STUDENT NAME: *Tychicus Chen*

PROJECT TITLE: Host-Viral Protein-Protein Interactions in Influenza virus Infections

STUDENT'S NAME: Tychicus Chen **SUPERVISOR'S NAME:** Dr. Kevin Coombs

DEPARTMENTAL AFFILIATIONS: Department of Medical Microbiology

SUMMARY:

Influenza virus infection remains a worldwide problem today. Pathogenicity can be attributed in part to the changing protein structure of the virus, allowing it to evade the host immune system. For example, the hemagglutinin (HA) and neuraminidase (NA) proteins used in the subtyping of influenza strains; their roles and interactions with host proteins having been extensively studied. Studying host-viral protein-protein interactions is important in understanding the virus replication process, and still there are many other key proteins worth investigating to further our knowledge in influenza research and potentially target new treatment. This research project will focus on the influenza virus protein NS1, a non-structural protein involved in the viral replication cycle, exploring host-viral protein-protein interactions throughout the replication process in an attempt to characterize the exact proteins NS1 requires during infection. The student will primarily be using a non-pathogenic influenza lab strain A/PR/8/34 (H1N1 subtype) with a mammalian cell line of the Madin-Darby Canine Kidney (MDCK) epithelial type. The objective of the student will be to learn proper and aseptic cell culturing technique, methods for protein analysis such as co-immunoprecipitation to "pull-down" proteins of interest using monoclonal antibodies directed against NS1, which the student will be purifying from murine lymphocytes, as well as polyacrylamide gel electrophoresis (SDS PAGE) to separate proteins, and finally western blotting. Any interacting proteins seen will then be sent for analysis using mass spectrometry to determine their exact identities for further study.

ACKNOWLEDGEMENTS:

Stipendiary support for the student was provided by Dr. Kevin Coombs, whose research is funded by a grant from CIHR.

_____ _____

Student's Signature **Supervisor's Signature**

STUDENT NAME: *Tychicus Chen*

INTRODUCTION:

The influenza virus is a serious and ubiquitous pathogen that accounts for over half a million deaths worldwide each year[1]. Due to its incredible genetic variability, it is difficult to predict new strains, making vaccinations less effective. The recent H1N1 pandemic caused several hospitalizations and death in all areas of the world, illustrating the devastating potential and just how unpredictable this virus can be, and the Center of Disease Control and Prevention (CDC) estimates the death toll from this pandemic alone to be over 400 thousand to date[2]. It is clear that influenza is still a serious concern and a logical target for medical research interest.

Influenza virus is classified under the family *Orthomyxoviridae* of which members have a characteristic segmented genome, consisting of 7-8 segments of (-) sense RNA[1,3,4]. In the case of Influenza A, these 8 genes encode 10 different proteins which allow the virus to replicate, invade host cells, and in some cases evade host immune defences [3,4,5]. Two particularly important and well characterized proteins in the Influenza A viruses are the hemagglutinin and neuraminidase proteins. There are currently 16 and 9 types of each protein known, respectively, and are useful in the subtyping and classification of the virus. Hence, Influenza A viruses are named based on their H/N combinations, such as the recent H1N1 "Swine Flu", or the H5N1 "Avian Flu".

What makes the influenza virus so unpredictable is its great genetic variability, attributed to the processes known as antigenic drift and antigenic shift[1,2,3]. Antigenic drift occurs due to the high error rate in the viral RNA polymerase, allowing for frequent mutations in the viral genome. Antigenic shift, on the other hand, refers to the mixing of gene segments between different influenza viruses within the same cell, reassorting to produce new hybrid viruses[5]. Vaccination strategies like the seasonal flu shot, therefore, are at the mercy of "predicting" which strains will be most likely to circulate, but this is not always effective as there is no way to know for sure and such a vaccine needs to be reformulated yearly.

The influenza virus replication cycle proceeds through entry, uncoating, nuclear import, production of viral mRNA and export, protein translation, protein processing , replication, production of progeny and export, and finally assembly and exit[5]. Two classes of antivirals are currently available for influenza treatment. The neuraminidase inhibitors, such as Tamiflu (oseltamivir phosphate) and Relenza (zanamavir), interfere with the virus exit mechanism[6]. The adamantane class of drugs, amantadine and rimantadine, interfere with the uncoating of the virus. However, once again due to the genetic variability and rapid mutation frequency, drug resistance becomes an issue in long-term treatment or pandemic situations, and new therapeutic approaches are always being sought.

This project will instead focus on a different kind of protein – the only non-structural protein the virus produces, so-called because it is produced during infection but does not exist in individual virions[3]. NS1 is a 26 kDa non-structural protein encoded on the 8th gene segment[1,3,4,7] and being uninvolved with the virus structure, is a key regulatory protein that has been shown to interact with RNA as well as protein, and most notably bind to dsRNA[8,9]. Some of the more specific regulatory roles include the inhibition of cellular host pre-mRNA splicing[10,11] and binding of host mRNA cleavage and polyadenylation specificity factors[12,13] which may also

inhibit the export of host mRNAs[13]. It has also been shown to interfere with several cell signalling pathways, such as binding to p85β to activate the PI3K/Akt pathway inhibiting apoptosis[14,15] and inhibiting downstream activation of interferon regulatory factor 3[16]. Finally, NS1 is also involved in evading the host immune system as well, through binding of the ubiquitin ligase tripartite motif-containing protein 25 to evade the host RIG-I viral RNA sensor[17], and suppressing the host antiviral state by inhibiting interferon production[18].

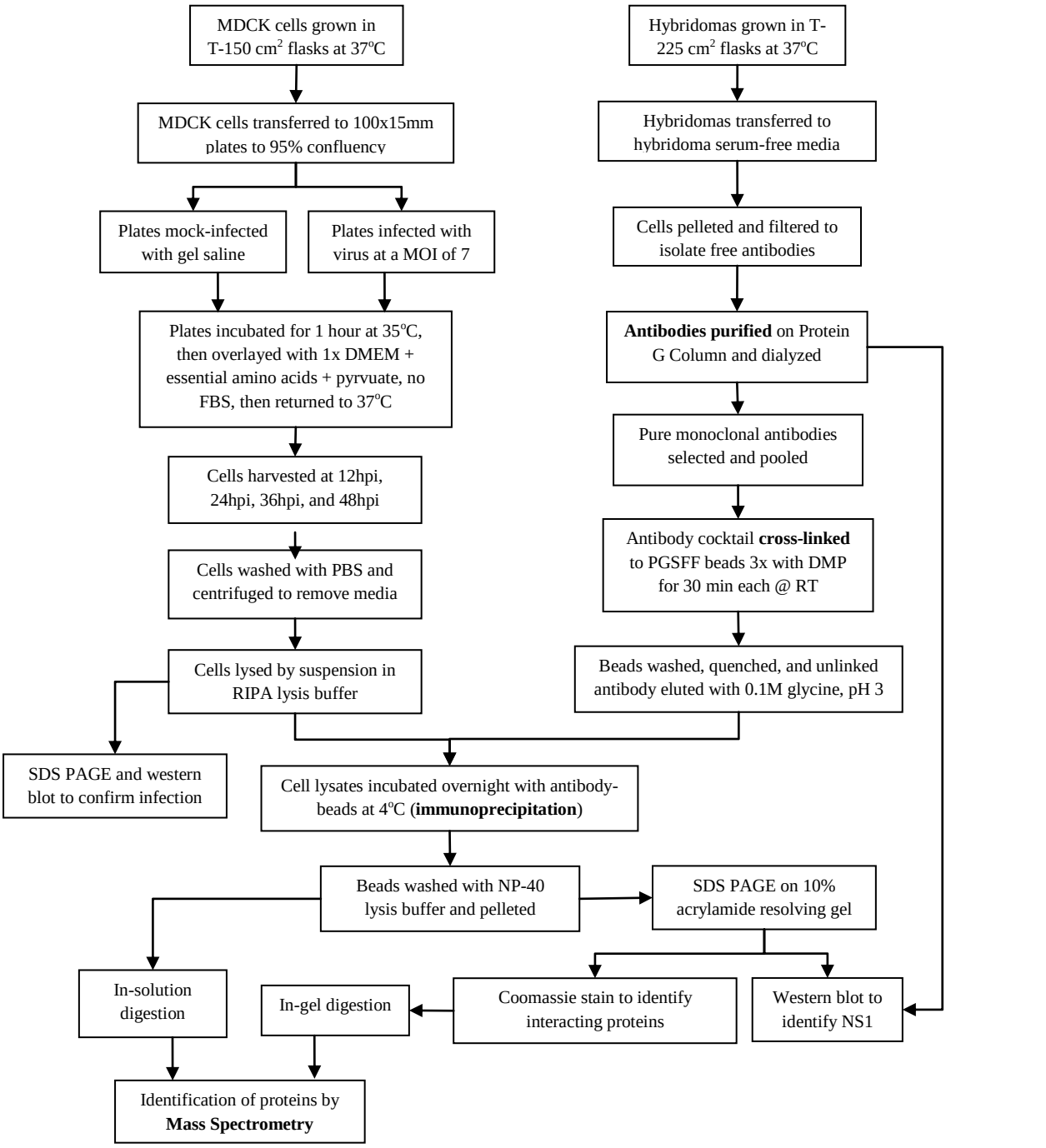
This project aims to characterize the many protein interactions between viral NS1 and host proteins. Recent studies have shown that NS1 interacts with the ribonucleoprotein complex, including another viral protein nucleoprotein, or NP, as well as CPSF30, a host RNA polymerase II[18,19]. Immunoprecipitation is a common technique used to draw relationships between proteins because their interaction brings them close together, allowing them to be precipitated together with a specific antibody. It is the goal of this study to determine interacting proteins that co-immunoprecipitate with NS1 and identify them using mass spectrometry for future studies.

MATERIALS AND METHODS:

In order to selectively investigate the NS1 protein – and any interacting proteins alongside of it – monoclonal antibodies directed against NS1 were grown. Hybridomas previously produced from mouse-origin antibody-producing B cells against NS1 and myeloma cells were grown to confluency in large T-225 cm^2 flasks, and cells were washed with serum-free RPMI buffer, centrifuged, and transferred to a hybridoma serum-free medium. Finally, free antibodies were separated from cellular material using centrifugation and the Millipore Express Stericup filter system. Filtered antibodies were purified by binding to a protein G column overnight at 4^oC, washing of the column with phosphate-buffered saline (PBS), and elution of bound antibodies using 0.1M glycine, pH 2.7. The eluted antibodies were collected from the column in fractions with each fraction screened for protein concentration using the Thermo Scientific NanoDrop 2000, selecting for highly concentrated samples which for the purposes of this study were chosen as fractions with a reading greater than 0.8 mg/mL. Selected fractions were transferred to dialysis tubing in PBS and dialyzed overnight at 4^oC to remove any remaining glycine. Protein content of final samples was determined using the Bio-Rad Protein Assay kit in a 96-well plate, read with a BioTek Synergy 4 spectrophotometer coupled with the Gen5 Data Analysis software at a wavelength of 540 nm. Samples were stored in aliquot at 4^oC for later use.

To investigate host-virus protein-protein interactions, cell cultures were grown and infected with influenza virus. The principle cell line used was derived from Madin-Darby Canine Kidney (MDCK) epithelial cells, which is a common standard for influenza studies. Cells were grown to 95% confluency in 150mm plates, infected at a multiplicity of infection (MOI) of 7, incubated at 35^oC for 1 hour to allow for adequate adsorption, and overlayed with Dulbecco's Modified Eagle Medium (DMEM) + essential amino acids + pyruvate, no FBS. Mock-infected cells were instead inoculated with an equal volume of gel saline and treated under the same conditions to serve as a control for normal host protein changes unrelated to infection. They were then harvested at various time points post-infection (12, 24, 36, and 48 hours) to investigate cellular proteome

changes across time. Two 150mm plates were used for mock infection and each time point and harvested by scraping into a conical tube along with media to ensure full collection of cells.



Flowchart summarizing experimental design

STUDENT NAME: *Tychicus Chen*

Cells were centrifuged and washed twice with ice-cold PBS and pelleted to be treated with radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitors (50mM Tris, pH 8.0; 100mM NaCl; 0.5% NP-40; phenylmethylsulfonyl fluoride to 50 µg/ml; tosyl-L-lysine chloromethyl ketone to 1 µg/ml; Leupeptin to 1 µg/ml; Pepstatin A to 1 µg/ml; Aprotinin to 0.1 U/ml) for 30 minutes on ice, and centrifuged to separate larger macromolecules and cell nuclei without the intention of disrupting any host-virus protein-protein interactions. The protein content of each lysate was determined using the Bio-Rad Protein Assay kit and analyzed spectrophotometrically at 540 nm using a BioTek Synergy 4 plate reader and the Gen5 Data Analysis software.

Influenza infection was to be confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) followed by western blotting on a nitrocellulose membrane with the previously prepared anti-NS1 monoclonal antibodies as the primary antibody, and a goat anti-mouse horseradish peroxidase (HRP) conjugated antibody as the secondary antibody, visualized with enhanced chemiluminescence (ECL) read by an Alpha Innotech FluorChemQ. A 10% acrylamide resolving gel was used for better separation of lower molecular weight proteins, to which 10ug of protein from each sample was loaded, and ran for 120V for 75 minutes.

To select for host proteins specifically interacting with viral NS1 protein, co-immunoprecipitation was employed for "pull down" out of solution. Of the purified monoclonal antibodies, representatives of both the IgG1 and IgG2 subclasses (κ light chain and λ light chain) were selected and pooled together to form a cocktail. These antibodies were to be cross-linked to Protein G Sepharose Fast Flow (PGSFF) beads, at their cited binding capacity of 6 mg/mL, using three applications of dimethylpimelimidate (DMP), pH 8, each over 30 minutes at room temperature, followed by washing with 0.2 M triethanolamine, quenching with 50 mM ethanolamine, and finally removal of any remaining unlinked antibody with 1 M glycine, pH 3. Beads were incubated with the prepared cell lysate at a ratio of 10µg of antibody-bead mixture to 500µg of cell lysate at 4°C overnight to allow for proper adherence, and then washed aggressively several times with NP-40 lysis buffer (50mM Tris, pH 8.0; 150mM NaCl; 1% NP-40; 50ug/mL PMSF; 1ug/mL aprotinin; 1ug/mL leupeptin; 1ug/mL pepstatin) with centrifugation between washes to separate uninteracting proteins from the protein-antibody-bead complexes. The final bead pellet was then resuspended in 1x Laemmli sample buffer and boiled for 10 minutes to elute bound proteins from the antibody-beads, centrifuged at 10,000x*g* for 5 minutes to remove the beads, and loaded onto a 10% polyacylamide gel in duplicate for protein separation. To one gel, generalized staining was done using Thermo Scientific GelCode Blue Stain Reagent to visualize all remaining proteins. The duplicate gel was transferred to a nitrocellulose membrane and probed with anti-NS1 antibody to show the presence of NS1 from the immunoprecipitation and for comparison with any GelCode Blue stained proteins other than the NS1. These stained proteins would be considered to have been interacting with NS1 and cut out for in-gel digestion followed by analysis by mass spectrometry. The immunoprecipitation could also be repeated but without boiling or SDS PAGE for in-solution digestion, and again analyzed with mass spectrometry. Identified proteins could then be considered as NS1-interacting proteins.

STUDENT NAME: *Tychicus Chen*

RESULTS:

The concentrations of selected purified monoclonal antibody samples, as determined by the Bio-Rad Protein Assay, are shown in Table 1. These samples were chosen for their high concentration of around 2 mg/mL in order to saturate the sepharose beads, as well as their representation of different IgG subclasses and light chains.

The course of influenza infection of the MDCK cells was followed visually during the 12, 24, 36, and 48 hour time points. As expected, cells began to die off as infection progressed, lifting off of plates and decreasing in number from the 24 hour time point and on (see Figure 1b). This was not seen in the mock-infected cells, which showed sustained viability even up to the 48 hour mark. Total protein was extracted from two confluent 150mm plates for mock infection and each time trial, and was also determined by the Bio-Rad Protein Assay method, as shown in Table 2.
All cells were initially plated and grown to confluency as consistently as possible before treatment (see Figure 1a), and as expected, the mock-infected cells yielded the highest protein concentration which declined as time post-infection increased, with the 48 hour time point containing the lowest amount of protein. This is by no means an accurate comparison of protein over time, but is useful in standardizing the amount of protein used later on.

Despite visual evidence of infection (Figure 1b), however, the western blot using the purified anti-NS1 antibodies was unable to confirm the presence of the viral protein in any of the cell lysates. No bands were seen in the western blot and so it was not included. The coomassie stained gel, on the other hand, is shown in Figure 2, illustrating the abundance of protein in each of the cell lysate samples. The western blot was repeated several times with the same result – no bands on the western blot with the purified anti-NS1 monoclonal antibodies and anti-mouse HRP secondary antibody.

The western blot for the immunoprecipitated protein samples also showed some anomalies (Figure 3). Again, there was no visible band for NS1, which is a 26 kDa protein. There were, however, very strong bands nearby at 25 kDa and as well as at around 50 kDa in all lanes, including the mock infected cell lysate. The light and heavy chains of IgG are about 25 kDa and 50 kDa, respectively, and considering that the IgG to be cross-linked to the sepharose beads for immunoprecipitation were of mouse origin, the anti-mouse HRP secondary antibody would target any unbound IgG in the sample, suggesting free IgG present in all samples, and will be explored further in the discussion. Lastly, the corresponding coomassie stain showed no other proteins aside from the same 25 kDa and 50 kDa bands in all lanes, suggesting the only protein remaining after immunoprecipitation was the IgG originally from the beads.

The presumably cross-linked antibody-bead mixture was loaded onto a separate gel with and without boiling to see if heating was the cause for the antibodies to elute from the beads. The results are seen in Figure 4, and show the presence of the IgG heavy and light chains regardless of boiling. This may suggest that the antibodies were never really cross-linked to the beads at all.

STUDENT NAME: *Tychicus Chen*

DISCUSSION:

Several areas in this project presented with difficulties that interfered with the proposed goal. The first issue pertains to the efficacy of the purified monoclonal antibodies. All purified antibodies were stored for long periods of time at 4^{o}C with no added preservatives. Because proteins are sensitive to freeze-thawing cycles, the antibodies were never frozen for storage in -80^{o}C. It is likely that the antibodies degraded and lost their activity after only a few short months. In future work, antibodies should be aliquoted into smaller amounts and stored in glycerol to be frozen at -80^{o}C, with only the required amount per application to be thawed and used immediately without the need for any refreezing. This was overlooked but would increase the preservation of the antibodies for long term storage and decrease the chances of protein degradation.

The second issue encountered in this project was with the cross-linking of the antibodies with the protein G sepharose beads. Dimethylpimelimidate is said to be an irreversible cross-linking agent, creating a covalent chemical bond, and so antibodies should not be expected to separate from the beads after they are bound for any reason. It could not be determined that boiling was a factor as free IgG was seen regardless of boiling (Figure 4), and so the success of cross-linking is brought into question. It could be that the cross-linking is susceptible to boiling after all, but without confirmation that the antibodies were ever linked to the beads this question remains unanswered. DMP is unstable in aqueous solution so care was taken to prepare fresh DMP solution each time, immediately before use. However it is possible that even the stock of DMP itself was defective, if for example the DMP was expired or subjected to moisture, reducing its value as a cross-linking agent. Thus, in future work, new reagent should be ordered or perhaps even a different cross-linking agent be considered. The possibility of free IgG leftover from the cross-linking procedure and contaminating samples should be minimal as the mixture was washed several times with 0.1M glycine, pH 3, with the purpose to elute weakly bound and uncross-linked antibody and remove it from the beads.

Because this project requires functional antibody in order to pull down the NS1 protein with interacting host proteins, as well as for probing in western blot, there are fundamental issues that need to be addressed before progress may continue. Moreover, antibody-bead cross-linking should be a crucial step as it allows the beads to be washed cleanly without the fear of protein loss, so it needs to be carried out successfully. The results presented are consistent with a non-functional antibody and ineffective antibody-bead cross-linking, preventing any further work from being done. Given more time, these issues need to be investigated and solved in order to proceed, with some starting suggestions given above.

STUDENT NAME: *Tychicus Chen*

FIGURES AND TABLES:

Antibody (from hybridoma array)	Subclass	Light chain	Purified Antibody
10c7	IgG2a	lambda	1.9 mg/mL
4e10	IgG1	kappa	1.9 mg/mL
5f4	IgG2b	kappa	2.0 mg/mL

Table 1: Selected antibodies for use with co-immunoprecipitation as well as primary probing for western blotting. These antibodies were selected for their high concentrations as well as their representation across IgG subclasses.

Lysate Sample	Protein Concentration
Mock	5.80 mg/mL
12hpi	5.82 mg/mL
24hpi	3.66 mg/mL
36hpi	3.15 mg/mL
48hpi	3.14 mg/mL

Table 2: Protein concentration of each cell lysate sample as determined by the Bio-Rad Protein Assay kit. A standard curve was produced using a 2 mg/mL bovine serum albumin (BSA) standard in serial dilution, and samples were read using a BioTek Synergy 4 plate reader at 540 nm and Gen5 Data Analysis software. Each sample harvested from two 150mm plates initially at 95% confluency (see Figure 1a).

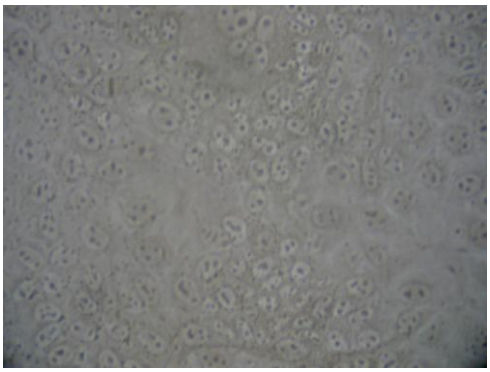


Figure 1a: MDCK cells grown in 150mm plates to 95% confluency in 1x DMEM + essential amino acids + pyruvate + 10% FBS.

STUDENT NAME: *Tychicus Chen*

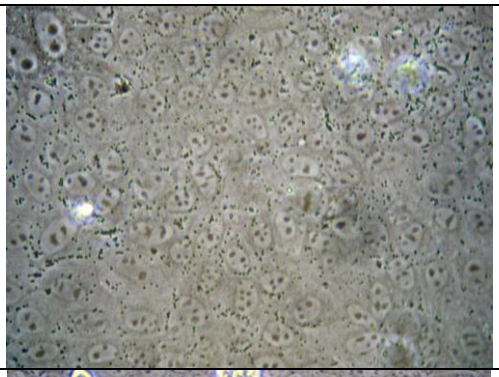
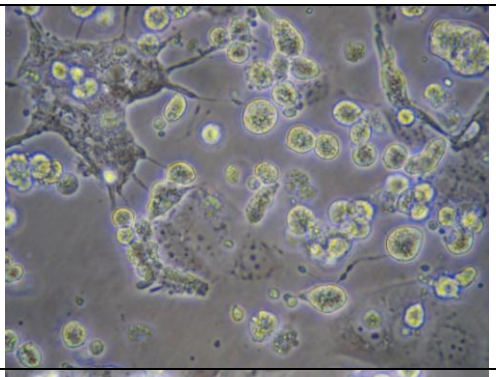
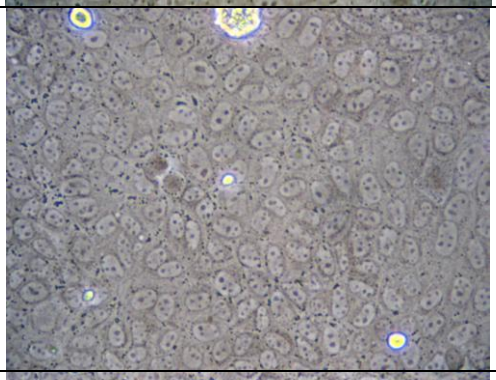
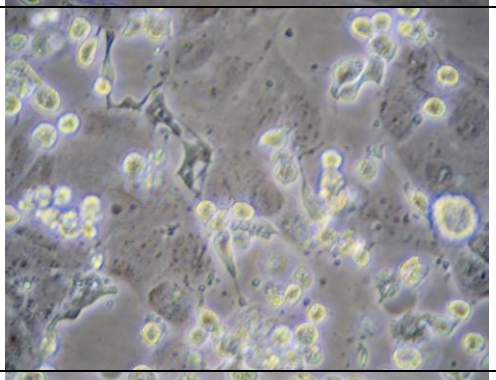
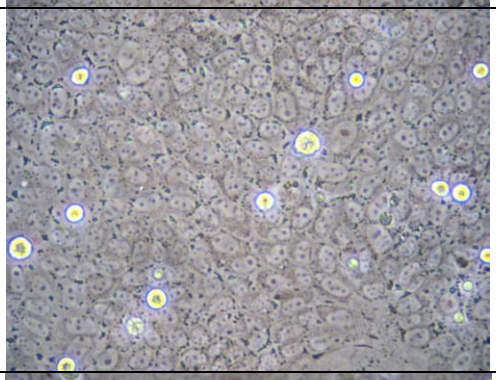
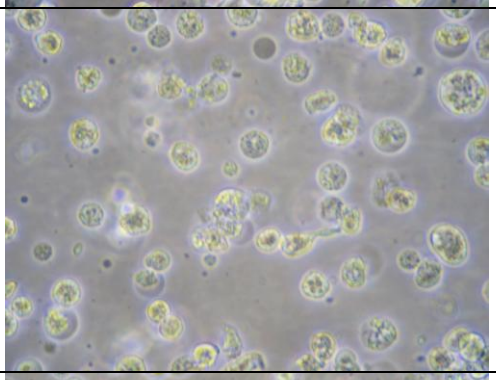
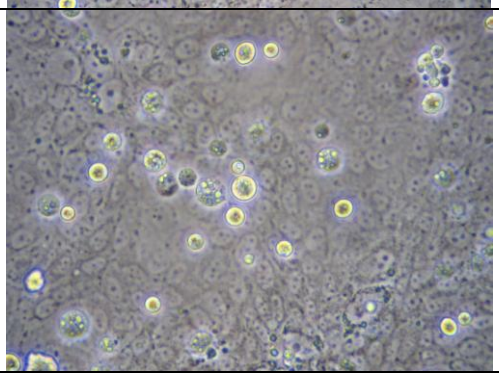
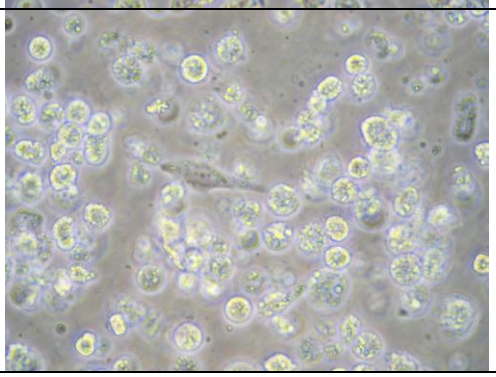
	Mock-Infected Cells	Infected Cells
12hpi		
24hpi		
36hpi		
48hpi		

Figure 1b: Cells mock infected with gel saline; and infected with Influenzavirus A/PR/8/34 at a multiplicity of infection of 7, grown in 1x DMEM + essential amino acids + pyruvate, no FBS. Images taken using a Nikon Eclipse TE2000-S at 400x zoom with camera adapter at 12, 24, 36, and 48 hours post-infection prior to harvesting.

STUDENT NAME: *Tychicus Chen*

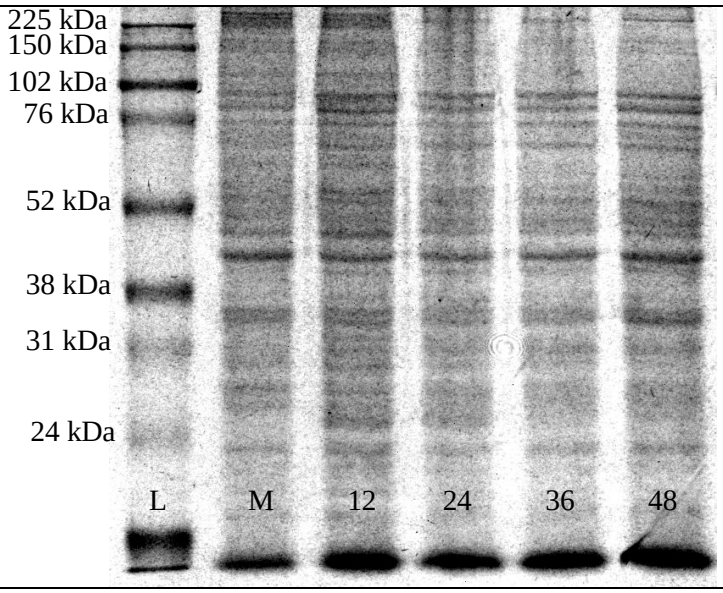


Figure 2: Cell lysates after mock infection (M), 12hpi (12), 24hpi (24), 36hpi (36), and 48hpi (48) loaded with 1x Laemmli buffer on a 10% acrylamide gel after boiling for 10 minutes. Gel stained directly using Thermo Scientific GelCode Blue Stain overnight and destained. GE Amersham Fluoresecent Rainbow Marker is shown (L).

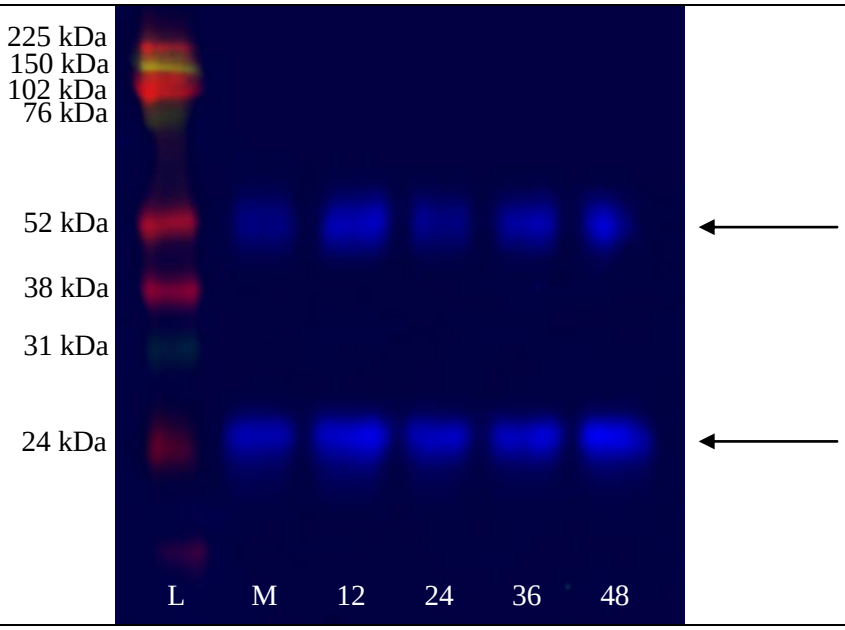


Figure 3: Immunoprecipitation products after incubating cell lysates from mock infection (M), 12hpi (12), 24hpi (24), 36hpi (36), and 48hpi (48) with antibody-beads overnight at 4^{o}C, centrifuging, washing with NP-40 lysis buffer, and loading onto a 10% acrylamide gel. Protein transferred to nitrocellulose membrane and treated first with 5f4 anti-NS1 monoclonal antibody followed by anti-mouse HRP antibody, visualized with enhanced chemiluminescence. The GE Amersham ECL Plex Fluoresecent Rainbow Marker is shown (L). Note again the strong bands at around 25 kDa and 50 kDa seen in both lanes (arrows), indicative of mouse IgG light chain and heavy chain, respectively.

STUDENT NAME: *Tychicus Chen*

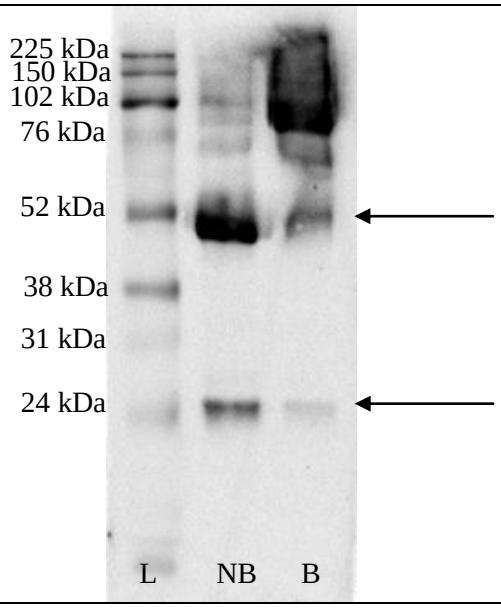


Figure 4: Antibody-bead mixture centrifuged and loaded on a 10% acrylamide gel without boiling (NB); boiled and centrifuged (B). Protein transferred to nitrocellulose membrane and treated with anti-mouse HRP antibody directly and visualized with enhanced chemiluminescence. The GE Amersham ECL Plex Fluoresecent Rainbow Marker is shown (L). Note the strong bands at around 25 kDa and 50 kDa seen in both lanes (arrows), indicative of mouse IgG light chain and heavy chain, respectively.

STUDENT NAME: *Tychicus Chen*

REFERENCES:

1. Wright, P. F. and R. G. Webster. 2001. Orthomyxoviruses, p. 1533-1579. In D. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia.
2. Center of Disease Control and Prevention. 2009. Online: http://www.cdc.gov. Accessed Jan 2010.
3. Lamb, R. A. and R. M. Krug. 2001. Orthomyxoviridae: The viruses and their replication, p. 1487-1531. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia.
4. Lamb, R. A. 1989. The Genes and Proteins of Influenza Viruses. Krug R.M., ed. New York, NY: Plenum Press; pp. 1-87.
5. Krug, R. M. 1989. The Influenza Viruses. Plenum Press, New York.
6. Ward, P., I. Small, J. Smith, P. Suter, and R. Dutkowski. 2005. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J. Antimicrob. Chemother. 55:i5- i21.
7. Inglis, S.C., T. Barrett, C. M. Brown, and J. W. Almond. 1979. The smallest genome RNA segment of influenza virus contains two genes that may overlap. P.N.A.S. 76: 3790-3794.
8. Hatada, E. R., and Fukuda, R.. 1992. Binding of influenza A virus NS1 protein to dsRNA in vitro. J. Gen. Virol. 73: 3325–3329.
9. Lu, Y., M. Wambach, M. Katze, and R. M. Krug. 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase (PKR) that phosphorylates the eIF-2 translation initiation factor. Virology. 214: 222–228.
10. Lu, Y., X. Y. Qian, and R. M. Krug. 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes Dev. 8:1817–1828.
11. Qiu, Y.,M. Nemeroff, and R.M. Krug. 1995. The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. RNA 1:304–316.
12. Li, Y., Z. Y. Chen, W. Wang, C. C. Baker, and R. M. Krug. 2001. The 3'-end-processing factor CPSF is required for the splicing of single-intron pre-mRNAs in vivo. RNA 7:920–931.
13. Krug, R. M., W. M. Yuan, D. L. Noah, and A. G. Latham. 2004. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. Virology. 309:181-189.
14. Hale, B. G., D. Jackson, Y. H. Chen, R. A. Lamb, and R. E. Randall. 2006. Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. Proc. Natl. Acad. Sci. U. S. A. 103:14194–14199.
15. Ehrhardt, C., T. Wolff, S. Pleschka, O. Planz, W. Beermann, J. G. Bode, M. Schmolke, and S. Ludwig. 2007. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J. Virol. 81:3058–3067.
16. Mibayashi, M., L. Martinez-Sobrido, Y. M. Loo, W. B. Cardenas, M. Gale, Jr., and A. Garcia-Sastre. 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. 81:514–524.\
17. Gack, M. U., R. A. Albrecht, T. Urano, K. S. Inn, I. C. Huang, E. Carnero, M. Farzan, S. Inoue, J. U. Jung, and A. Garcia-Sastre. 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe 5:439–449.

STUDENT NAME: *Tychicus Chen*

18. Marion, R. M., T. Zurcher, S. de la Luna, and J. Ortin. 1997. Influenza virus NS1 protein interacts with viral transcription-replication complexes in vivo. J. Gen. Virol. 78(10):2447-51.
19. Robb, N. C., G. Chase, K. Bier, F. T. Vreede, P. C. Shaw, N. Naffakh, M. Schwemmle, and E. Fodor. 2011. The Influenza A NS1 Protein Interacts with the Nucleoprotein of Viral Ribonucleoprotein Complexes. J. Virol. [Epub ahead of print].