UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Screen for Truncated Forms of Influenza A NS1 Protein that Inhibit the Activity of Full-Length NS1.

A Thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

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The Thesis of Alexander Michael Sarkisian is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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"Ignorance more frequently begets confidence than does knowledge: it is those who know little, and not those who know much, who so positively assert that this or that problem will never be solved by science"

~ Charles Darwin

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LIST OF ABBREVIATIONS

NS1 Non-structural protein 1

PCR Polymerase Chain Reaction

DN Dominant Negative

$NS1^{DN20}$ One of the NS1-DN candidates carrying a deletion of the residues 129-215 in the NS1 protein

$NS1^{DN25}$ One of the NS1-DN candidates, sequence unknown

EMS Ethyl Methanesulfonate

Δ2-3 Delta 2-3 transposase

PBST 1000:1 mixture of PBS:Tween

BSA Bovine Serum Albumin

hs-Gal4 Heat-Shock-Gal4

dsRNA Double-Stranded RNA

wt Wild-type

LIST OF SYMBOLS

mL= Milliliters

µL = Microliters

kD = KiloDaltons

mM = Millimolar

Δ = Delta

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ABSTRACT OF THE THESIS

A Screen for Truncated Forms of Influenza A NS1 Protein that Inhibit the Activity of Full-Length NS1.

by

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Master of Science in Biology

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Professor Ethan Bier, Chair

Major pandemics of Influenza A have caused the deaths of several million people in the past century alone. Discovering treatments to combat influenza A infection could save many lives in the event of an outbreak. We are attempting to generate new ways of treating influenza by creating dominant negative inhibitors to the influenza non-structural protein, NS1, which has an important role in suppressing the host immune response and is critical for viral replication. We have generated a stock of *Drosophila melanogaster* carrying a genomic insertion of a NS1 transgene flanked by an N-terminal HA tag and a C-terminal Myc tag. Expression of NS1 conditionally in the wing using the yeast derived UAS/Gal4 system causes a visible wing phenotype including expansion

between wing veins L3 and L4 and partial loss of L4. We have conducted a screen in which the mutagen delta 2-3 transposase was used to create deletions and truncations directed to the NS1 transgene in flies. From this screen, we have isolated two putative NS1 DN alleles that produce truncated NS1 proteins and can suppress the wing phenotype caused by the expression of the wild-type copy. Furthermore, these alleles produce no phenotype when expressed on their own indicating a low probability of serious off target effects if used as treatments in humans.

INTRODUCTION

Influenza pandemics have resulted in the loss of several million lives in the past century alone. Every year, seasonal influenza epidemics cause 3-5 million cases of severe illness and as many as 500,000 deaths worldwide (Carrat and Flahault 2007). In addition to widespread morbidity and mortality, it is estimated that the annual economic impact of influenza in the US ranges from 12-14 billion USD (Carrat and Flahault 2007). There is a clear need for more comprehensive and effective methods of vaccination and treatment for influenza infection. Currently used vaccines must be constantly reformulated since the viral replication process results in a continual change in the epitope sequence. In addition, the appearance of resistance of the H1N1 virus to commonly used antiviral drugs oseltamivir and zanamivir raises much uncertainty about the availability of effective treatments during future flu seasons (Hansen et. al 2010).

Influenza viruses constitute the genus *Orthomyxovirus*, which consists of three virus types (species): A, B, and C (Couch 1996). Furthermore, influenza A viruses are subtyped based on the sequence of the surface antigens, Hemagglutinin (HA) and Neuraminidase (NA) (Carrat and Flahault 2007). Influenza A and B viruses are the only species known to infect humans, with influenza A typically being more virulent than influenza B. For example, the three pandemics of the 20th century: the 1918 Spanish flu, 1957 Asian flu, and 1968 Hong Kong flu pandemics were caused by influenza A viruses. (Neumann and Kawaoka 2006; Carrat and Flahault 2007).

The influenza A genome is comprised of ten genes (PA, PB1, PB2, NP, HA, NA, M1, M2, NS1, NS2/NEP) encoded in eight strands of negative-sense, single stranded RNA (Fuji et. al 2009). The proteins present on the surface of the virus are HA, M2, and NA, which are required for attachment, entry, and budding from the host respectively (Hedestam 2008). Many vaccines currently used consist of inactivated virus, allowing the body to produce antibodies to HA (Ellebedy and Webby 2009). These vaccines are currently the most effective treatment available, but can be too strain-specific. One way to generate a broadly active treatment would be to disrupt a required function of one or more of the influenza proteins that are conserved across many, if not all, strains rather than targeting an epitope sequence. However, such methods of treatment are currently unavailable (Karlsson-Hedestam et. al 2008).

Each year, the World Health Organization makes recommendations about the contents of the influenza vaccine to ensure that the strains in the vaccine match the most prevalent circulating strains and drift variants (Carrat and Flahault 2007). However, the processes of antigenic drift and occasionally antigenic shift lead to the continual production of new strains of influenza virus, making the protective effects of vaccination very short-lived (Mueller et. al 2010). Antigenic drift which is caused by changes in the viral genome, particularly HA and NA, by an error prone viral RNA polymerase can occur as frequently as each time the virus replicates (Carrat and Flahault 2007). Antigenic shift occurs when viral RNA strands from multiple strains mix within a host and create a novel hybrid virus. Sometimes, this leads to highly pathogenic viruses that can cross the

species border. It is estimated that an antigenic shift event happens three times every 100 years, which is consistent with the three pandemic-causing antigenic shifts that took place in the last century (1918, 1957, and 1968) (Carrat and Flahault 2007).

Aside from antigenic drift and shift, influenza viruses have evolved other mechanisms that allow the virus to evade the host's immune response. The NS1 protein, in particular has been shown to play a major role in the suppression of the host immune response during infection, with its ability to inhibit functions of both the innate and adaptive branches of the immune system (Fernandez-Sesma 2007). This includes early blocking of the IFN-independent antiviral response, suppression of the IFN-dependent host response, and inhibiting the activation of PKR, a proapoptotic kinase typically activated by dsRNA (Krug et. al 2003). NS1 can also inhibit the processing and polyadenylation of cellular mRNAs by binding to the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) and polyA binding protein, halting cellular protein synthesis including the synthesis of antiviral factors and frees the host ribosomes to be utilized for viral RNA translation (Ehrhardt et. al 2010). NS1 has also been shown to inhibit activation of Akt/PKB, a proapoptotic factor, and PI3K- signaling which is involved in survival, metabolism, proliferation, and immunity. (Ehrhardt and Ludwig 2009).

In addition to its effects on the host's machinery, there is also increasing evidence supporting a role for NS1 in the export of viral mRNAs, making it a critical component of the viral life cycle (Schneider and Wolff 2009). It's diverse

roles throughout infection and the high degree of conserved regions in the NS1 protein make it an attractive target for the development of new influenza treatments (Darapaneni et. al 2009).

In this study, we have used Drosophila melanogaster to screen for dominant negative (DN) inhibitors of NS1with the goal that these peptides can be developed into novel treatments for most influenza strains (methods adapted from Guichard et. al, 2002). We have generated flies carrying a genomic insertion of a NS1 transgene flanked by an N-terminal HA tag and a C-terminal Myc tag. Expression of NS1 conditionally in the wing using the yeast derived UAS/Gal4 system causes a visible wing phenotype indicative of enhanced Hedgehog signaling, including expansion between wing veins L3 and L4 and partial loss of L4 (Figure 1).

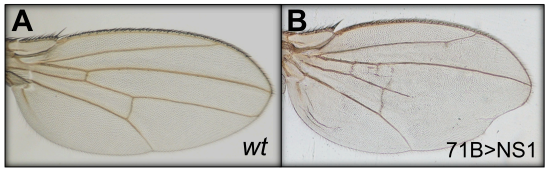

Figure 1. Expression of NS1 in the fly wing gives an observable phenotype. (A) Wild-type wing. (B) Wing phenotype resulting NS1 expression under the control of wing-specific gal4 driver 71B.

The mutagen Δ2-3 transposase was used to create random deletions and truncations directed to the NS1 transgene in flies and progeny carrying both the wild type and mutagenized alleles were screened for suppression of the wing phenotype. Additionally the mutagen Ethyl Methanesulfonate (EMS), which causes random point mutations, was used to screen for DN candidates with point mutations the NS1 transgene. From a Δ2-3 screen we have isolated and

characterized a truncated form of NS1 ($NS1^{DN20}$) that suppresses the wild type NS1-dependent wing phenotype. Furthermore, this allele produces no phenotype when expressed alone, indicating a low probability of serious off target effects if used as a treatment in humans.

MATERIALS AND METHODS

Δ2-3 Mutagenesis Screen

$$\text{(Step 1) w;}\ \frac{NS1}{TM6B}\ \times\ \frac{\Delta 2-3}{TM6B}$$

$$\text{(Step 2)}\ w;\frac{NS1}{\Delta 2-3}\ \times\ w;\ \frac{NS1}{cyo};\ \frac{71B}{TM2}$$

$$\text{(Step 3)}\ NS1;\ \frac{NS1^{DN}}{71B}\ \times\ w;\ \frac{sp}{cyo};\ \frac{TM2}{TM6B}$$

$$\text{(Step 4)}\quad \frac{NS1\ or+}{sp\ or\ cyo};\ \frac{NS1^{DN}}{TM2\ or\ TM6B}\ \times\ w;\ \frac{sp}{cyo};\ \frac{TM2}{TM6B}$$

$$\text{(Stock)}\ w;\ \frac{sp}{cyo};\ \frac{NS1^{DN}}{TM2\ or\ TM6B}$$

Scheme 1. Δ2-3 Mutagenesis Scheme. Males carrying the UAS-NS1 transgene were crossed to females carrying Δ2-3 transposase. F1 progeny that carried both Δ2-3 and UAS-NS1, and thus had the potential to generate mutant NS1 alleles, were crossed to flies carrying UAS-NS1 expressed under the control of the wing-Gal4 driver 71B. F2 progeny which carried both the wild type and potentially mutated alleles of NS1 were screened for rescue of the wing phenotype. Those that had a reduced phenotype were isolated as candidates expressing a dominant negative and were then crossed to a balancer stock (Step 3). The 71B GAL4 driver was eliminated by selecting against progeny with a dark red eye color for step 4. Flies without 71B were again crossed to a balancer stock (Step 4) to eliminate the wt NS1 allele (flies with wt NS1 have yellow eye color) and isolate the DN in a balanced stock. This screen was carried out twice, with approximately 100 crosses each time.

Ethyl Methanesulfonate Mutagenesis Screen

Ethyl Methanesulfonate (EMS) is a mutagen that creates random point mutations. 6 µl of EMS was diluted into 2.4 mL of 5% sucrose. Starved Males carrying the UAS-NS1 transgene were put in vials containing tissue paper soaked with 1.2 mL of the EMS solution and allowed to feed overnight. A total of 3 EMS

mutagenesis screens were carried out, with each screen containing 60-100 crosses.

$$\text{(Step 1)}\ w;\ \frac{NS1}{cyo} \quad EMS\ Treatment$$

$$\text{(Step 2)}\ w;\ \frac{EMS-NS1}{cyo}\ \times\ w;\ \frac{NS1}{cyo};\ \frac{71B}{TM6B}$$

$$\text{(Step 3)}\ w;\ \frac{EMS-NS1}{NS1};\ 71B\ \times\ 71B$$

$$\text{(Step 4)}\ w;\ NS1^{DN};\ 71B\ \times\ w;\ \frac{sp}{cyo};\ \frac{TM2}{TM6B}$$

$$\text{(Stock)}\ w;\ \frac{NS1^{DN}}{cyo}$$

Scheme 2: EMS Mutagenesis Scheme. Males carrying the UAS-NS1 transgene were treated with EMS (Step 1) and then crossed to females carrying UAS-NS1 under the control of the wing-specific driver 71B (Step 2). The F_1 progeny that had wings displaying a reduced phenotype were isolated and crossed to flies carrying 71B to segregate the DN allele from the wild-type NS1 allele by screening for the absence of a wing phenotype (Step 3). Flies in which the combined expression of the mutated and wt NS1 resulted in a rescue of the NS1 wing phenotype were considered to have a dominant negative allele (referred to as $NS1^{DN}$ beginning at step 4). The F_2 progeny were crossed to a balancer stock to make a balanced $NS1^{DN}$ stock and progeny were selected that didn't have the dark red eye color, indicating the absence of 71B. All crosses were carried out in a 25^0 C incubator.

Drosophila Genomic DNA Preparations

Fly genomic preparations which were used for PCRs were done according to the Berkeley Drosophila Genome Project (BDGP) protocol (Rehm 2008).

Wing Imaginal Disc Immunohistochemistry:

All wing imaginal disc dissections were done on ice, in a fixing solution consisting of one part 8% formaldehyde and three parts Brower fix (.15M pipes, 3mM MgSO4, 1.5 mM EGTA, 1.5% NP40; pH to 6.9). The discs were fixed for one hour at 4 degrees Celsius. The fix was then removed and the discs washed with PBST. Discs were blocked in PBS with .1% Tween20 (PBST) with 5% BSA for one hour at room temperature. Discs were then incubated with primary antibodies diluted in PBST with 5% BSA for a minimum of 2 hours. Following 3 washes in PBST, discs were incubated with fluorescent Secondary antibodies diluted in PBST with 5% BSA for 1-2 hours and kept in the dark. Following 4 washes, Wings were mounted using Aqua-poly/mount non-fluorescing mounting medium (Polysciences, Inc, Warrington, PA). Slides were visualized using confocal microscopy.

Western Blotting

Hs-Gal4; UAS-NS1 and hs-Gal4; UAS-NS1^{DN} were heat shocked at 37° C for two hours. Ten flies of each type were homogenized in 100 µL of PBST, centrifuged at 14,000 RPM for 5 minutes, and the supernatant collected. 33 µL of Nupage® LDS sample buffer (Invitrogen, Carlsbad, CA) and 13.3 µL of reducing agent were added to the supernatant, boiled for 5 minutes and 15 µL was loaded onto a 12% polyacrylamide gel. The gel was run at 200 Volts in Nupage® MOPS SDS Running Buffer (Invitrogen, Carlsbad, CA). Transfer to nitrocellulose membrane was conducted at 45 Volts for 90 minutes in Nupage® Transfer Buffer (Invitrogen, Carlsbad, CA). The membrane was blocked with PBST with 3% milk for 1 hour then incubated with rat anti-HA (1:500), and rabbit anti-Myc (1:100) primary antibodies for 2 hours. After four washes in PBST with 3% dry milk, the

membrane was incubated with mouse anti rat-488 and mouse anti rabbit-594 fluorescent secondary antibodies. After four washes in PBST with 3% dry milk and one wash in PBS the membrane was visualized using a Typhoon™ scanner (GE Healthcare, Piscataway, NJ).

Inverse PCR and Sequencing of NS1^{DN} Alleles

To sequence the DN transgenes generated by Δ2-3, we used a digestion/ligation strategy (Scheme 3) to circularize genomic DNA pieces followed by inverse PCR. Genomic DNA was digested with EcoRI and ligations were done according to the BDGP protocol (Rehm 2008). Inverse PCR was done using primers directed to the UAS that proceeded in opposite directions such that the entire circularized piece of DNA would be amplified. This enabled us to obtain the sequence of the genomic DNA adjacent to the UAS indicating where the transgene had inserted. Using a primer to the UAS and a primer directed to a known genomic sequence downstream of the insertion site, we were able to find the sequence of the truncated NS1 allele.

The inverse PCR protocol was 95° for 5 minutes, followed by 35-40 cycles of 95° for 30 seconds, 52° for 45 seconds, then 72° for 1 minute. PCR products were run on a 1% agarose gel in order to ensure the presence of the proper PCR products. Prior to sequencing, PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). DNA sequencing was performed by the DNA Sequencing Shared Resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant # 2 P30 CA023100-23.

PCR Site Directed Mutagenesis

Site directed mutagenesis was performed by subjecting a vector containing the NS1 allele to a PCR with custom primers (Allele Biotechnology, San Diego, CA) that allowed for the amplification of only the first 387 base pairs of the NS1 gene (encoding the first 129 amino acids of the NS1 protein). The truncated NS1 PCR product was cloned into pENTR vector and transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen Corp., Carlsbad, CA). Transformed cells were grown up overnight on selective plates. We used Gateway cloning to recombine the pENTR-NS1DN with a vector containing p-element sites for germline transformation as well as a UAS sequence upstream of the recombination site. This construct was injected into fly embryos to generate genomic transformants carrying the truncated allele downstream of UAS.

RESULTS

From the 2 initial screens that employed Δ2-3 mutagenesis, 8 DN candidates were recovered and made into balanced stocks. We chose to focus on two stocks with white eyes ($NS1^{DN20}$ and $NS1^{DN25}$) because loss of the mini-white gene, which was downstream of UAS-NS1 in the transgene construct injected, indicated a deletion/truncation event must have taken place. Our efforts were later narrowed down to only candidate $NS1^{DN20}$ and data for $NS1^{DN25}$ can be found in the appendix.

To confirm dominant negative activity of the candidate alleles, flies from the DN stocks were crossed to flies carrying the wing-Gal4 driver 71B and a copy of the wild-type NS1 allele. The mutant NS1 allele in $NS1^{DN20}$ showed clear suppression of the activity of NS1 when co-expressed in the fly wing (Fig. 2C). Additionally, the expression of $NS1^{DN20}$ alone produced no wing phenotype (Fig. 2B), indicating a low probability of serious side effects in humans.

Figure 2. $NS1^{DN20}$ suppresses the function of the wild type NS1 in the fly wing. (A) The phenotype resulting from the expression of wild-type NS1 driven by the wing GAL4 driver 71B shows an increase in distance between wing veins L3 and L4 and a partial loss of L4. (B) Expression of $NS1^{DN20}$ causes no visible changes in the wing morphology. (C) $NS1^{DN20}$ shows a clear suppression of the NS1 wing phenotype when co-expressed with NS1.

Western blot confirmed a truncation of NS1 in $NS1^{DN20}$ but we had no success in visualizing NS1 proteins isolated from the $NS1^{DN25}$ stock or other DN candidates that were abandoned (see western for $NS1^{DN25}$ in appendix).

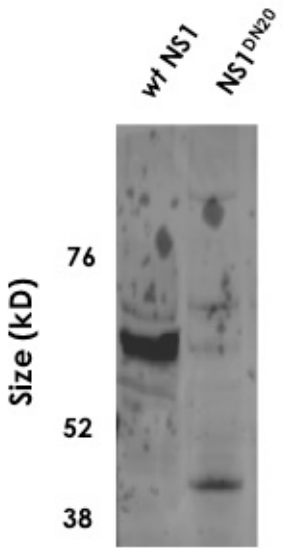

Figure 3. Western blot showing NS1 protein extracted from flies expressing wild type NS1 and $NS1^{DN20}$ blotted with an HA antibody directed to the N-terminal tag of the transgene. This blot shows truncation of approximately 20 kD in the NS1 protein of $NS1^{DN20}$.

Wing imaginal discs from 3rd instar larvae expressing wt NS1 and $NS1^{DN20}$ were stained with antibodies to the N-terminal HA and C-terminal Myc tags as shown in Fig. 4, $NS1^{DN20}$ has lost the Myc tag, confirming a truncation in the dominant negative allele.

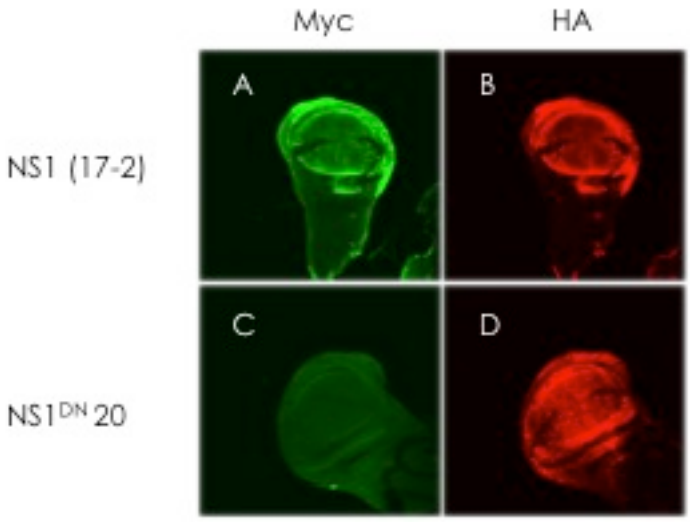

Figure 4. Immunohistochemistry confirming truncation in $NS1^{DN20}$ by the loss of the C-terminal Myc tag. (A-D) Larval wing imaginal discs expressing wild type NS1 (A, B) or $NS1^{DN20}$ (C, D) under the control of 71B were stained for the C-terminal Myc (A,C) and N-terminal HA (B,D) tags to mark NS1 protein.

Genomic DNA was isolated from flies carrying the $NS1^{DN20}$ allele the transgene was amplified by PCR. Upon sequence, we detected that $NS1^{DN20}$ contained a truncation from residues 130-215. A de novo stock was generated carrying this truncation downstream of the UAS sequence and tagged N-terminally with HA and C-terminally with Myc. Flies expressing UAS-NS1Δ130-215 displayed a normal wing phenotype when expression of the NS1Δ130-215 was driven by 71B (Fig. 5C) Additionally, the protein encoded by this allele acted as a dominant-negative suppressor of NS1, evidenced by the lack of the NS1 phenotype in flies expressing both the NS1 and NS1Δ130-215 proteins (Fig. 5B)

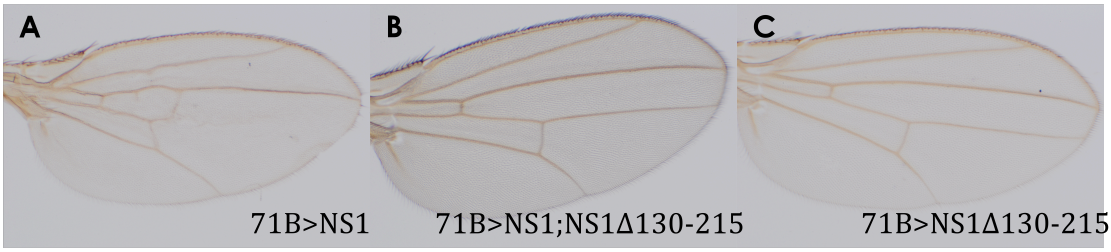

Figure 5. NS1Δ130-215 protein acts as a dominant negative inhibitor of NS1 in the fly wing. A, Wings expressing the *wt* NS1 protein driven by 71B. B, NS1Δ130-215 inhibits the NS1 phenotype when co-expressed in the fly wing. C, NS1Δ130-215 does not cause a wing phenotype when expressed in the fly wing with 71B.

DISCUSSION

With the 2009 pandemic of the H1N1 influenza A strain it is clear that current methods of vaccination are no match for this rapidly evolving virus. One of the biggest challenges in influenza vaccine development is that potent vaccines generally target the most variable proteins of the virus (Ellebedy and Webby, 2009). Increased vigilance and surveillance have proved to be helpful ways to averting pandemics, as was the case in the 1997 outbreak of H5N1 ('bird flu') in Hong Kong, (Shortridge et. al 2000), but this is simply not enough. Once a pandemic is recognized it can take several months for a vaccine to become widely available. Until reliable methods of vaccination that provide long term protection against the majority of influenza strains and drift variants, there is a need for improving treatment in those who are infected by the virus. Current treatment methods also target highly variable proteins of influenza. We were able to use a Δ2-3 transposase mutagenesis screen to generate a dominant-negative (DN) allele of NS1 that inhibited the function of the wild-type NS1 alleles in the fly wing.

The precise mechanism of the inhibition of NS1 by $NS1^{DN20}$ is still under investigation; it may be that this truncated form of the proteins acts by a combination of the following mechanisms. One possibility is that the protein fragment forms a stable heterodimer with full-length NS1 protein, reducing the level of wild-type dimers and thereby blocking its activity. It is also possible that the $NS1^{DN}$ peptides are able to sequester one or more of the downstream targets of the NS1 protein, preventing the functional NS1 protein from interacting with its

downstream target(s).

Recently, it has been shown that influenza A viruses lacking the NS1 gene are unable to form viral progeny (Wacheck, et. al 2010). Thus, it is likely that a block of the activity of NS1 in vivo would lead to a similar effect, functionally crippling the virus from producing more progeny and spreading within the host. By blocking the functions of NS1, DN proteins would also enable the host immune system to perform its usual functions in response to infection.

Recent advances and innovations in the field of protein therapeutics have increased the potential for treating sick humans with proteins such as dominant negatives. Additionally, with lung epithelial tissue as the primary site of infection in humans, intranasal delivery would be a very attractive method of peptide delivery. Intranasal delivery has proven to be effective for delivery of peptides including human growth hormone, and calcitonin (Steyn 2010; Plessis 2010). At 22 kD, human growth hormone has a size similar to that of NS1 (25 kD). Although other properties also play a role in the efficiency of peptide absorption across lung epithelium, the size of NS1-DN peptides would not likely be a issue.

There are nearly two hundred different proteins currently produced as therapies and treatments (Carter 2011). As reviewed by Leader et. al (2008), the advantages to using proteins over small molecule drugs as therapy are numerous. One important advantage is the specificity of proteins' activity, which makes adverse effects, including immune responses less common (Leader et. al 2008). Additionally, the clinical development and FDA approval time is on average one year faster for protein therapeutics than small-molecule drugs

developed between 1980 and 2002 (Leader et. al 2008). From an economic standpoint protein therapeutics can be more profitable to pharmaceutical companies because the unique form and functions of proteins make broader patent protection possible (Leader et. al 2008).

We have demonstrated that *Drosophila* can be used to generate inhibitory proteins. There is a large potential for therapeutic peptide development using *Drosophila* genetics, and this study is just another example that demonstrates the broad utility of the fruit fly as a model genetic organism for studying human disease.

FURTHER WORK

There is a large therapeutic potential for the treatment of influenza with dominant negative NS1 peptides. Further tests would need to be done to confirm the activity of $NS1^{DN}$ peptides in a mammalian host model. Also it would be important to test the efficacy of DN peptides across strains with varying NS1 sequences.

Additionally, work to develop effective methods of peptide delivery could greatly enhance the potential benefit of protein therapeutics as treatments for a wide array of diseases include both genetic and infections disease. It is likely that $NS1^{DN}$ proteins could be effectively engineered to be delivered intranasally to patients infected with influenza A virus. New methods of peptide delivery including Pheroid™ technology have proven effective for nasal delivery of peptides such as human growth hormone and calcitonin (Steyn 2010; Plessis 2010). Pheroid™ technology is a recently patented drug-delivery system that uses submicron lipid based structures, which have been proposed to improve the transcellular transport of molecules (Steyn 2010). Current methods of protein modification and protein engineering can be utilized to increase factors such as the protein efficacy and half-life, making protein therapeutics an attractive form of treatment.

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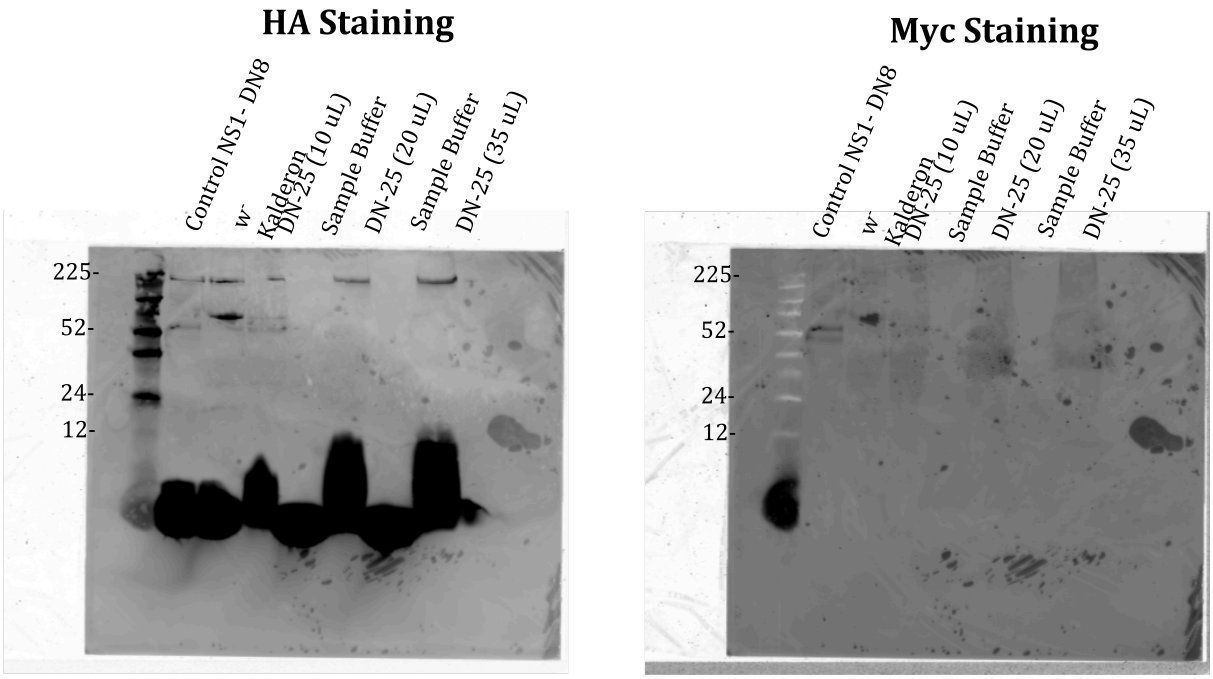
APPENDIX



Immunoblotting showing protein labeled with anti-HA and anti-Myc antibodies. It can be seen that the protein from candidate NS1^{DN25} is not visible; therefore we did not continue to pursue characterizing this candidate.

NS1^{DN25} suppresses the NS1 phenotype. A) Candidate NS1^{DN25} shows suppression of the NS1 phenotype when expressed in the fly wing under the control of 71B. B) Candidate NS1^{DN25} also shows no phenotype when expressed alone.