as phosphorylation sites. Mutating T286 to alanine of the P protein reduced the minigenome activity about 2-fold, while mutating S36 or S126 to alanine did not affect the minigenome activity. Further studies of mutations at T286 found that P-T286D showed very low activity and P-T286E showed no activity at all in the minigenome system. Incorporation of T286A into the virus genome (rPIV5-P-T286A) resulted in a virus that grew slower and to a lower titer (10-fold reduction compared with PIV5). Viral protein expression and viral RNA synthesis in rPIV5-P-T286A infected cells were delayed compared with PIV5. Using a transcription-only minigenome system, I found that T286A/D/E mutations affected viral transcription. Biochemical studies showed that P-T286A/D/E mutations did not affect NP-P-L complex formation. While I failed in obtaining rPIV5-P-T286E virus, several revertant viruses were obtained, all of which showed defects in both the minigenome system and viral growth.
Figure 3-1. Effect of P mutants on PIV5 minigenome system. (A). Coomassie blue stained gel showing the P protein used for mass spectrometry. (B). Mass spectra showing phosphorylation of T286. The bottom panel shows the digested and TiO₂ enriched fraction which contained phosphorylated threonine at amino acid 286. Phosphorylation of T286 can be proved by: (1) y7-98, a neutral loss of H₃PO₄ from y7; (2) mass difference between y7 and y6: 182.09, which is equal to the mass of phosphorylated threonine. (C). Minigenome activity of P-T286A. Increasing amount of P or P-T286A was transfected together with other plasmids as described in the material and methods. The minigenome activity was measured and normalized as the ratio of R-luc activity to FF-luc activity. Western blotting was performed to detect the expression levels of the NP and the P or P-T286A. (D, E). The function of P-S36A or P-S126A in the minigenome system.
Figure 3-2. Effect of P mutations at T286. (A, B, C). The minigenome activity of P-T286S, P-T286D or P-T286E was compared with P wild type. The expressions of NP and P or P-T286S/T286D/P-T286E are shown at the bottom of each figure. (D). Circular dichroism analysis of P mutants. The P protein fused with a His-tag at its N-terminus (His-P), His-P-T286A, His-P-T286D, and His-P-T286E were purified from E.coli. 10 µM of P or P mutants in 10 mM KH$_2$PO$_4$-KCl buffer (pH 7.0) were measured on Jasco-J715 spectropolarimeter.
A

PIV5: ......aat gtt act gtg gaa......
......N V T V E......

rPIV5-P-T286A: ......aat gtt gct gtg gaa......
......N V A V E......

rPIV5-P-T286E: ......aat gtt gaa gtg gaa......
......N V E V E......

284 285 286 287 288

B

![Graph showing viral titer over days post infection with PIV5 and rPIV5-P-T286A comparing viral titer at various days post infection. The graph indicates a significant difference (P<0.01) between the two groups.](image-url)

P<0.01
Figure 3-3. Effect of P-T286A on viral growth. (A). Schematic of mutant viruses rPIV5-P-T286A and rPIV5-P-T286E. The changes of the nucleic acid and amino acid residues were highlighted in the box. For rPIV5-P-T286A virus, the whole genome was sequenced and no other mutation was found. (B). Growth curve of PIV5 and rPIV5-P-T286A in MDBK cells at a MOI of 0.01. Three separate experiments were performed (P <0.01 for all time points). (C). Growth curve of PIV5 and rPIV5-P-T286A in MDBK cells at a MOI of 5. P value was calculated from three replicates.
A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>NP</th>
<th>P</th>
<th>V</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIV5</td>
<td>4h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T286A</td>
<td>4h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

- Mock
- PIV5
- T286A

<table>
<thead>
<tr>
<th>Hours post infection</th>
<th>Mean fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>12</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

P < 0.01
P < 0.05
C

![Bar graph with mean fluorescence intensity](image)

P < 0.01

D

<table>
<thead>
<tr>
<th></th>
<th>4h</th>
<th>8h</th>
<th>12h</th>
<th>16h</th>
<th>20h</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIV5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T286A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Western blot analysis](image)

NP
P
V

β-Actin
Figure 3-4. Viral protein expression in PIV5-P-T286A infected cells. (A). Western blotting. The viral protein expression in PIV5 or rPIV5-P-T286A infected HeLa cells at a MOI of 5 was compared at different time points. T286A means rPIV5-P-T286A virus. (B). Flow cytometry. PIV5 or rPIV5-P-T286A infected HeLa cells at a MOI of 5 were processed for flow cytometry at different time points. Mean fluorescence intensity (the P and the V proteins) was used to show viral protein expression level (y axis). The Error bars represented the SEM of 4 replicates. (C). Flow cytometry data in PIV5 or rPIV5-P-T286A infected HeLa cells at a MOI of 1 at 10 hpi. (D). Similar western blotting result in BSR-T7 cells. (E). Flow cytometry data in BSR-T7 cells at a MOI of 5. (F). Similar flow cytometry data in BSR-T7 cells at a MOI of 1 at 10 hpi.
Figure 3-5. Viral RNA synthesis in PIV5-P-T286A infected cells. (A). Viral mRNA levels at a MOI of 5. MDBK cells were infected with PIV5 or PIV5-P-T286A at a MOI of 5. Total RNAs were purified and used for RT. To measure mRNA levels, oligo d(T) and primers targeting the HN region (BH193 and BH194) and a FAM dye were used for real time PCR. Genome level of PIV5 or rPIV5-P-T286A at 2 hours post infection was used as the baseline for normalization. (B). Genome levels at a MOI of 5. Oligo BH191 complementary to a region within M gene was used for reverse transcription. Genome level at 2 hpi for each virus was used as a baseline for normalization. (C). The Ratio between viral mRNA and genome RNA based on (A) and (B). (D). Viral mRNA levels at a MOI of 1. The experiment was performed similar to (A). (E). Genome levels at a MOI of 1.
Figure 3-6: Viral transcription activity of P mutations at T286. (A) P or P-T286A was transfected together with other plasmids for the transcription-only minigenome system. Dual luciferase assay was carried out 2 days post transfection. The relative luciferase activity was shown as the ratio of R-luc activity to FF-luc activity. (B, C). The function of P-T286D or P-T286E was compared with P wide type in PIV5 transcription-only minigenome system, similar to (A).
Figure 3-7. The NP-P-L complex formation. (A). Interaction between the NP and the P/P mutants. BSR-T7 cells were transfected with NP together with P or P mutant. After labeling with $^{35}$S-Cys/Met, the cells were lysed and the supernatants were incubated with Pk antibody or NP antibody for Co-immunoprecipitation. (B). IP-IB data to show the interaction between Flag-L and P/P-T286A/P-T286E/P-T286D. Pk antibody was used for IP and mouse anti-Flag was used for IB. (C). Tetramer formation of the P or P mutants. The transfected BSR-T7 cells metabolically labeled with $^{35}$S-Met/Cys. The cells were incubated with DSP to cross link disulfide bonds and stabilize P tetramer. The lysates were incubated with Pk antibody and resolved in SDS-PAGE with or without DTT. The gel was dried and the proteins were visualized with a Typhoon 9700.
Figure 3-8. Effect of revertant mutations. (A). Growth curve of rPIV5-P-T286A/V/D/K compared with PIV5 in MDBK cells at a MOI of 0.01. The whole genomes of revertant viruses for T286A, T286V, T286D, or T286K were sequenced and used for growth curve analysis. (B). Minigenome activity of P-T286K was compared with P wild type. Western blotting was performed to show the input amounts of NP and P or P-T286K. (C). Minigenome activity of P-T286V was compared with P wild type.
PIV5 P protein from infected HeLa cells was digested with trypsin and the phosphopeptides were enriched by TiO$_2$ and performed for LC-MS/MS. Mass spectrometry of two individual experiments were analyzed and the phosphorylation sites or peptides are shown. “+” means positive; “-” means negative.

<table>
<thead>
<tr>
<th>Phosphorylation site</th>
<th>Peptide</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS157</td>
<td>ENPIATSPSPIIDEFK</td>
<td>+, +</td>
</tr>
<tr>
<td>pT286</td>
<td>IMDPGVPSSNVPTVFDVRK</td>
<td>+, +</td>
</tr>
<tr>
<td>pS126</td>
<td>TLPSGPSPYKGVK</td>
<td>+, -</td>
</tr>
<tr>
<td>pS36</td>
<td>LIETGLNTVEYFTSQVTGTGPSSLGK</td>
<td>-, +</td>
</tr>
<tr>
<td>Unknown two sites (293-331)</td>
<td>TLSNHAVVVPESFNDSFLTQSEDVISLDELARPTATSVK</td>
<td>-, +</td>
</tr>
</tbody>
</table>

Table 3-1. Summary of LC-MS/MS results
Three individual rescuing were performed and 26 plaques in total were picked and amplified in MDBK cells. The supernatant was used for viral RNA extraction, RT-PCR and the PCR products were sequenced. The differences in nucleic acid residues and amino acid residues are shown.

<table>
<thead>
<tr>
<th>Theoretical (286)</th>
<th>Nucleic acid</th>
<th>Amino acid change</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAA (E)</td>
<td>AAA</td>
<td>E-K</td>
<td>9/26</td>
</tr>
<tr>
<td>GAA (E)</td>
<td>GCA</td>
<td>E-A</td>
<td>4/26</td>
</tr>
<tr>
<td>GAA (E)</td>
<td>GTA</td>
<td>E-V</td>
<td>2/26</td>
</tr>
<tr>
<td>GAA (E)</td>
<td>GAC</td>
<td>E-D</td>
<td>4/26</td>
</tr>
<tr>
<td>GAA (E)</td>
<td>ACT</td>
<td>E-T</td>
<td>1/26</td>
</tr>
<tr>
<td>GAA (E)</td>
<td>Mixture</td>
<td>Mixture</td>
<td>6/26</td>
</tr>
</tbody>
</table>
CHAPTER 4: SUMOYLATION OF THE P PROTEIN AT K254 IS IMPORTANT FOR PIV5 GROWTH
### 4.1 Abstract

The P protein of PIV5 is an essential cofactor of the viral RNA dependent RNA polymerase. Phosphorylation of the P protein can positively or negatively regulate viral gene expression, depending on the precise phosphorylation sites. Sumoylation, a process of adding SUMO to protein post-translationally, plays a critical role in regulating protein function. In this study, I have found that the P protein of PIV5 was sumoylated with SUMO1 in both transfected cells and infected cells. K254 residue of the P protein is within a consensus sumoylation motif. Mutation of the P protein at K254 to arginine (P-K254R) reduces PIV5 minigenome activity as well as sumoylation level of the P protein. Incorporation of K254R in the recombinant PIV5 (rPIV5-P-K254R) resulted in a virus that grew slower and to a lower titer than wild type PIV5; had lower levels of viral RNA synthesis and protein expression than wild type PIV5, suggesting that sumoylation of the P protein at K254 is important for the growth of PIV5. Biochemical studies did not reveal any defect in the interactions of P-K254R with the NP and the L proteins, or in formation of a homotetramer. We propose that sumoylation of the P protein at K254 may regulate PIV5 gene expression through a host protein.
4.2 Introduction

Small ubiquitin-related modifier, or small ubiquitin like modifier (SUMO) was first identified as a protein that is covalently linked to a target protein post-translationally in 1996 (81). Many proteins including RanGap1, Sp3, ERK, P53, IκBα, PIAS, STAT1, MDA5 and RIG-I have been found covalently linked to SUMO through a process called sumoylation (34, 79, 84, 97, 100, 105, 117, 131). Sumoylated proteins are involved in various cellular processes including transcriptional regulation, nuclear-cytosolic transport, protein stability, stress response, signal transduction, DNA repair and cell cycle (3, 13, 38, 54, 121, 124). The reversible sumoylation is carried out similarly to ubiquitination through activation, conjugation and ligation (38, 54). Immature SUMO is cleaved by SUMO specific isopeptidases (Sentrin specific proteases, SENP) to expose its C-terminal Gly-Gly motif to become a mature SUMO. Activation of a mature SUMO protein, carried out by the SUMO specific E1 activating enzyme, forms a SUMO-adenylate conjugate, as an intermediate. SUMO is then transferred to E2 conjugating enzyme such as Ubc9 for SUMO1. Finally, E2 conjugating enzyme transfers SUMO to a substrate, in which E3 ligase may facilitate the reaction and determine the specificity (38). Unlike ubiquitination, poly-SUMO1 chain does not occur at a single site because there is no lysine within SUMO1 protein as an acceptor for additional SUMO (113). In addition, sumoylation is not known to mediate protein degradation (38).

SUMO proteins are about 100 amino acid residues in length and 12kDa in size. There are four SUMO isoforms encoded by human genome, SUMO1, SUMO2, SUMO3 and SUMO4.
SUMO1, SUMO2 and SUMO3 are ubiquitously expressed but SUMO4 is not. It is unclear whether SUMO4 can be processed to its mature form in vivo (38). The mature forms of SUMO2 and SUMO3 share 97% identity, but only 50% identity with SUMO1 (38). As a result, SUMO1 and SUMO2/3 are conjugated to different target proteins and serve different functions. While SUMO1 is conjugated to target protein as a monomer, SUMO2/3 can be conjugated as a monomer or a polymer (113).

Among the hundreds of sumoylated proteins identified so far, most of them are nuclear proteins (16, 54). Sumoylated viral proteins are mostly from DNA virus such as IE2p86 and IE1p72 of human cytomegalovirus (HCMV) (15, 49, 62), E1, E2 and L2 capsid protein of human Papillomavirus (HPV) (80, 95, 125). For RNA viruses, sumoylation of viral proteins have also been detected. Sumoylation of Gag protein of human immunodeficiency virus (HIV) may affect HIV infectivity (43). Sumoylation of CA protein of Moloney Murine Leukemia virus (MMLV) has been found to be important in early events of virus infection (133). The NS1 protein of most influenza virus strains is sumoylated, which enhances NS1 stability and promotes rapid growth of the virus (129). It is known that viral proteins can promote or reduce sumoylation of host proteins (16, 56, 123). For example, VP35 protein of Ebola Zaire virus has been found to block type I interferon (IFN) production by increasing sumoylation of PIAS1 (Protein Inhibitor of Activated STAT1) (21). It was reported that L3 endoprotease of adenovirus, I7 protein of poxvirus, and S273R protein of African swine fever virus (ASFV) have sequence and functional similarity to SUMO specific protease Ulp1 (yeast homolog of SENP, which can process SUMO1 precursor and remove SUMO from substrates), indicating that these viral proteases may function in processing sumoylation and
desumoylation of viral or host proteins. (65, 123),

Parainfluenza virus 5 (PIV5) is a prototypic member of paramyxovirus family, which contains many important human and animal pathogens including mumps virus (MuV), measles virus (MeV), Sendai virus (SeV) and emerging Hendra virus (HeV) and Nipah virus (NiV) (58). The negative stranded RNA genome of PIV5 is composed of seven genes and yet encodes eight known proteins (58). The viral RNA dependent RNA polymerase (rRdRP) is consisted of the large protein (L) and the phosphoprotein (P). The L protein has the enzymatic activities capable of initiation, elongation and termination of viral RNA synthesis as well as adding 5’ cap structure and 3’ poly (A) sequence to the viral mRNA. The P protein is the cofactor for the polymerase, playing an essential role in regulating viral RNA synthesis (58). The P protein is heavily phosphorylated and phosphorylation status regulates the function of the P protein in viral RNA synthesis (36, 109, 115). In this study, I have investigated the role of the P protein sumoylation in regulating viral gene expression.
4.3 Materials and methods

4.3.1 Plasmids, viruses and cells

Human SUMO1, SUMO2, and SUMO3 genes were purchased (Open Biosystems) and cloned into pCAGGS vector with or without a Flag tag at the N-terminus. Plasmids encoding the P protein, P-K254R, His-P, His-P-K254R and full-length genome for rPIV5-P-K254R, were made similarly as previously described (47, 109, 115). rPIV5-P-K254R virus was recovered from the plasmid as previously described (109). The whole genome of the mutant virus rPIV5-P-K254R was confirmed by sequencing. All cell lines used in this study were described at chapter 2.

4.3.2 Immunoblotting

To detect sumoylation of the P protein, Flag-SUMO1, Flag-SUMO2 or Flag-SUMO3 were cotransfected with the P into BSR-T7 cells. At 20-24 hours post transfection, the cells were lysed with WCEB (115), the lysates were centrifuged and the supernatants were mixed with the same volume of 2X SDS loading buffer, heated at 95°C for 5 minutes and resolved in 10% SDS-PAGE. The proteins were transferred onto methanol soaked PVDF (Millipore) membrane. The membrane was incubated with mouse anti-P (Pk) antibody or mouse anti-Flag antibody, followed by incubation with anti-mouse secondary antibody labeled with HRP. After wash, the PVDF membrane was incubated with ECL Advance Substrate (GE Healthcare) and scanned using Kodak Image Station 440. Similar immunoblotting
experiment was performed using SUMO1/2/3 without Flag tag (Figure 4-4B). To detect viral protein expression, MDBK cells were infected with PIV5 or rPIV5-P-K254R at a MOI of 3. The cells were lysed with WCEB at different time points. The proteins were resolved in SDS-PAGE followed by transfer as above. Anti-NP and Pk (recognize both P and V protein) antibody from mouse were used to detect viral proteins NP, P and V. β-actin was used as protein loading control.

4.3.3 Immunoprecipitation-Immunoblotting (IP-IB)

To further confirm sumoylation of the P protein, SUMO1 and P were transfected separately or together into BSR-T7 cells. The cells were lysed with WCEB and the supernatants were incubated with mouse anti-P and protein G-sephorase beads at 4°C for 2-3 hours. The IP products were washed three times with WCEB and mixed with protein loading buffer, followed by SDS-PAGE. The Proteins were detected using immunoblotting as described above. Three individual experiments were performed and the relative level of the P protein sumoylation was normalized and quantified. To detect sumoylation of the P protein in infected cells, HeLa cells were mock infected or infected with PIV5 or rPIV5-P-K254R at a MOI of 5. At 24 hours post infection, the cells were lysed with WCEB, and similar IP-IB experiments were performed as described above. To compare the interaction between the L protein and P/P-K254R, 3 µg Flag-L was cotransfected with 1 µg P or P-K254R. Pk antibody was used for immunoprecipitation followed by immnublotting with Flag antibody. Aliquots of the cells lyastes were used for immunoblotting to show the input amounts of Flag-L and P/P-K254R.
4.3.4 PIV5 minigenome system and dual luciferase assay

PIV5 minigenome system used in this study was described before and above (109). Briefly, increasing amounts (0.01-0.16 µg/well, 24-well plate, 4 replicates for each condition) of the P or P-K254R was transfected together with other plasmids (0.2 µg pSMG-Rluc, 0.2 µg NP, 0.3 µg L, and 1 ng FF-Luc) into BSR-T7 cells. After 20-22 hours, the cells were lysed with passive lysis buffer and one tenth of the lysate from each well were used for dual luciferase assay (Promega). The y-axis, relative luciferase activity was normalized as the ratio of Renilla luciferase (R-Luc) activity to Firefly luciferae (FF-Luc) activity. Aliquots of the cell lysates from the transfected cells were used for immunoblotting to detect the input amounts of NP and P/P-K254R. For PIV5 transcription-only minigenome system, a mutant minigenome plasmid pSMG-m-Rluc carrying deletion in the region essential for PIV5 replication was used (115). The experimental process is similar to that of PIV5 minigenome system but the dual luciferase assay was carried out at two days post transfection.

4.3.5 Protein purification and circular dichroism

Circular dichroism (CD) was carried out using His-P and His-P-K254R purified from E.coli. Briefly, P or P-K254R with 8 histidine (His) at the N-terminus in pET15b vector were transformed into BL21(DE3)PolyS competent cells. A single colony for each protein was selected and grown in LB medium with ampcillin (50ng/ml) and chloramphenicol (34ng/ml). When OD 600 of the bacteria was within the range from 0.5 to 0.6, IPTG was added into the
bacteria culture at a final concentration of 1 mM to induce protein expression for four hours at 37°C. Proteins were purified using Nickel (Ni) charged resin and examined by SDS-PAGE and Coomassie blue staining. The purified proteins were desalted and resuspended in 10 mM KH$_2$PO$_4$-10 mM KCl (pH 7.0). 300 µl of 10 µM of His-P and His-P-K254R were measured on Jasco-J715 spectropolarimeter using 0.1 cm path-length cuvette (39). Three measurements were taken for each sample and the average of milidegrees (mdeg) was shown as the y-axis.

4.3.6 Growth curve and plaque assay

MDBK cells in 6-well plates were infected with PIV5 or rPIV5-P-K254R at a MOI of 0.01. The supernatants were collected at 0, 1, 2, 3, 4 and 5 days post infection and centrifuged to remove cell debris. For high MOI infection, MDBK cells in 6-well plates were infected with PIV5 or rPIV5-P-K254R at a MOI of 3, and the supernatants were collected at 0, 12, 24, 36, and 48 hours post infection. BHK cells in 6-well plate were infected with the virus stocks in serial dilution (1:10 to 1:10$^7$). After two hours, the inoculuses were removed and replaced with 5ml DMEM containing 2% FBS, 10% TPB, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1% low melting agarose. The plaques were counted 4-5 days post infection. Three replicates for each time point were collected for statistical analysis.

4.3.7 Flow cytometry

To further compare viral protein expression levels in PIV5 and rPIV5-P-K254R-infected cells, flow cytometry was performed as previously described (71, 109, 111). MDBK cells
were mock infected or infected with PIV5 or rPIV5-P-K254R at a MOI of 3. The cells were collected at different time points. The cells were fixed with PBS-0.5% formamide and resuspended in 0.5 ml DEMM-FBS (1:1). 1.5 ml 70% ethanol was added to permeabilize the cells. After washed with PBS, the cells were incubated with Pk antibody (anti-P/V) and secondary antibody against mouse labeled with FITC. The mean fluorescence intensity was measured using LSRII flow cytometer (BD). Similar flow cytometry was performed in MDBK, HeLa, and BSR-T7 cells at 10 hpi at a MOI of 1.

4.3.8 Reverse transcription and real time PCR

MDBK cells in 6-well plate were infected with PIV5 or rPIV5-P-K254R at a MOI of 3 or 0.5. Total RNAs from the infected cells were extracted using RNeasy Mini Kit (Qiagen) at different time points. One tenth of the total RNAs from each sample were used for reverse transcription (RT) using Superscript III reverse transcriptase (Invitrogen). Oligo (dT)$^{15}$ was used in RT to detect viral mRNA level; BH191 annealing to the M gene of the genomic RNA was used in RT to measure viral genome level. 1% of the cDNAs from RT were used for real time PCR reaction on Step one Plus Real Time PCR System as described before (115). Relative levels of viral mRNA and viral genome were determined by calculating $\Delta$Ct, and normalized with the level of the input genome defined as the viral genome level at 2 hpi. Three replicates for each sample were used for statistic analysis.
4.3.9 Immunoprecipitation and DSP crosslinking

To compare interaction between the NP and the P/P-K254R, BSR-T7 cells in 6 cm plates were transfected with 1 µg NP together with 1 µg P or P-K254R. After 18-20 hours, the cells were metabolically labeled with $^{35}$S-Met/Cys for 3 hours. The labeled cells were lysed with WCEB and immunoprecipitated with either anti-P antibody or anti-NP antibody. The IP products were washed and resolved in SDS-PAGE. The gel was dried and the proteins were visualized by using Typhoon 9700 (GE healthcare). To detect tetramer formation of P or P-K254R, the transfected BSR-T7 cells labeled with $^{35}$S-Cys/Met for 3 hours at 37°C. The labeled cells were incubated with 1 mM DSP in PBS-0.5% NP40 to cross link the disulfide bond as chapter 3. After crosslinking, the cells were lysed with WCEB and immunoprecipitation was performed as described above using anti-P antibody. After washing, half of the IP products were mixed with protein loading buffer without DTT to detect P tetramer, and half of the IP products were mixed with protein loading buffer with 200 mM DTT to show P band after removing the crosslinking. The mixture was resolved in 10% SDS gel and the proteins were visualized by Typhoon 9700 (GE Healthcare).
4.4 Results

4.4.1 The P protein is sumoylated by SUMO1 but not SUMO2 or SUMO3

To investigate whether the P protein is sumoylated, P and Flag-SUMO1, Flag-SUMO2, or Flag-SUMO3 were cotransfected into BSR-T7 cells and modification of the P protein was examined using immunoblotting with Pk antibody (anti-P). A slower moving band was detected, consistent with the size of the P protein plus 12 kDa, indicating that the P protein might be sumoylated by SUMO1 (Figure 4-1A). Interestingly, there was no corresponding band in P and SUMO2, or P and SUMO3 cotransfected cells, suggesting that P may not be sumoylated by SUMO2 or SUMO3. Similar immunoblotting experiment was performed using P and SUMO1, SUMO2 or SUMO3 cotransfected cells (Figure 4-1B), which gave us similar result. To further confirm that the slower moving P band was indeed sumoylated P protein, IP-IB experiment was performed using mouse anti-P antibody for IP and rabbit anti-SUMO1 (Santa Cruz Biotechnology) for IB. The slower moving band was recognized by both anti-P antibody and anti-SUMO1 antibody; the size between the P protein band and the slower moving band was about 12 kDa, indicating that the P protein is sumoylated by SUMO1 (Figure 4-1C). Additional sumoylation products were also detected in the lane of P plus SUMO1 (Figure 4-1A and C). Since Poly-SUMO1 chain cannot form at the same site, there are likely multiple sumoylation sites within the P protein. Sumoylation of endogenous P protein was also detected in PIV5 infected cells using a similar IP-IB approach (Figure 4-1D).
4.4.2 Identification of one consensus sumoylation site within the P protein

Many sumoylated proteins share a consensus motif: $\Psi$KXD/E, with a hydrophobic amino acid residue $\Psi$ at $-1$ place and an acidic amino acid residue at $+2$ place; $+1$ place can be any amino acid residue (54, 96). K254 of the P protein fits the consensus motif (Figure 4-2A). To investigate whether K254 of the P protein is indeed a sumoylation site, K254R mutation of the P protein was generated and analyzed. K254R mutation reduced sumoylation level of the P protein by about 40% (Figure 4-2BC), indicating that K254 is a sumoylation site of the P protein. The statistically significant reduction of sumoylation level of P-K254R indicates that there are other sumoylation sites within the P protein, consistent with the observation in Figure 4-1.

4.4.3 K254R mutation reduces PIV5 minigenome activity

To examine whether sumoylation of the P protein plays a role in regulating PIV5 gene expression, the effect of K254R mutation of the P protein was studied using a PIV5 minigenome system. P-K254R significantly reduced the minigenome activity (Figure 4-3A), suggesting that sumoylation of the P protein at K254 affects PIV5 gene expression. To investigate the possibility that the negative impact of P-K254R mutation in viral gene expression may be due to a change of protein secondary structure, circular dichroism was performed to compare the secondary structure of P-K254R with the P protein purified from E. coli. Those two proteins showed identical absorption from wavelengths 190 to 260nm (Figure 4-3B), suggesting K to R mutation at amino acid residue 254 did not affect the
secondary structure of the P protein.

**4.4.4 K254 of the P protein is a sumoylation site in PIV5 infected cells**

To examine the role of sumoylation of the P protein in virus infection, K254R mutation was incorporated into PIV5 to generate a recombinant virus (rPIV5-P-K254R) (Figure 4-4A). The genome of the mutant virus rPIV5-P-K254R was sequenced and no other amino acid residue change was detected. The sumoylation level of P-K254R in rPIV5-P-K254R infected cells was compared with that of the P protein in PIV5 infected cells. There was a lower level (20% reduction) of P-K254R sumoylation in rPIV5-P-K254R virus infected cells, indicating that K254 is a sumoylation site in PIV5 infected cells (Figure 4-4BC).

**4.4.5 rPIV5-P-K254R grows slower and to a lower titer than PIV5**

It is very important to confirm the findings from minigenome system in real virus infection. Growth of rPIV5-P-K254R virus was characterized with low MOI infection (MOI=0.01) (Figure 4-5A) and high MOI infection (MOI=3) (Figure 4-5B) in MDBK cells. rPIV5-P-K254R grew slower and to a lower titer (about 10 fold reduction) than PIV5, suggesting that mutation at K254 of the P protein caused defect in the growth of PIV5.
4.4.6 K254R mutation reduces viral protein expression

To study whether the defect in rPIV5-P-K254R growth is due to the defect in viral protein expression, levels of viral protein expression in rPIV5-P-K254R infected cells were compared to PIV5. At a MOI of 3, a lower level of viral protein expression in rPIV5-P-K254R infected cells was found at 8 hours post infection as well as at later time points by immunoblotting and flow cytometry (Figure 4-6AB). At a low MOI infection (MOI=1), rPIV5-P-K254R also showed significant defect in viral protein expression at 10 hours post infection (Figure 4-6C). In addition, the defect of rPIV5-P-K254R in viral protein expression was also detected in HeLa and BSR-T7 cells, indicating that the phenotype is not cell line specific (Figure 4-6DE).

4.4.7 rPIV5-P-K254R has lower levels of vial mRNA and genome RNA

To further examine the mechanism of the defect in rPIV5-P-K254R growth, quantitative real time PCR (qRT-PCR) were used to compare the levels of viral mRNA and viral genome RNA in MDBK cells. At a high MOI infection (MOI=3), rPIV5-P-K254R had a lower level of viral mRNA at 8 hpi (about 8 fold reduction) and at later time points (more than 10 fold reduction) (Figure 4-7A). The genome RNA level was also reduced in rPIV5-P-K254R infected cells at 8 hpi and at later time points (about 4 fold reduction) (Figure 4-7B). The ratio of viral mRNA to genome RNA was less in rPIV5-P-K254R infected cells (Figure 4-7C), indicating that K254R mutation affects viral transcription. Similar real time PCR results were observed at low MOI infection (MOI=0.5). (Figure 4-7DE), suggesting that K254R
mutation reduces viral RNA synthesis.

4.4.8 K254R mutation affects viral transcription

To further confirm that K254R mutation of the P protein affects viral transcription, a transcription-only minigenome, which is only functional in viral transcription but not replication, was used to compare the transcription activity. P-K254R reduced the activity of the transcription-only minigenome (ANOVA, P<0.01), indicating that P-K254R is defective in viral mRNA transcription (Figure 4-8).

4.4.9 P-K254R does not affect NP-P, P-L interaction and tetramer formation

The interactions of NP and P, P and L, as well as P tetramer formation are essential for PIV5 RNA synthesis. To study the mechanism for the defect of P-K254R, the NP and P/K254R interaction, the L and P/P-K254R interaction, were examined using co-immunoprecipitation and IP-IB in transfected cells (Figure 4-9AB). P-K254R can bind to the NP and the L proteins, similar to wide type P. In addition, P-K254R formed a tetramer like wild type P (Figure 4-9C), indicating that there is no defect in P-NP, P-L interactions and tetramer formation of P-K254R.
4.5 Summary

Sumoylation is a protein post-translational modification that plays an important role in regulating the function of target proteins. The P protein of PIV5, a prototype of paramyxovirus was found to undergo sumoylation by SUMO1 in both transfected cells and virus-infected cells. Sumoylation of the P protein by SUMO2 or SUMO3 was not detected. K254 of the P protein was identified as one sumoylation site, though there are likely other sumoylation sites with the P protein. Mutating the P protein at K254 to arginine reduced sumoylation level of the P protein. P-K254R reduced the minigenome activity about 3-5 fold. Incorporation of K254R mutation into the virus (rPIV5-P-K254R) reduced virus growth and resulted in a virus growing to a lower titer (about 10 fold reduction) than wild type PIV5. Viral protein expression and viral RNA synthesis in rPIV5-P-K254R infected cells were dramatically reduced compared with PIV5 infected cells, suggesting that sumoylation of K254 of the P protein is important for PIV5 gene expression. Using transcription-only minigenome system, I found that K254R mutation affected viral transcription. Biochemical studies did not reveal any defect in NP-P-L complex formation by K254R mutation of the P protein. It is possible that sumoylation of the P protein at K254 plays an important role in regulating PIV5 gene expression through a host protein.
A

GFP  P  F-S1  F-S1 +P  F-S2  F-S2 +P  F-S3  F-S3 +P

- Sumo-P
- P
- F-S1/2/3

B

GFP  P  SUMO1  P+SUMO1  SUMO2  P+SUMO2  SUMO3  P+SUMO3

52 kDa-
38 kDa-

- SUMO-P
- P

IB: anti-P
cell lysate
Figure 4-1. Sumoylation of the P protein by SUMO1. (A). Immunoblotting. P was cotransfected with Flag-SUMO1 (F-S1), Flag-SUMO2 (F-S2), or Flag-SUMO3 (F-S3) into BSR-T7 cells. After 24 hours, the cells were lysed and aliquots of the supernatant were resolved in SDS-PAGE. Anti-P antibody (Pκ) or anti-Flag was used for immunoblotting. (B) Immunoblotting in P and SUMO1/2/3 without Flag tag cotransfected cells. (C). IP-IB of transfected cells. Pκ antibody (Pκ, from mouse) was used for IP and anti-SUMO1 (from rabbit) was used for IB (Left part). Aliquot of the cell lysates were used for immunoblotting with anti-P antibody (right part). (D). IP-IB of infected cells. HeLa cells were mock infected or infected with PIV5. At 24 hpi, the cells were lysed and similar IP-IB was performed.
A

Sumoylation motif: \( \Psi K X D/E \)

PIV5 P: \( 1 \ K \ N \ D \)

254

B

[Diagram showing protein gel results for GFP, SUMO1, P, P+SUMO1 K254R, K254R+SUMO1, IP: anti-P, IB: anti-SUMO1, SUMO-P, IB: anti-P cell lysate, SUMO-P, P]
Figure 4-2. **K254 of the P protein is one sumoylation site.** (A). Alignment of sumoylation consensus motif and neighboring sequences of K254 of PIV5 P protein. (B). Sumoylation of P-K254R. K254 was mutated to arginine (R) and P-K254R was used for IP-IB to compare the sumoylation level to wild type P. (C). Quantification of the sumoylation levels. Data of three individual experiments from (B) were used for quantification and statistic analysis. The ratio of the single-sumoylated P protein to total P protein was set to 1 for wild type P protein and the relative sumoylation level of P-K254R was normalized to wild type P. P value was calculated using t-test.
Figure 4-3. P-K254R reduces PIV5 minigenome activity. (A). Minigenome activity of P-K254R. Increasing amount of P-K24R or the P protein was used in the minigenome system. Immunoblotting was performed to show the input amounts of NP, P or P-K254R. (B). CD analysis of His-P and His-P-K254R purified from E. coli.
A

PIV5: ......cag acc atc **aag** aat gac ata......
...... Q T I **K** N D I ......

PIV5-P-K254R: ......cag acc atc **agg** aat gac ata......
...... Q T I **R** N D I ......

254

B

Figure 4-4. Sumoylation of K254 in PIV5 infected cells. (A). Schematic of the mutation site. The differences in nucleic acid residues and amino acid residue between PIV5 and rPIV5-P-K254R virus were shown. (B). Sumoylation of P-K254R in infected cells. HeLa cells were mock infected or infected with PIV5 or rPIV5-P-K254R at a MOI of 5. At 24 hours post infection, the cells were lysed and the supernatants were used for IP-IB. (C). Quantification of the sumoylation levels. Three individual experiments of (B) were performed for quantification and statistic analysis.
Figure 4-5: Growth curve of rPIV5-P-K254R. (A). Growth curve of rPIV5-P-K254R was compared with PIV5 at a MOI of 0.01. “K254R” means “rPIV5-P-K254R. (B). Growth curve of rPIV5-P-K254R at a MOI of 3 was compared with PIV5 at different time points.
Figure 4-6. Viral protein expression levels in rPIV5-P-K254R infected cells. (A). Immunoblotting. MDBK cells were infected with PIV5 or rPIV5-P-K254R at a MOI of 3. The cells were collected at different time points and used for immunoblotting using anti-NP and Pk (anti-P/V) antibodies. β-actin was used as protein loading control. K254R means rPIV5-P-K254R virus. (B). Flow cytometry. MDBK cells were mock infected or infected with PIV5 or rPIV5-P-K254R at a MOI of 3. Flow cytometry was performed to compare viral protein expression at different time points using Pk antibody. (C, D, E). Flow cytometry in MDBK, HeLa and BSR-T7 cells. Similar experiments were performed at a MOI of 1 in MDBK, HeLa and BSR-T7 cells at 10 hpi.
**Figure 4-7. Viral RNA levels in rPIV5-P-K254R-infected cells.** (A). Viral mRNA levels after high MOI infection. MDBK cells were infected with PIV5 or rPIV5-P-K254R at a MOI of 3. The cells were collected at different time points for RNA extraction and real time PCR. Oligo d(T) was used for RT. The relative mRNA levels were normalized with the input genome defined as the viral genome at 2 hpi. (B). Viral genome levels after high MOI infection. The same RNA from (A) was used for RT using BH191 annealing to the M gene of the viral genome. The cDNA was used for real time PCR analysis. The genome level at 2 hpi was used as the baseline for normalization. (C). The ratio between viral mRNA and genome RNA based on (A) and (B). (D). Viral mRNA levels after low MOI infection. MDBK cells were infected with PIV5 or rPIV5-P-K254R at a MOI of 0.5. Total RNA was extracted from the cells at different time points for RT and real time PCR analysis. (E). Viral genome levels after low MOI infection. The same RNA from (D) was used for RT using BH191 to measure viral genome levels.
Figure 4-8. P-K254R affects viral RNA transcription. P-K254R was compared to wild type P using transcription-only minigenome system. The plasmids were transfected similarly to normal PIV5 minigenome system. Dual luciferase assay was carried out at 2 days post infection. Aliquots of the cell lysates were used for immunoblotting with anti-NP and anti-P antibodies.
**Figure 4-9. Interactions of P-K254R with NP, L and itself.** (A). Interaction between NP and P/P-K254R. BSR-T7 cells were transfected with NP along with P or P-K254R and metabolically labeled with \(^{35}\text{S-Met/Cys}\). The labeled cells were lysed for immunoprecipitation using anti-P antibody or anti-NP antibody. (B). Interaction between L and P/P-K254R. BSR-T7 cells were transfected with Flag-L along with P or P-K254R. The transfected cells were lysed for IP using anti-P antibody and IB using anti-Flag antibody. Aliquots of the cells lysates were used for immunoblotting to show the input amounts of L and P/P-K254R. (C). Tetramer formation of P/P-K254R. The transfected cells were labeled with \(^{35}\text{S-Met/Cys}\) and DSP was used for crosslinking followed by immunoprecipitation using anti-P antibody. Half of the IP products were mixed with protein loading buffer without DTT, and the other half were mixed with protein loading buffer with DTT.
CHAPTER 5: CONCLUSIONS AND DISCUSSION
5.1 Conclusions

5.1.1 PLK1 down-regulates PIV5 gene expression

The P proteins in paramyxoviruses are essential components of the viral RNA dependent RNA polymerase. We have reported for the first time that phosphorylation of PIV5 P protein directly regulates PIV5 gene expression (Chapter 2). We have also identified a host kinase, Polo-Like-Kinase 1 (PLK1), that binds to the P protein at S(pS157)P. PLK1 inhibition increased viral gene expression; PLK1 over-expression inhibited viral gene expression; and PLK1 directly phosphorylated the P protein in vitro and in vivo, indicating that PLK1 down-regulates viral gene expression by phosphorylating the P protein. Further studies have identified S308 as a PLK1 phosphorylation site within the P protein. Mutant viruses containing either PLK1 binding site (rPIV5-V/P-S157A) or PLK1 phosphorylation site (rPIV5-P-S308A) show elevated viral gene expression. The two mutant viruses increase CPE caused by apoptosis and induce production of cytokines including IFN-β and IL-6, suggesting that PIV5 may use PLK1 to limit its gene expression therefore preventing host innate immune responses.

5.1.2 Phosphorylation at T286 is important for PIV5 growth

Since the P protein is heavily phosphorylated in PIV5 infected cells, it is still believed that phosphorylation of the P protein at other sites may play positive roles in viral gene expression. To address that question, mass spectrometry was performed using the P protein
purified from PIV5 infected cells. Several phosphorylation sites or putative phosphorylation sites were identified, including S157, T286 as well as S36, S126 and a long peptide (293-331) which contains two phosphorylation sites. Mutation of S36 and S126 to alanine of the P protein did not affect the minigenome activity. Mutation of the P protein at T286 to alanine, aspartic acid reduced the minigenome activity and P-T286E showed no activity at all. Incorporation of T286A into the recombinant virus (rPIV5-P-T286A) showed delayed viral RNA synthesis, viral protein expression and viral growth. The results of biochemical studies suggest that the defects of T286 mutations are not due to the NP-P-L complex formation. While I failed in rescuing rPIV5-P-T286E virus, several revertant viruses were obtained, all of which carried mutations solely at T286. All the revertant viruses showed defects in viral growth compared with PIV5.

5.1.3 Sumoylation of K254 of the P protein is important for PIV5 growth

Sumoylation is a protein post-translational modification, playing a critical role in regulating protein function. The P protein of PIV5 has been found to undergo sumoylation by SUMO1 in both transfected cells and infected cells (Chapter 4). K254 of the P protein has been shown to be one of the sumoylation sites. P-K24R, having similar secondary structure as P wild type, significantly reduced the minigenome activity. Mutant virus containing K254R mutation (rPIV5-P-K254R) grew slower and to lower titer than PIV5; viral RNA synthesis and viral protein expression in rPIV5-P-K254R infected cells were dramatically reduced. Based on the transcription-only minigenome system, P-K254 was found to affect viral transcription. We propose that sumoylation of the P protein at K254 may regulate PIV5 gene
expression possibly through a host protein. This is a novel finding that the P protein of paramyxovirus can be sumoylated and that sumoylation may play a role in regulating paramyxovirus RNA synthesis.
5.2 Discussion

5.2.1 Phosphorylation in viral gene expression: negative role

The role of the P protein phosphorylation in paramyxovirus has been an enigma. It was initially suggested that phosphorylation of the P protein was important for viral RNA synthesis. However, recent studies on SeV and RSV seemingly suggest that phosphorylation of the P proteins of paramyxoviruses does not have a role in viral gene expression. It is possible that the critical phosphorylation sites within the P proteins were not identified in these earlier studies. While it is true that some of the phosphorylation sites may be superfluous and may not have a role in viral gene expression, it is difficult to imagine that none of the phosphorylation sites within a heavily phosphorylated P protein have any role in its function. Even though mutation of known phosphorylation sites reduces phosphorylation of SeV and RSV P by 90%, it is possible that the remaining residues that are phosphorylated (even though they only count for about 10% of total phosphorylation) are critical for viral gene expression.

In this work, I have identified a host kinase, PLK1, which phosphorylates PIV5 P protein and mapped both its binding (SSP motif at S157) and phosphorylation (S308) sites within the P protein. I have demonstrated using a recombinant virus that S308 of the P protein is important in regulating viral gene expression. Interestingly, PLK1 phosphorylation down-regulates viral gene expression, contrary to our expectation that phosphorylation may be essential for viral gene expression. Previously, we have found that AKT1, a serine/threonine
kinase, plays a critical role in viral gene expression (110). However, direct interaction between P and AKT1 as well as phosphorylation site of AKT within the P protein has not been reported. Further studies of phosphorylation of the P protein will be needed to identify possible phosphorylation sites within P that are critical for viral gene expression.

Previously, we showed that S157 was phosphorylated and contributed to the phenotypes of rPIV5-CPI+ (115). However, it was formally possible that the V protein, which also contains the S157 mutation, contributed to the CPI+ phenotypes since V and P are identical in the first 164 amino acid residues. In this work, I have identified a single amino acid residue (S308) within the P protein as a PLK1 phosphorylation site. Mutating this phosphorylation site has the same effect on viral gene expression as the CPI+ P mutations, further confirming our previous report that the increased gene expression from rPIV5-CPI+ is due to a P protein that is more efficient in facilitating viral RNA synthesis. Since the S308A mutation is within the unique P sequence while the V remains intact. It may seem counterintuitive that a virus would down-regulate its own gene expression by maintaining binding and phosphorylation sites for PLK1. However, higher PIV5 gene expression is associated with the induction of cytokine expression (IFN-β and IL-6) and cell death, which in turn limits virus replication and spread. IFN-β is a cytokine playing important role in triggering immune response against virus (77, 87). IL-6 is a pro-inflammatory cytokine to stimulate immune response (28, 73). Therefore, viruses that down-regulate their gene expression via PLK1 phosphorylation of the P protein would have advantages in viral transmission over viruses with defective PLK1 binding or phosphorylation sites (Figure 5-1). Since increased cell death and cytokine expression induced by virus infection limit virus replication, it is possible that targeting
PLK1 can enhance host innate immune responses, leading to a novel strategy to control virus infection. The lower viral gene expression after PLK1 phosphorylation may also favor persistent viral infection. Interestingly, about half the strains of PIV5 have an S residue at position 157 (22).

PLK1 can phosphorylate proteins with a PLK1 binding domain as well as proteins associated with PLK1-binding proteins. Thus, it is possible that PLK1 down-regulates viral gene expression through phosphorylating a P-binding protein. However, the S308A mutant, which still binds PLK1, has the same viral gene expression phenotype as PLK1 binding site (S157) mutants, indicating that P is not merely a scaffold for PLK1 to down-regulate viral gene expression, but is the target of PLK1 itself. The exact mechanism of how the phosphorylation by PLK1 at residue S308 affects the function of P remains to be determined. The P protein does not have a catalytic domain but it plays an essential regulatory role in viral gene synthesis. The P protein is known to interact with viral proteins NP and L. Binding and phosphorylation by PLK1 do not appear to have an impact on the interactions between P and the NP or the L. Previously, it has been reported that rPIV5-CPI- has an increased viral RNA genome synthesis while viral mRNA transcription is not affected (115). It is possible that phosphorylation at S308 changes the conformation of P to make it less efficient in facilitating viral RNA genome synthesis. It is possible that phosphorylated P at S308 affects RNA binding and increases the rate of RNA synthesis. It is also possible that phosphorylation of P at S308 may affect its ability to interact with a yet-to-be identified host protein, which in turn regulates viral gene expression.
A role of PLK1 in infection by other viruses has been reported recently. Expression of human T-lymphotropic virus type-I protein p30 reduces expression and phosphorylation of PLK1 (27) while PLK1 is up-regulated in CD4+ T cells from rhesus macaques infected with simian immunodeficiency virus (19) as well as in cells expressing E6/E7 of human papillomavirus (89). PLK1 can directly phosphorylate pp65, a dispensable gene of human cytomegalovirus (37). However, none of these studies has demonstrated a direct role of PLK1 in viral gene expression and the biological consequence of these studies are not clear. Recently, Ludlow et al reported that the V protein of NiV contains a PLK1 binding site within the shared region of the NiV P protein and that the PLK1 binding site is also involved in interacting with STAT1 protein, a key protein in IFN signaling (76). However, the biological consequence of this PLK1 interaction is not clear. Since Nipah virus is a biosafety level 4 pathogen, examining the role of PLK1 in the context of Nipah virus infection is difficult. Ours is the first report of PLK1 playing a direct role in virus replication.

The PLK1 binding site of the P protein of PIV5 is within the shared region of the V and P protein; thus, the PIV5 V should bind PLK1 as well. It is well known that the V protein blocks interferon signaling by causing degradation of STAT. However, it is unlikely this PLK1 binding site of V protein is involved in interferon signaling since the mutations at the S157 residue have no effect on IFN signaling (23). It is possible that PLK1 plays additional roles in viral life cycle. For instance, PIV5 V protein is known to slow down the cell cycle (69), so it is tempting to hypothesize that PLK1 is involved in the regulation of cell cycle by the V protein.
A cursory examination of the P proteins of paramyxoviruses indicates that most contain SSP (or STP) sequence motifs; some of the P proteins have more than one SSP sequence motif. For instance, NiV P has two SSP sequences. However, it is not known whether those S/T residues are phosphorylated. Since efficient PLK1 binding requires the middle S/T residue to be phosphorylated (31), it is not clear whether these SSP sequences are truly SSP motifs and bind PLK1. It is possible that the role of PLK1 in viral gene expression is universal for all paramyxoviruses. On the other hand, it is equally possible that PLK1 plays different roles for different paramyxoviruses. These questions warrant further investigation.

5.2.2 Phosphorylation in viral gene expression: positive role

I have identified a phosphorylation site within the P protein of PIV5, T286 (Chapter 3). Mutating the T residue at 286 to A, resulting in a P protein that was less efficient than wild type P (about half) in a minigenome system and had delayed and slowed growth rate when incorporated into a recombinant virus, indicating that phosphorylation of the P protein of paramyxovirus plays an important role in PIV5 growth. This is the first time that an important role of a phosphorylation site within the P protein in viral gene expression has been demonstrated. Take together with the work on the role of phosphorylation at S157 of P of PIV5 in down-regulating viral gene expression, the results indicate that P protein phosphorylation does play a role in regulating paramyxovirus gene expression: phosphorylation can up regulate as well as down regulate viral gene expression.

Besides S157 and T286, there are other phosphorylation sites. While mutating putative sites
S36 and S36 had no impact on the minigenome activity, indicating that these sites may be incidental if they are indeed phosphorylation sites. There are likely other phosphorylation sites within the P protein. The peptide from amino acid residue 293 to 331 contains two putative phosphorylation sites according to mass spectrometry result. In addition, the mass spectrometry analysis only covered about 74.2% of the P protein. Further analysis of temporal regulations of the known phosphorylation sites as well as identifying novel critical phosphorylation sites within the P protein will provide new knowledge on regulation of viral gene expression.

While the work here indicates that the phosphorylation of T286 is important for efficient viral RNA synthesis, the exact mechanism is not clear. Mutating of T286 to D or E, a phosphomimic residue, resulted in a mutant more defective, to the mutant T286A, either because T286D and T286E did not mimic T286 phosphorylation, or T286D and T286E did mimic T286 phosphorylation, but over-phosphorylation was defective to the P protein. We speculate that phosphorylation of T286 plays a critical role in association of the P protein to another protein or RNA template. The P protein is known to interact with other viral proteins, NP and L as well as form homotetramer. Mutating the T286 residue had no detectable effect on its association with NP or L. Mutant P proteins formed homotetramer as efficient as wild type P, indicating that the partner protein for the function of T286 is a host protein. This is consistent with the data on revertants from PIV5-T286E rescue. If the residue T286 were essential for its interaction with other viral proteins to have a role in viral RNA synthesis, compensatory mutations would have risen in other viral proteins. Instead, the only mutations we have observed were in the P protein at the exactly the site, indicating that phosphorylation
of T286 is likely important for interacting with a host protein. It is known that P protein interacts with AKT1, a serine/threonine kinase. However, no difference in interactions between wild type P or P mutants and AKT1 have been detected (data not shown), indicating that a host protein other than AKT1 may play a role in regulating viral RNA synthesis. We propose that the phosphorylation of T286 enhances association of a host protein, which results in modification of the P protein itself or P-associated viral proteins NP and L.

Another possible mechanism is that the binding between the P protein and RNA template may be regulated by the phosphorylation status of the P protein. While T286A is neutral, T286E is negatively charged, T286 can be neutral before phosphorylation or after dephosphorylation, or negatively charged after phosphorylation. In other word, the negative charge of pT286 is reversible, while T286E is constitutively negatively charged. In addition, P can form a tetramer, so another possible difference between T286 phosphorylation and T286E is that only partial P in the tetramer is phosphorylated, thus the number of the negative charge is different from that of T286E. Since it was reported that P could bind to RNA in vitro (70), P protein phosphorylation at T286 may play a role in regulating the binding between P-L complex and the RNA template (negative charge). It is possible that before phosphorylation at T286, P facilitates the binding between P-L complex and the RNA template; after phosphorylation at T286, P protein promotes the movement of the P-L complex or the release of newly synthesized RNA. P-T286A and L complex may bind RNA genome too tight, which is not optimal for the polymerase complex movement or RNA release; For P-T286E, due to its heavily negative charge, The polymerase complex may fail to bind RNA genome, thus no function in PIV5 RNA synthesis. Future works will focus on
these two possibilities to study the mechanism.

It is not clear which kinase phosphorylates the P protein at T286. Previously, AKT is known to play an essential role in PIV5 gene expression (110). However, T286 is unlikely to be an AKT phosphorylation site. AKT substrate contains positively charged residues such as lysine or arginine at -3 and -5 place (1). In addition, ATK inhibitor IV reduced rPIV5-P-T286A protein expression as well as phosphorylation of P-T286A (data not shown). A candidate kinase for phosphorylating the P protein at T286 is CK2. T286 (TVED) is within the context of a CK2 consensus phosphorylation motif [pS/pT]XX[E/D] (83). CK2 inhibitor reduced virus replication in HeLa cells (data not shown). In vitro kinase assay showed that CK2 phosphorylated the P protein of PIV5 purified from E.coli (data not shown). However, CK2 phosphorylated P-T286A at a similar level as the wild type P. It is possible that there are several potential CK2 sites: mutating one site did not make a significant difference in the overall phosphorylation level of the P protein. Previous studies suggested that CK2 phosphorylated the P protein from RSV, measles virus, Rinderpest virus and Borna disease virus (26, 55, 82, 103). Due to numerous subunits and isoforms of CK2, and ubiquitous potential CK2 sites, work on the role of CK2 has not been conclusive. Further studies are needed to clarify the role of CK2 as well as to identify host kinases that phosphorylates the P protein at T286 and other phosphorylation sites.
5.2.3 Sumoylation in viral gene expression

Sumoylation is an important protein post-translational modification and is involved in many essential cellular processes. I have found that PIV5 P protein, a viral protein from a non-segmented, negative-strand RNA virus, was sumoylated by SUMO1, but not SUMO2 or SUMO3 (Chapter 4). The mechanism of preference of SUMO1 over SUMO2/3 is not clear. Interestingly, the V protein, which shares the N-ternimus with the P protein, can also be recognized by Pk antibody; the NP and L protein can be coimmunoprecipitated with the P protein, but sumoylation of the NP, the L or the V protein was not detected. Based on a consensus sumoylation motif and confirmed by IP-IB experiment, I have identified K254 of the P protein as a sumoylation site. Poly-sumoylation of the P protein was found and K254R mutation reduced the sumoylation level of the P protein, suggesting that there are other sumoylation sites within the P protein. Further studies need to identify those extra sumoylation site(s) and their function. It is possible that sumoylation of the P protein at different sites play different roles in PIV5 gene expression. Sumoylation of the P protein at K254 is suggested to be important for PIV5 minigenome activity as well as PIV5 growth, indicating that sumoylation of the P protein regulates PIV5 gene expression. I also found that sumoylation of the P protein at K254 affected viral RNA transcription, which may lead to a reduced level of viral replication in infected cells. However, we cannot exclude the possibility that sumoylation of the P protein at K254 also directly affects viral RNA replication as well. Future work will address this question by developing new strategies.

Among the effects of sumoylation, transcriptional regulation is an important one. In
eukaryotes, sumoylation usually has an inhibitory effect on gene transcription. Mutations at the sumoylation sites of transcription factors Elk, Sp-3, STAT-1 or P300 lead to transcription activation (54). Although the steady-state sumoylation level is less than 5% of the given protein, most transcription factors can be significantly activated when sumoylation sites were mutated (38). SUMO can also have positive effects on transcription through activating β-catenin activated factor Tcf-4 (130). For viruses, most studies suggest that sumoylations of viral proteins play positive roles in viral gene expression. Bovine papillomavirus (BPV) E1 protein can be sumoylated at K154 and mutation of K154R results in sequestration of E1 in the cytoplasm therefore loss of replication capacity (95); Sumoylation of human papillomavirus (HPV) E2 proteins affect its activity in both transcriptional activation and repression (126). Sumoylation of HCMV IE2p86 protein was important for IE2 mediated transactivation (49).

Despite the different roles of sumoylation of host proteins and viral proteins in transcription, the underneath mechanism may be similar. Sumoylation may function through modulating protein-protein interaction or altering substrate conformation (54). The SUMO dependent transcriptional repression is likely due to SUMO-dependent recruitment of downstream effect proteins. For example, only sumoylated p300 can recruit HDAC6, a transcriptional repressor (40). Interestingly, HDAC6 can also interact with PIAS proteins, which also bind directly to SUMO and sumoylated proteins (54, 74). Therefore, sumoylation may be important in the complex formation of transcription factors. Similarly, one possible mechanism for the effect of viral protein sumoylation on viral transcription could be the recruitment of downstream effect proteins, either from the virus or from the host cells. Our study has shown that P-
K254R had no defect in interacting with NP and L protein or in homotetramer formation of the P protein. We propose that sumoylation of the P protein at K254 regulates PIV5 gene expression through interacting with yet to be identified host protein(s).
Figure 5-1: A working model for regulation of PIV5 gene expression by PLK1. For wild type PIV5, PLK1 binds to SSP motif (156-158) within the P protein, and then phosphorylates the P protein at S308, resulting in inhibition of PIV5 gene expression. The reduced viral gene expression supports efficient virus replication, but enables virus to avoid the induction of cell death and cytokine expression. For rPIV5-CPI+, SSP is mutated to SFP, preventing the binding of P to PLK1 and, thus, no phosphorylation of S308 by PLK1. As a result, viral gene expression is higher than that of PIV5, and cell death as well as cytokine production is induced, resulting in limiting replication and spread of virus. However, when PLK1 is over expressed, PLK1 can interact with S157F weakly through STP centered at T108 and reduces P-S157F mini-genome expression moderately. For rPIV5-P-S308A, while the SSP motif is intact and P-S308A still binds to PLK1; however, there is no phosphorylation of 308. This mutant virus, similar to rPIV5-CPI+, shows elevated viral gene expression and increased induction of cell death.
REFERENCES


28. **Dillon, P. J., and G. D. Parks.** 2007. Role for the phosphoprotein P subunit of the


77. Luthra, P., D. Sun, R. H. Silverman, and B. He. 2011. Activation of IFN-β expression by a viral mRNA through RNase L and MDA5. Proc Natl Acad Sci U S A
166


94. **Randall, R. E., and A. Bermingham.** 1996. NP:P and NP:V interactions of the


110. **Sun, M., S. M. Fuentes, K. Timani, D. Sun, C. Murphy, Y. Lin, A. August, M. N. Teng, and B. He.** 2008. Akt plays a critical role in replication of nonsegmented


Vita

Dengyun Sun

EDUCATION

Ph.D Cell and Developmental Biology, Pennsylvania State University (2006-2011)
M.S. Plant Biology, Nanjing University, Nanjing, China (2003-2006)
B.S. in Biology, Anhui Normal University, Wuhu, China (1999-2003)

PUBLICATIONS

1. Sun D, Xu P, He B. Sumoylation of the P protein at K254 plays an important role in growth of parainfluenza virus 5 (PIV5). Under revision.

ACADEMIC HONORS AND AWARDS

Travel Award, American Society for Virology, 29th Annual meeting (2010)
Travel Award, American Society for Virology, 28th Annual meeting (2009)
Pennsylvania State University Graduate Fellowship Award (2006)