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The Influenza A virus leads to yearly epidemics and occasional world-wide pandemics, as with the Spanish Influenza of 1918. The frequent mutation rate of the virus mandates that new vaccines be created often. Additionally, acquired resistance to antiviral drugs makes them less effective over time. Cellular targets, that have a much lower rate of mutation, provide possible targets for new therapies that can withstand viral genetic drift. The goal of this study was to identify possible cellular targets which modulate the function of the M2 viral protein, and therefore affect the replication cycle. To obtain this goal, the second-site modifier screen in *Drosophila melanogaster* was employed to test approximately 1,200 gene disruptions for their effects on M2 activity. To first establish the model system as a reliable testing tool, flies expressing M2 were exposed to amantadine, a known M2 blocker. It was shown that M2 functions as an ion channel in the fly, as in human hosts; and, that amantadine blocks this activity, thus supporting our use of the model system. Subsequently, the mutant stocks were screened for changes in rough eye phenotype of M2 expressing flies, followed by control verifications. Of the stocks screened 9 candidates were selected for future studies. These genes represent possible cellular targets for future antiviral therapies.

INHIBITION OF INFLUENZA A VIRAL REPLICATION BY ACTIVITY MODULATION OF THE M2 VIRAL PROTEIN

by

Jennifer Berrier Kincaid

A Thesis Submitted to
The Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Greensboro
2010

Approved by

_____
Committee Chair

To

Mom, Dad, Jessica and

My Wonderful Husband Jason,

For Their

Love, Support and Encouragement

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at the The University of North Carolina at Greensboro.

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Date of Acceptance by Committee

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CHAPTER I

INTRODUCTION

Influenza A is a member of the family of single-stranded, negative-sense RNA viruses *Orthomyxoviridae*. The genome of the virus is comprised of 8 RNA segments which code for proteins needed for viral entry and assembly, including surface receptors, components of the RNA polymerase, and other proteins with various functions (see Figure 1). Three of these proteins are the membrane-bound surface proteins Hemagglutinin (HA), Neuraminidase (NA), and Matrix Protein 2 (M2); proteins that are key players in the initiation and propagation of infection. The glycoprotein hemagglutinin is a surface receptor, that binds sialic acid on the surface of target cells, and subsequently plays a role in the fusion of the viral membrane with that of the host endosome following endocytosis. Neuraminidase, also a glycoprotein, is involved in viral release from the host cell; the enzymatic activity of NA is to cleave viral HA from the sialic acid of the host post-budding thereby releasing the viral particles. The third membrane protein, M2, has been shown to have multiple roles in virus production including acidification of the virion interior which allows release of the viral genome after membrane fusion, and stabilization of HA during transport in the trans-Golgi network prior to the assembly process for some, but not all, Influenza A strains (Palese and Shaw, 2007; Betakova, 2007) (Figure 2).

Infection with the Influenza A virus begins once the virus particle is taken up by the host cell by receptor-mediated endocytosis (Figure 2). Once inside the endosome, the low pH of the organelle triggers a conformational change in the viral surface protein HA, leading to the fusion of the viral and endosomal membranes. Several HA molecules, working together, form a pore giving access for genome release. The RNA genome within the virion is associated with proteins, the primary being NP, forming the RNP complex. This complex is tethered to the M1 protein, which lies below the viral membrane. Prior to genome release, the M2 protein pumps H^+ from the endosome into the virus interior. This change in pH within the virion causes the dissociation of the RNP complex from the M1 protein. After fusion and pore formation by HA, the RNP complex is available for transport to the nucleus to begin viral transcription and replication. The genome is first transcribed into positive-sense RNA (cRNA), which is then used in the production of the negative-sense RNA (vRNA). Next, the new virions must be assembled, packaged, and released from the host cell. Viral proteins direct the export of the genome (RNP complex) from the nucleus by way of the cellular nuclear export pathway. Sorting signals on the viral proteins, including HA and NA, direct them through the Golgi network to the apical surface of the host cell. Upon budding, the neuraminidase protein cleaves sialic acid from the surface HA, releasing the new virus particles (Palese and Shaw, 2007; Betakova, 2007). The pH of host cell compartments is a key factor in *Orthomyxoviridae* infections, most notably in viral uncoating (Palese and Shaw, 2007); therefore, changes in pH can prevent or reduce virus production (Sugrue, et al., 1990). The key mediator of pH for the Influenza A virus is the M2 protein (Palese

and Shaw, 2007; Pinto and Lamb, 2007); which has been found to be necessary for viral replication (Takeda et al., 2002).

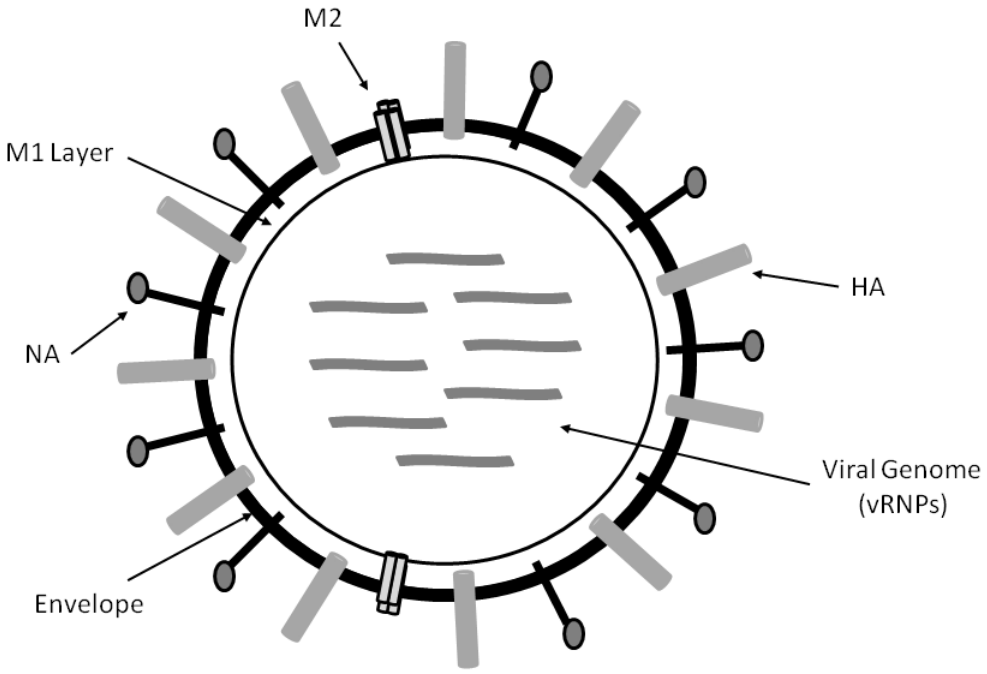

Figure 1. The Influenza A virus. Viral particles consist of an RNA genome (vRNPs), and viral proteins including the three surface proteins: Hemagglutinin (HA), Neuraminidase (NA), and Matrix Protein 2 (M2).

The M gene on the seventh RNA segment encodes both the M1 and M2 proteins, the latter translated from a spliced mRNA. M2 is an integral membrane protein composed of four helices (tetrameric). The ninety-six amino acids that comprise the protein constitute an N- terminal ectodomain (23 residues), a transmembrane domain (19 or 20 residues), and a C-terminal cytoplasmic domain (53 or 54 residues) (Lamb et al., 1985; Pinto and Lamb, 2007; Betakova, 2007). While expressed in large numbers on the

surface of infected cells (Lamb et al., 1985), there are relatively few M2 proteins within the virion membrane, approximately 20-60 per particle (Lamb et al, 1994; Betakova, 2007). The various roles of M2 were brought to light during studies involving other Influenza proteins such as HA and M1, and from amantadine-resistant strains.

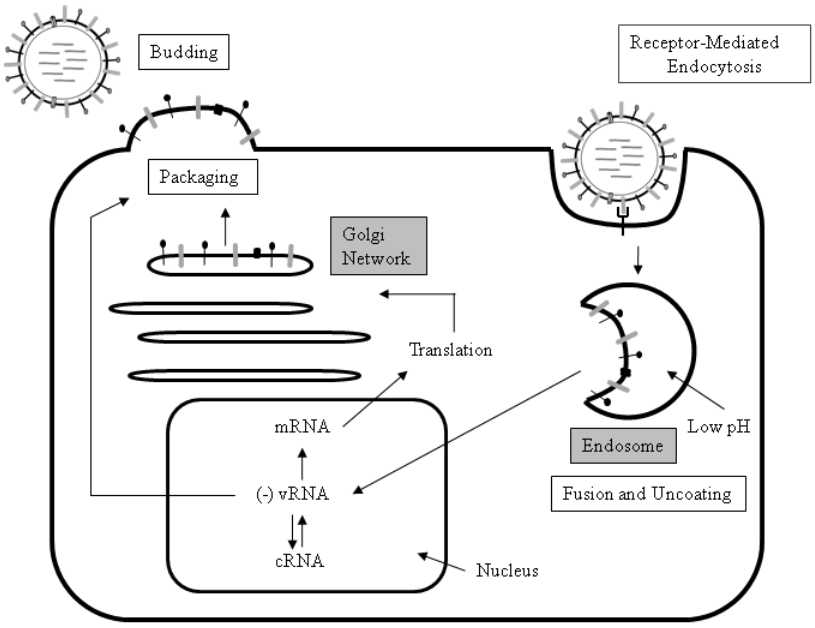

Figure 2. Influenza A virus replication cycle. The processes of viral entry, replication and release of new influenza virus particles from the host cell are shown. Boxes with no fill indicate some of the major steps in the cycle. Boxes with gray indicate the two known areas of M2 function within the cell.

The effect of the drug amantadine, and the derivative rimantadine, on prevention of Influenza infections has been known for over forty years. These antivirals block the M2 channel, and therefore inhibit the flow of H^+ ions (Wright, et al., 2007). Work involving amantadine-resistant virus strains has shown that resistance is linked to

mutations in four amino acid residues of the transmembrane domain of M2, suggesting a role for M2 in viral replication (Hay et al., 1985).

Amantadine has been studied in conjunction with other Influenza A proteins in order to understand viral processes. The HA protein undergoes a conformational change in response to the low pH of the endosomal lumen, which facilitates viral uncoating (Palese and Shaw, 2007). Treatment with amantadine results in host cell expression of HA in this changed form for certain strains (H7 and H5), indicating exposure to low pH post-translation. The link between amantadine and M2 suggests that M2 may be involved in regulating the pH of cellular compartments during viral assembly (Sugrue et al., 1990). In further support of this idea, Ciampor and colleagues (1992a, 1992b) have found that this conformational change occurs soon after HA leaves the Golgi complex, implying that this change occurs during transport of HA through the trans-Golgi network; and, that the vesicles in this network are increased in acidity in amantadine-treated cells. Thus, for some Influenza A strains, M2 is involved in preserving HA in its native form so that it is functional in the mature virion. An additional role for M2 is suggested from studies involving the structural protein, M1.

Uncoating of the viral genome is initiated by a conformational change in HA initiated by low endosomal pH (Palese and Shaw, 2007). For the viral genome to be transported to the nucleus for replication, the M1 protein, which is believed to connect the viral membrane with the vRNPs (viral NP and RNA), must be removed (Martin and Helenius, 1991; Helenius 1992). Zhirnov (1990) found that this event is pH dependent,

requiring a low pH (pH 5.0-6.0). The connection to M2 was made when it was discovered that amantadine treatment blocked this dissociation, and thus inhibited the viral genome from reaching the nucleus (Martin and Helenius, 1991). M2 is, therefore, thought to function in acidification of the viral interior to allow for release of the vRNPs from the M1 protein in preparation for membrane fusion and release (Martin and Helenius, 1991; Helenius, 1992; Lamb et al., 1994; Betakova, 2007). Voltage studies revealed a possible mechanism for this process.

That M2 was a transmembrane protein that functioned in pH regulation during different stages of virus infection led Pinto and coworkers to investigate the possible presence of ion channel activity. Voltages across *Xenopus laevis* oocytes expressing wild-type M2 were greater than control cells expressing M2 transmembrane domain mutants. Additionally, amantadine blocked this voltage increase in cells expressing wild-type M2, but not in cells expressing mutant M2 that are resistant to amantadine (Pinto et al., 1992). It was also established that the activation of this ion channel activity was not due to membrane voltage, but to low pH (Pinto et al., 1992; Wang et al., 1993; Shimbo et al., 1996; Mould et al., 2000), and is inactivated by high pH (Pinto and Lamb, 2007). Various ions have been investigated, including Na^+ (Pinto et al., 1992; Chizhmakov et al., 1996), K^+, Cl^- (Chizhmakov et al., 1996), NH_4^+ (Mould et al., 2000), and H^+ (Pinto et al., 1992, Chizhmakov et al., 1996; Shimbo et al., 1996; Mould et al., 2000), with the conclusion that the primary ion involved in M2 function is H^+. This ion conductance is reduced by a decrease in internal (C-terminus) pH (Shimbo et al., 1996); and, ion selectivity can be altered by changes in the Histidine 37 residue that is located

within the transmembrane domain (Mould et al., 2001; Pinto and Lamb, 2007; Betakova, 2007). The conductance of other ions (cations) counter to H^+ cannot be completely ruled out, as this activity seems necessary in order to maintain an acceptable membrane potential (Lamb et al., 1994; Shimbo et al., 1996). No such activity by M2, nor by HA or NA, is currently known.

The cytoplasmic tail of M2 has recently been the focus of much research which suggests it is important for protein function. Some truncations, but not all, in the tail can reduce the channel activity (Tobler et al., 1999). Furthermore, the cytoplasmic domain has been implicated in assembly and budding via an interaction with M1. Various mutations in the tail of M2, which are shown to be linked to interactions with M1, result in a decrease in viral production (McCown and Pekosz, 2006; Chen et al., 2008). While the ion channel activity of M2 is its primary function, it appears that the cytoplasmic tail helps mediate this behavior.

The frequent mutation rate of the surface proteins HA and NA, which are the primary targets of current vaccines and antiviral therapies, leads to resistance; yearly vaccines, and new antivirals, must be developed to combat this problem. The rate of antigenic drift of these proteins is believed to be less than 1% per year. While seemingly low, this frequency is enough to initiate epidemics from new variants every few years, which, in turn, require modifications to the existing vaccines (Wright et al., 2007). While M2 is somewhat more conserved across the strains of Influenza, there is currently no vaccine available that targets this protein, and the antiviral drugs, amantadine and

rimantadine, which block activity are also subject to resistance by some mutant strains, which show no decrease in infectivity (Pinto and Lamb, 2007). Furthermore, some common strains, including the H5N1 variants, as well as all Influenza B and C viruses, are resistant to amantadine (Wright et al., 2007). Therefore, there exists a need for the development of new therapies that would be effective against all strains of Influenza, and not lose efficacy as the virus mutates.

Although not currently a common model system for studying human viruses, *Drosophila melanogaster* has been used in recent years as a tool for understanding viral mechanisms. Such studies include protein-protein interactions involved in disorders associated with HIV/AIDS (Battaglia et al., 2001), the role of cytomegalovirus (HCMV) on embryonic development (Steinberg et al., 2008), Epstein-Barr (EBV) interactions with host proteins (Adamson et al., 2005), and functional analysis of the SARS protein 3a (Wong et al., 2005). Adamson, studying EBV, and Wong, studying SARS, both utilized the *GMR-Gal4* system which allows for eye- specific expression of a protein of interest (Hay, et al., 1994). This system provides a fast and efficient means to screen for second site modifiers of a particular phenotype. *GMR*, Glass multimer reporter, is a sequence that binds the eye-specific protein Glass. Binding of Glass to *GMR* drives the expression of Gal4 in the eyes of the fly. In turn, Gal4 binds to the UAS, upstream activating sequence, of the second construct resulting in M2 transcription (see Figure 3). *GMR-Gal4* driven expression of M2 in the eye results in a rough-eye phenotype (unpublished, Adamson) (Figure 3 and 4). Enhancers and/or Suppressors of the phenotype can be found by performing crosses containing the *GMR-Gal4: UAS-M2/+* system with flies

containing mutations in genes of interest. These second-site modifiers represent hypothetical cellular targets for modulation of M2 activity. By manipulating these targets, it may be possible to prevent or impair Influenza A infections.

In this study, the use of *Drosophila melanogaster* as a tool for studying Influenza is investigated. Once supported, genetic screening using M2-expressing flies was carried out to identify proteins that potentially interact with M2, and are therefore possible targets for future antiviral research.

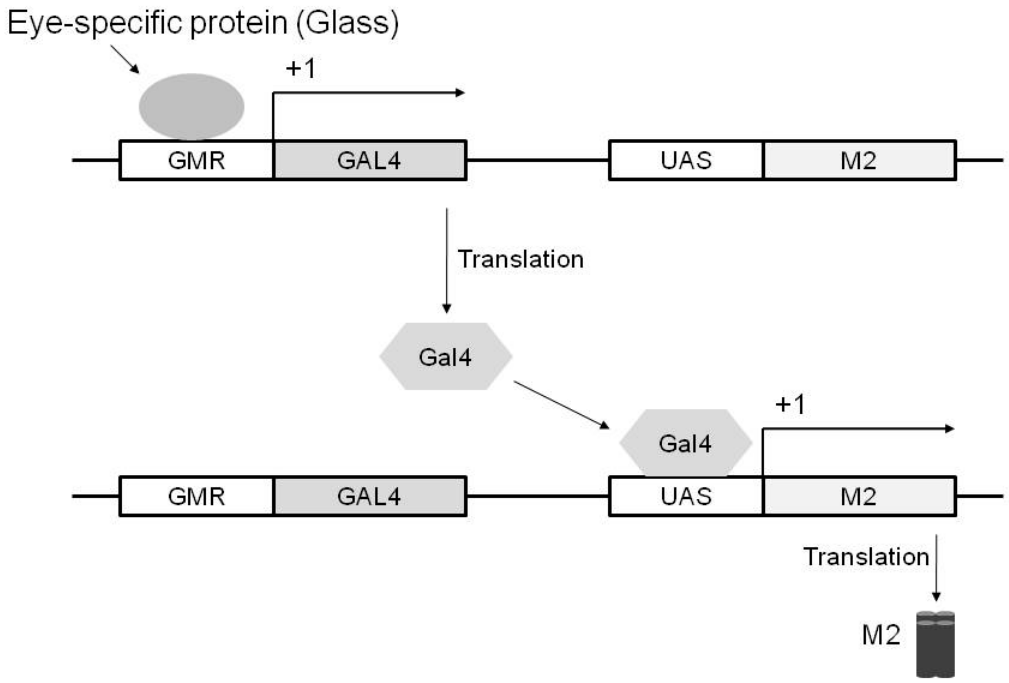

Figure 3. The *GMR-Gal4: UAS-M2/+* system. Glass (an eye specific protein) binds to GMR driving Gal4 expression. The Gal4 protein binds to the UAS region driving M2 expression.

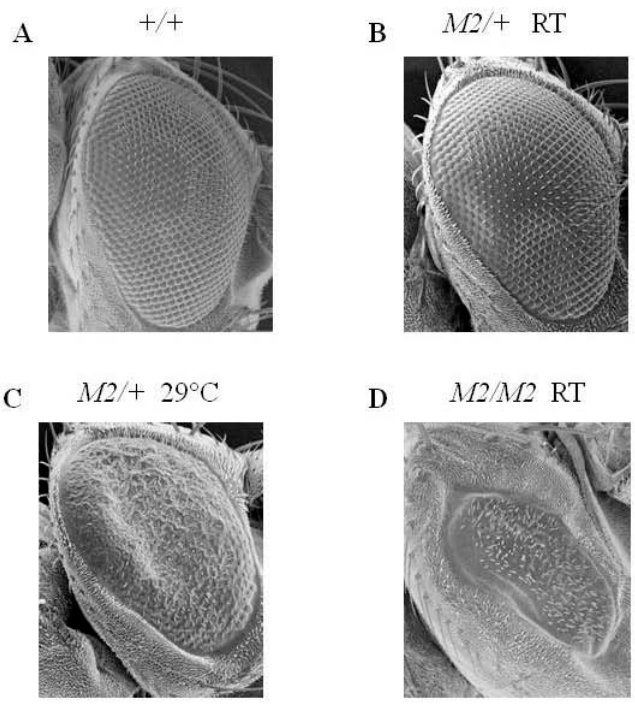

Figure 4. ***Drosophila melanogaster*** **eye phenotypes.** A) Flies with a wild-type phenotype (*GMR-Gal4/+*) (no M2 construct). B) Flies that are heterozygous for M2 raised at room temperature (RT). C) Flies that are heterozygous for M2 raised at 29°C. D) Flies that are homozygous for M2 raised at room temperature. The M2 phenotype is temperature dependent (29°C), but is made more severe when the fly is homozygous (i.e. it is dose-dependent).

CHAPTER II

MATERIALS AND METHODS

Fly culture

Flies expressing M2 under the *GMR-Gal4: UAS-M2/+* system were maintained at room temperature in vials containing a food medium of cornmeal, molasses, agar, and dead yeast (with the addition of methyl 4-hydroxybenzoate as a mold inhibitor).

Amantadine treatment for phenotype analysis

Second instar larvae from the *GMR-Gal4: UAS-M2/+* line were placed on fly food with the daily addition of amantadine hydrochloride (Sigma) dissolved in water at one of five concentrations (0 μg/ml, 10μg/ml, 20μg/ml, 30μg/ml, 40μg/ml). Larvae were kept at 29°C throughout the procedure, until eclosion. Upon eclosion, flies were scored for the number and severity of rough eye phenotypes over the total number of flies.

Amantadine treatment for pH analysis

Second instar larvae of the genotypes *C135-Gal4/+; UAS-M2/+* and *C135-Gal4/+* were treated daily with 30 μg/ml amantadine hydrochloride (Sigma) in water (prepared from 2mg/ml stock) until third instar was reached. Control larvae from each genotype received sterile water only, no amantadine. All vials were kept at 29°C until third instar at which point SNARF-1 staining was performed.

SNARF-1 analysis

Third instar larvae of the genotypes *C135-Gal4/+* (control) and *C135-Gal4/+; UAS-M2/+* were dissected in S2 cell medium. Once dissected the tissues were moved to fresh S2 medium, which contained 10μM SNARF-1 (Invitrogen, C1271), and incubated for thirty minutes at room temperature. Following incubation, tissues were rinsed with S2 medium and imaged via confocal microscopy (Olympus FV500). Samples were excited using an argon laser at 488nm, and emissions were analyzed at both 560nm and 660nm. Olympus Fluoview software allowed for intensity measurements at those wavelengths, and the ratio of 660nm/560nm was calculated.

M2 modifier fly screen

Virgin female flies (3) from the *GMR-Gal4: UAS-M2/+* line were collected and crossed to males (3) from a mutant stock from the Bloomington EP line series, P{EP gy2} (provided by Hugo Bellen's lab). Mutant stocks contained P elements, which contain a UAS promoter region. The P elements are inserted upstream from a gene and cause over-expression, are inserted into the gene causing dysfunction during transcription, or are located on the opposite strand from the gene and cause the expression of an antisense RNA that may interfere with transcription or translation of the protein (insertion location depends on the particular stock, Figure 5). Female *GMR-Gal4:UAS-M2/+* and mutant stock crosses were incubated at room temperature for two days, after which they were moved to 29°C (M2 is temperature sensitive at second instar) until eclosion. Adult flies were then screened for eye phenotype. Flies (*GMR-Gal4:*

UAS-M2/ P{EP gy2}) were scored for enhancement or suppression of the M2 phenotype (Figure 6A).

Verification of fly crosses

Putative modifiers were initially verified by crossing the EP line mutant stock flies (males) to a nonrelated stock (females) that contained the UAS system driving the expression of a different viral protein, BRLF1 from Epstein-Barr virus (*GMR-Gal4: UAS-BRLF1/+*). Those that did not alter eye phenotype in the BRLF1 screen were then tested for M2-dependence by crossing the EP line mutant stocks to *GMR-Gal4/+* stock flies which do not contain *UAS-M2*. Flies were kept at 29°C starting two days after crossing. Upon eclosion flies were scored for eye phenotype (Figure 6B and 6C).

Candidate stock database analysis

Data regarding candidate stocks obtained for all screens were found on FlyBase (www.flybase.org), unless otherwise noted. Human homolog searches were conducted using the Nucleotide Blast function at NCBI (www.ncbi.nlm.nih.gov).

Scanning electron microscopy

Eye phenotypes of amantadine series-treated flies and those bearing second-site modifiers were visualized in detail using SEM at magnifications between 250 and 600X (Hitachi S-4800). Prior to visualization flies were mounted and sputter coated (Pelco Model 3, 91000) with gold for 2 minutes.

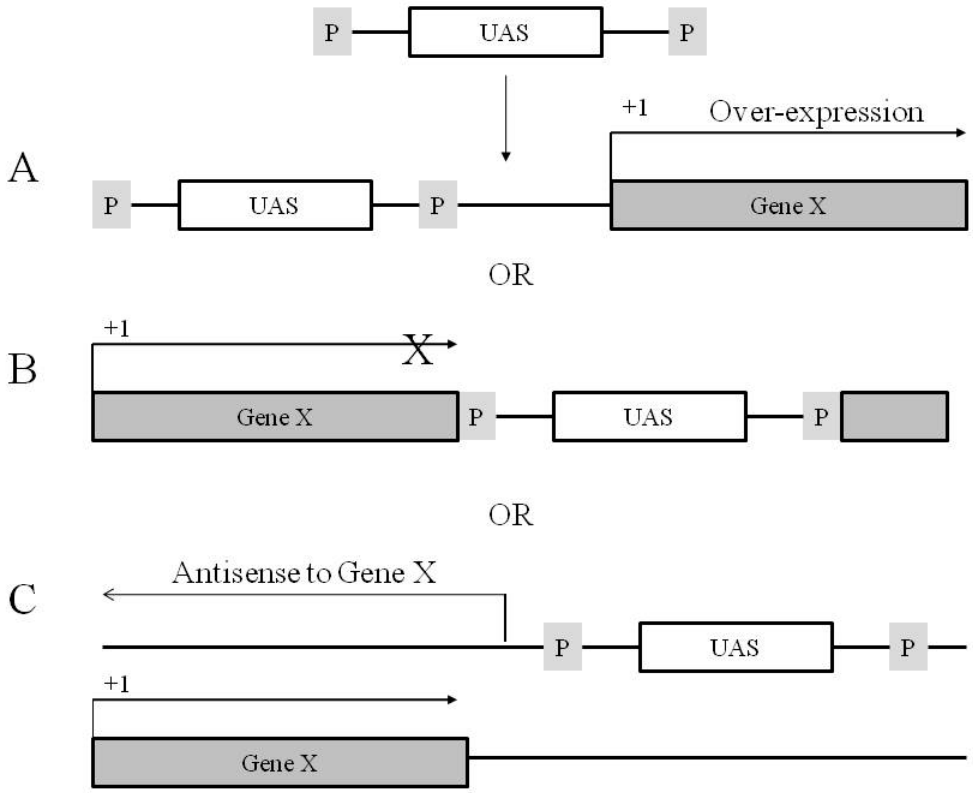

Figure 5. The EP Line: Gene Disruption Project. The P element containing the upstream activating sequence (UAS) is inserted into chromosome 2 in one of three ways. A) The P element is inserted upstream of "gene X" and leads to over-expression. B) The P element is inserted within "gene X" and either interferes with transcription, or alters the protein product. C) The P element is located on the opposite strand, running antiparallel, and leads to the expression of an antisense RNA that may interfere with transcription or translation of "gene X".

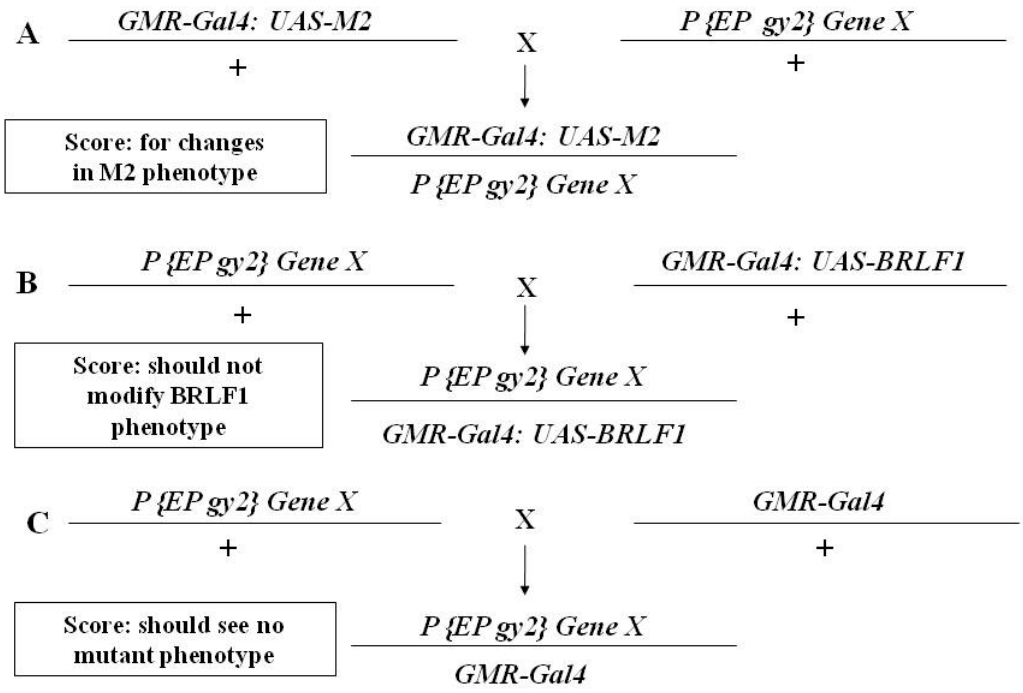

Figure 6. M2 genetic screen and verifications. A) Flies containing the *GMR-Gal4: UAS-M2/+* constructs were crossed to stocks from the EP line which contained a disruptive P element (see Figure 5). The box indicates the objective for the screen, alterations of the M2 phenotype, which suggests a possible interaction with M2. B) Initial verification of Modifiers in A by crossing of EP line stocks to flies containing a double construct system using BRLF-1, an Epstein-Barr protein. C) Second verification by crossing of EP line stocks, which passed initial verification, to flies containing *GMR-Gal4* construct only. Boxes for B and C indicate the proposed outcome for flies demonstrating a novel interaction with M2.

CHAPTER III

RESULTS

Amantadine reverses the M2 rough eye phenotype

Previously in our lab, a fly line was created which allowed the expression of the Influenza A viral protein M2 in the eye cells of *Drosophila melanogaster*. This line contains the construct *GMR-Gal4* which drives a second construct *UAS-M2* resulting in M2 transcription and translation (Adamson, unpublished). In order to assess the usefulness of *Drosophila melanogaster* as a tool for studying Influenza, flies expressing the viral protein M2 were exposed to the antiviral drug amantadine hydrochloride at varying concentrations. Amantadine is a known M2 inhibitor, which blocks ion conductance, and thus M2-induced changes in pH (Wright, et al., 2007). Therefore, changes in the rough eye phenotype due to M2 expression should be reversible via amantadine treatment. As shown in Figure 7, flies exposed to 0 μg/ml of amantadine produced an M2 eye phenotype. With increasing concentrations of amantadine the M2 phenotype was increasingly suppressed to that of near wild-type. Additionally, the percentage of flies that expressed the rough phenotype decreased with each increase in concentration (Table 1, Figure 8). This demonstrates that amantadine reverses the effect of M2 in this expression system, as it does in cell culture systems (Ciampor, et al., 1992a; Ciampor, et al., 1992b; Wang, et al., 1993). That amantadine, a channel blocker, suppresses the M2 phenotype suggests that the viral protein is functioning as a proton-

pump in the eye cells of *Drosophila* as in traditional hosts. This supports the use of *Drosophila* as a model system for human virus research.

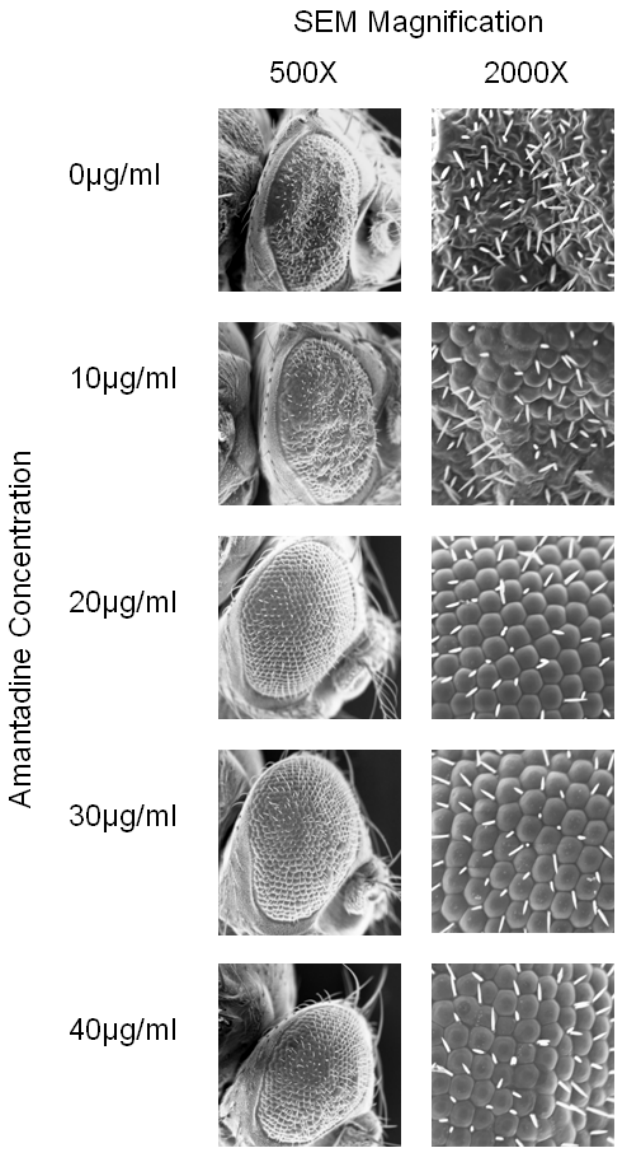

Figure 7. Amantadine reverses the M2 rough eye phenotype. M2 (*GMR-Gal4: UAS-M2/+*) flies at the second instar stage were placed on fly food supplemented daily with differing concentrations of amantadine hydrochloride. Upon eclosion, adult flies were screened for rough eye phenotype. The rough eye phenotype for each concentration is shown.

Table 1. Amantadine reverses M2 rough eye phenotype in *Drosophila*.

Amantadine Concentration	# rough/total	% rough
0 μg/mL	26/26	100%
10 μg/mL	22/31	71%
20 μg/mL	13/32	41%
30 μg/mL	10/28	35%
40 μg/mL	3/32	9%

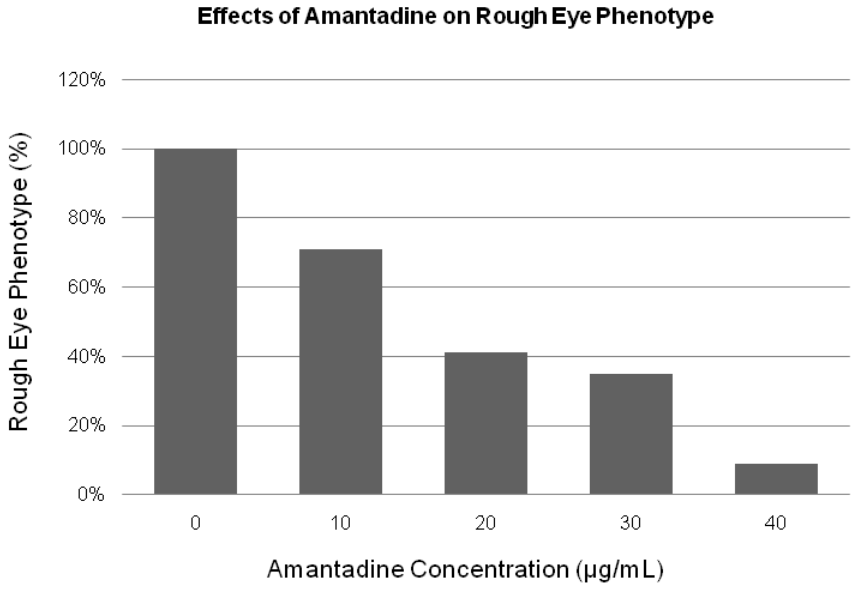

Figure 8. Amantadine reduces percentage of M2 rough eye phenotype. The percentage of adult flies expressing a rough eye phenotype post-amantadine treatment is shown for varying concentrations. The remaining percentage of flies for each concentration expressed a wild-type phenotype.

Amantadine treatment reduces vesicular alkalinity caused by M2

To further verify that M2 functions as a modulator of pH in *Drosophila*, the intracellular pH indicator SNARF-1 was used to analyze changes in vesicular pH. It was hypothesized that M2 functions as a proton-pump to increase the pH in intracellular

compartments in *Drosophila*, and that amantadine increases the acidity by blocking this activity. It has previously been shown in our lab that when M2 was expressed in the fat bodies of third instar larvae, vesicles were more basic than control groups not expressing M2. In the current study larvae expressing M2 in the fat bodies (*C135-Gal4/+; UAS-M2/+*), and control larvae not expressing M2 (*C135-Gal4/+*), were fed amantadine or sterile water (control) daily until third instar, at which point they were dissected for microscopy.

The indicator SNARF-1 is excited at a wavelength of 488nm, but is affected by pH. An acidic environment produces emissions at 560nm, while basic pH is detected at 660nm. The ratio between the two emissions can thus be used as an indicator of changes in pH. Using confocal microscopy it was shown that M2 expressing larvae (*C135-Gal4/+; UAS-M2/+*, no amantadine) had increased vesicular pH over larvae not expressing M2 (*C135-Gal4/+*, no amantadine) (Figure 9A and C). This coincides with our previously obtained results (Adamson, unpublished). Additionally, larvae expressing M2 and treated with amantadine showed a decrease in pH over larvae expressing M2 that did not receive amantadine (Figure 9C and 9D). As shown in Table 2, emissions at 660nm, which detects basic pH, decreased in M2-expressing fat body cells treated with amantadine, compared to those not treated. The ratio of 660nm/560nm also decreased; this change represents the effect of amantadine on reducing M2 activity in *Drosophila*. These results support the hypothesis that M2 functions as proton pump in *Drosophila* to regulate pH, and that it responds to amantadine treatment as in cell culture, resulting in reduced vesicular pH.

It is interesting to note that larvae which expressed M2, and had not been treated with amantadine, were bloated as compared to M2 larvae which had received treatment (not shown). Since *C135-Gal4* also drives expression in intestinal cells, the resulting M2 activity presumably created a proton imbalance that affected osmosis, allowing for increased water absorption. This demonstrates that the effect of M2 as an ion channel is not restricted to fat body tissue; the ion channel activity occurs in other tissues as well.

Gene disruptions lead to modification of the M2 rough eye phenotype

To identify host proteins that could potentially affect M2 activity, stocks from the EP lines were obtained. The approximately 1,200 stocks each contained a P element on the second chromosome resulting in the disruption of a particular gene. The P elements contain an upstream activating sequence (UAS), and are inserted in one of three positions. Insertion within a gene is expected to interfere with transcription or alter the protein product (Figure 5B), while insertion upstream of a gene is expected to cause over-expression (Figure 5A). The third position is on the opposite strand from the gene, and runs antiparallel (Figure 5C); this position is expected to lead to the transcription of the antisense causing reduced transcription or translation. Flies from the mutant stocks were crossed to flies from the *GMR-Gal4: UAS-M2/+* line and allowed to mature. Subsequently, adults were screened for modifications in the M2 rough eye phenotype (Figure 6A). Several stocks were identified that showed either suppression or enhancement (Table 3, Appendix A). Each result was scored for modification. Scores were based upon degree (slight to severe) of the modification, and rough percentage of

progeny that showed the modification. The alteration in the M2 phenotype by these particular gene disruptions suggests possible modulation of M2 activity, whether direct or indirect, by the affected proteins.

Some candidate genes also modify BRLF1 activity

To show that the modification is due to an interaction with M2 specifically, stocks for candidates obtained from the initial cross with M2 were crossed to a line containing the same construct system, but a different viral protein. For this system, females from the *GMR-Gal4: UAS-BRLF1/+* fly line were crossed to males from the candidate stocks. The BRLF-1 protein is found in Epstein-Barr virus, and plays a role in initiation of the EBV lytic cycle by activating early genes (Quinlivan, et al., 1993). It was expected that if a modified phenotype is a result of a candidate protein-M2 interaction, the modification observed previously would not be observed when a different protein is driven by the *GMR: UAS* system (Figure 6B). Therefore, possible true modifiers of M2 will not modify the *GMR-Gal4: UAS-BRLF-1/+* eye phenotype (differs slightly from M2 phenotype, not shown). Progeny from the BRLF-1 verification crosses were screened for changes in phenotype. Those that modified in the same direction, enhanced or suppressed, are listed in Table 4 (Appendix A). Since these gene disruptions modified both viral proteins they do not suggest a specific interaction with M2, and were therefore rejected as possible candidates for future studies.

GMR-Gal4 screen provides possible targets for future antiviral studies

Prior to beginning the second verification screen, the remaining stocks were assessed based on protein function and the processes in which they are known to be involved based on the information provided in FlyBase (www.flybase.org). Proteins that are primarily involved in protein translation, RNA Polymerase II activity, and RNA/DNA binding were not investigated further as they may affect M2 levels and not necessarily M2 function. Additionally, some proteins that function in processes that seemingly have no connection were excluded, such as those involved in oogenesis, gonad development, and behavior. Stocks that were chosen for investigation were crossed to a fly line containing only the *GMR-Gal4* construct. Since these flies do not contain M2, it was expected that disrupted genes involved in M2 activity modulation would not modify the *GMR-Gal4* wild-type phenotype (Figure 6C). Therefore, stocks that did not modify the wild-type phenotype were identified as shown in Table 5 (Appendix A). These genes represent proteins that could, as suggested by the results from the three screens, modulate M2 either directly or indirectly. These genes were checked for human homologs using FlyBase; those possessing known homologs are listed in Table 5, along with the function of the human protein. Genes that reported no homolog in FlyBase were searched using NCBI Nucleotide Blast search. Only one additional homolog was found, that for *Vha68-1*. Genes that have no human homolog were excluded here as targets for future investigations based on their inability to affect M2 in human hosts. Nine genes were selected from the remaining candidates for studies in vertebrate systems; the *Drosophila* genes selected are *Den1* (enhancer), *Gapdh1* (suppressor), *snama* (suppressor), *Pi3K21B*

(enhancer), *CG30122* (enhancer), *Trap1* (suppressor), *Vha68-1* (enhancer), *Vha44* (enhancer), and *CG9339* (enhancer). The M2 modified phenotypes for these stocks are shown in Figure 10.

Table 2. Vesicular pH decreases in M2 expressing larvae treated with amantadine.

C135-Gal4/+; UAS-M2/+	**n[+]**	**660nm (alkaline) emission[§] (light intensity units)**	**ratio 660/550nm[§*]**
- amantadine	25	5.08×10^6	2.74
+ amantadine	22	3.71×10^6	2.64

[+] number of fat body cells analyzed per condition
* The higher the ratio, the more basic the vesicular pH
§ averages are shown, values are significant according to Student's T test, $P < 0.05$

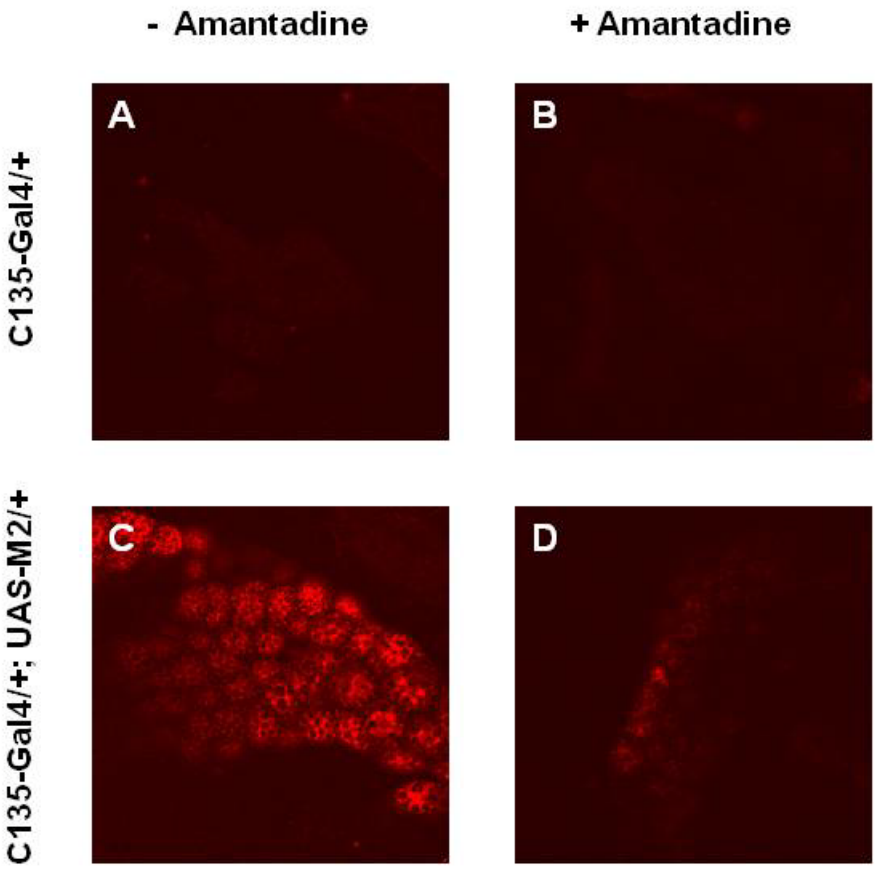

Figure 9. Amantadine treatment reduces alkaline vesicular pH. Fat body tissue from third instar larvae of the genotypes *C135-Gal4/+* (A and B) and *C135-Gal4/+; UAS-M2/+* (C and D) are shown, treated with either amantadine (+) or water (-). Red staining represents basic pH (660nm emissions), and is shown to be localized to the intracellular vesicles.

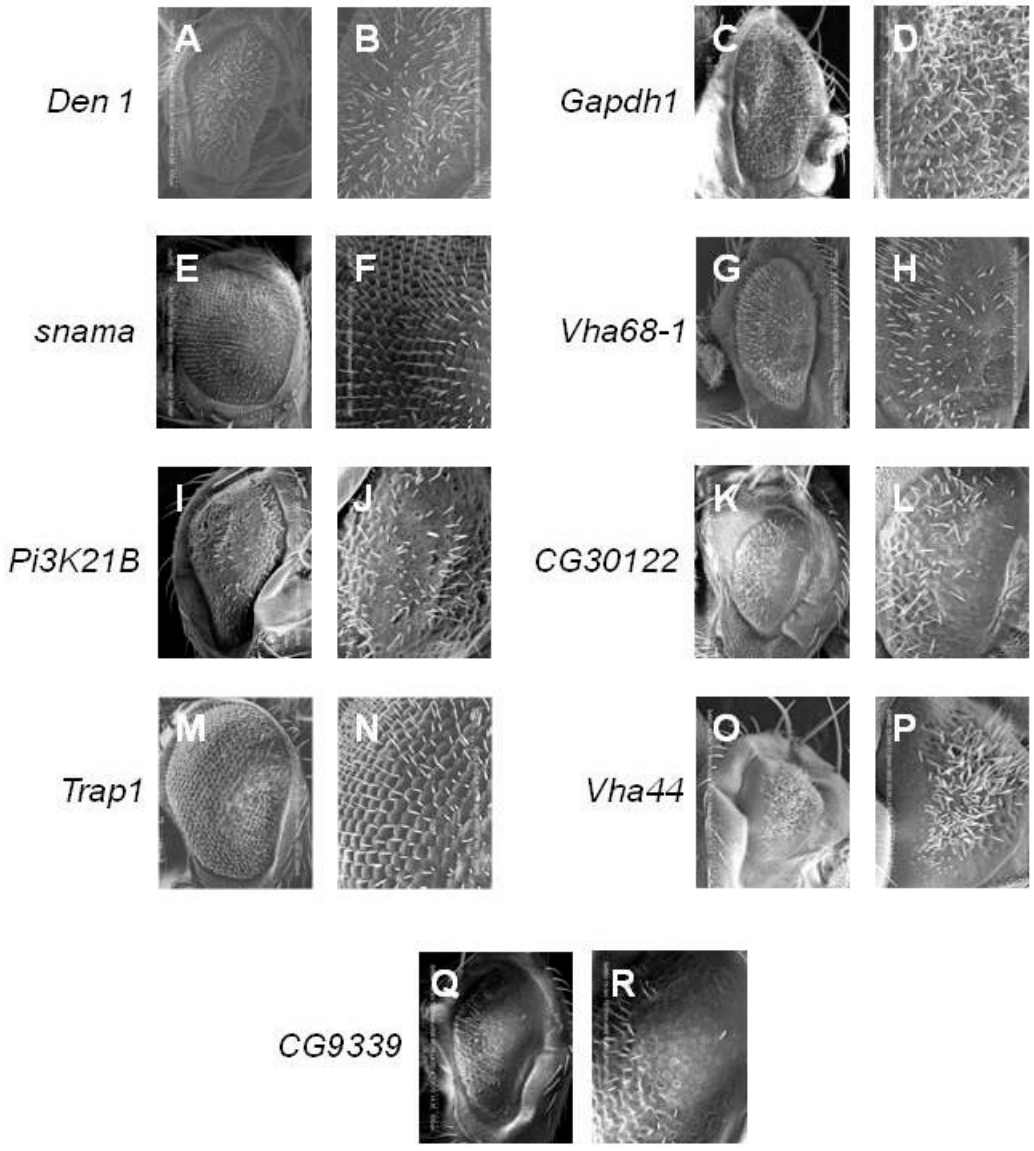

Figure 10. Candidate *Drosophila* genes modify M2 eye phenotype. SEM of mutant stocks containing disrupted genes which alter the eye phenotype produced by M2 expression. All flies were raised at 29°C and possess both M2 and the inserted P element. A) *Den 1*mutant at 300X. B) *Den 1* mutant at 600X . C) *Gapdh1* mutant at 250X. D) *Gapdh1* mutant at 600X. E) *snama* mutant at 250X. F) *snama* mutant at 600X. G) *Vha68-1* mutant at 300X. H) *Vha68-1* mutant at 600X. I) *Pi3K21B* mutant at 300X. J) *Pi3K21B* mutant at 600X. K) *CG30122* mutant at 300X. L) *CG30122* mutant at 600X. M) *Trap1* mutant at 300X. N) *Trap1* mutant at 600X. O) *Vha44* mutant at 300X. P) *Vha44* mutant at 600X. Q) *CG9339* mutant at 300X. R) *CG9339* mutant at 600X. Note that *Gapdh1*, *snama* and *Trap1* are suppressors of the M2 phenotype while the remaining candidates enhance.

CHAPTER IV

DISCUSSION

The aim of this study was to further investigate cellular interactions with the Influenza A viral protein M2 using a model system, *Drosophila melanogaster*, that has recently emerged as a useful tool in current viral research. The first goal was to determine if M2 behaves as an ion channel, and modulates pH, in *Drosophila* as it does in vertebrate hosts. In addition to showing a rough eye phenotype, the fly line expressing M2 (*GMR-Gal4: UAS-M2/+*) has been previously shown by our lab to localize to the membranes of *Drosophila* eye cells (Adamson, unpublished). Confocal microscopy of the imaginal discs of third instar larvae also demonstrated that M2 localizes to the apical surface of cells (Adamson, unpublished). This is in agreement with the apical localization in MDCK epithelial cells observed by Hughey and colleagues (1992). Additionally, the pH of intracellular compartments was found to be higher in M2 expressing flies, than in wild-type (*C135-Gal4/+*) (Figure 9A and 9C, also Adamson, unpublished). While this change in pH suggests ion channel activity by M2 in *Drosophila*, this activity was verified using the known M2 channel blocker amantadine (Hay, et al., 1985). With increasing concentrations of amantadine the rough eye phenotype exhibited by M2 expressing flies was reversed to near wild-type. The percentage of flies with the rough eye phenotype decreased as well. These results

suggest that M2 functions as an ion channel in the eye tissue of *Drosophila*, and this activity can be modulated by interfering with channel activity.

The effect of M2 activity in the trans-Golgi network is a decrease in acidity, thereby protecting the HA surface protein from a premature conformational change (Ciampor, et al., 1992a). The finding that M2 in *Drosophila* leads to an increase in pH in intracellular compartments (presumably including the Golgi network, based on confocal microscopy), and that the M2 eye phenotype is reversed by amantadine treatment led to the question of whether amantadine can reverse the M2-induced effects on compartmental pH. Using the driver system *C135-Gal4*, M2 was expressed in the fat bodies of larvae (*C135-Gal4/+; UAS-M2/+*), and compared to control larvae not expressing the protein (*C135-Gal4/+*). After treatment with either amantadine or water, the fat bodies were examined for vesicular pH by SNARF-1 analysis. M2 expressing larvae, fed only water, had increased compartmental pH versus non-expressing larvae (Figure 9A and 9C). And, larvae that express M2 show a decrease in pH upon amantadine treatment (Figure 9C and 9D, Table 2). These results suggest that the M2 construct successfully produces functional M2 with ion channel activity that functions to regulate vesicular pH like that of vertebrate hosts; and, importantly, that our *Drosophila* system is a useful investigative tool for studying viral-host interactions.

The next goal was to use the M2 fly line to screen for dominant modifiers of M2, using changes in the rough eye phenotype as indicators of possible interactions. The initial screen for these second-site modifiers included approximately 1,200 stocks

containing a mutation, via P element insertion, on the second chromosome. These stocks either over-expressed or under-expressed a gene based upon the insertion site of the P element. Many stocks were identified whose mutations either suppressed or enhanced the rough eye of M2 flies (Table 3). The functions of these genes varied widely including those involved in development and morphogenesis, cell communication, metabolic processes, behavior, biosynthesis of molecules, molecular binding, transcription and translation.

This field was narrowed using the *GMR-Gal4: UAS-BRFL1/+* fly line. Flies whose phenotype was similarly modified in the presence of a protein other than M2 suggest that the change was not due to M2, but to some other unknown factor involving the UAS system. Since these flies could not reliably be tested further, they were removed from consideration in future studies.

The second verification was used to rule out modifications resulting from the *GMR-Gal4* driver. Prior to this verification, continuing stocks were reviewed to determine which were of interest based on their functions. Genes involved in general replication, transcription and translation were generally excluded. These proteins may interact with M2; however, further analysis would be required to determine if their effects are due to changes in levels of M2 rather than modulation of activity. The remaining stocks were screened against *GMR-Gal4* flies which contained no M2. Stocks that retained the wild-type *GMR-Gal4* phenotype represent those that are not affected by *GMR-Gal4* alone, also suggesting that the initially observed phenotype was due to an

interaction, or lack of, with M2. In using *Drosophila* as a model organism to study human viruses it must be taken into consideration that not all genes within the fly genome have human homologs. If no homolog exists, the interaction observed with M2, while interesting, is of no relevance for antiviral studies since it cannot occur in the human host. Genes with human homologs are thus candidates for future studies in vertebrate hosts with the goal of the development of new antivirals. Out of this screen 19 genes, which have homologs, were identified that modulated the M2 eye phenotype, and required the presence of M2 for this effect. Nine of these genes were selected for future studies: *Den1*, *Gapdh1*, *snama*, *Pi3K21B*, *CG30122*, *Trap1*, *Vha68-1*, *Vha44*, and *CG9339*.

The proton-pump activity of the M2 protein presents a possible mechanism by which to prevent viral replication. Blockage of the cellular vacuolar proton ATP-ase (V-ATPase) results in increased pH within the lumen of endosomes. This increase in pH has been shown to prevent viral release from these vesicles (Guinea and Carrasco, 1995). Since M2 is activated by the acidity of the vesicles in which it travels, it dependent on this cellular protein for activation of channel activity (Wang, et. al, 1993; Guinea and Carrasco, 1995). Therefore, the finding that two of the candidate genes are V-ATPase subunits (*Vha68-1* and *Vha44*) helped to further validate the genetic screen as a method for identifying M2 modulators. As would be predicted, the disruption of the genes (P element insertion) led to an enhancement of the rough eye phenotype (Figure 10G, 10H, 10O and 10P).

Vacuolar ATPases are proton pumps found within the internal membranes of all eukaryotic cells; and, function in the acidification of vesicular compartments by means of H^+ transport across the membrane using energy derived from ATP hydrolysis. This acidification plays an important role in many functions within the cell, including macromolecule degradation, release of internalized ligands from their receptors, and cytosolic pH regulation. Other functions are cell-type specific (Beyenbach and Wiezczorek, 2006). The pump is composed of two domains termed V_1 and V_0. The V_1 domain consists of eight subunits (A-H) (Figure 11). Three units of both A and B form the head of the cytoplasmic V_1. Subunit A is responsible for the binding of ATP and subsequent enzymatic activity; subunit B, which has no enzymatic activity, is also required for nucleotide binding. Subunits C-H form the rotational and peripheral stalks that link V_1 to V_0 (Forgac, 1999; Nishi and Forgac, 2002; Beyenbach and Wieczorek, 2006). The V_0 domain contains at least six subunits which function in proton transport. All subunits appear to be necessary for pump assembly, except subunit H; and, both V_1 and V_0 domains are needed for complete function (although V_0 can assemble within the membrane without V_1) (Nishi and Forgac, 2002). Levels of pumps are not necessarily constant within the membranes, and the two domains can dissociate. This dissociation is controlled by several factors including glucose levels and hormones (Forgac, 1999; Beyenbach and Wieczorek, 2006). Additional regulation of intact pumps is attributed to disulfide bond formation between cysteine residues, changes in coupling efficiency, and proteins (activator and/or inhibitors, and other transporters) (Nishi and Forgac, 2002).

The function of pH regulation by cellular V- ATPases offers a possible target for the modulation of M2 function via cellular components. Previously in our lab several of the V-ATPase subunits were investigated also using a genetic screen. In Figure 11, all subunits were tested. Those that enhanced M2 are shaded. In particular, mutant subunits A, B, and C gave the strongest enhancement (Adamson, unpublished). These results coincide with the findings here that regulation of the V-ATPase affects M2 activity. The possibility exists that while reduction in V-ATPase function enhances M2, over-expression, or increased numbers of assembled pumps, may serve to inhibit M2. In modulating the V-ATPase for use as a therapy, however, it must be considered that blocking the pump in the trans-Golgi will enhance M2, but the opposite is true in the endosome. As there was no viral infection via endocytosis in the *Drosophila* system, only the trans-Golgi interactions between M2 and V-ATPase are demonstrated here. Removal of pump function in the endosome will suppress M2 as it will be exposed to higher pH values and presumably not activate. Antivirals developed using this structure as a way to modulate M2 must consider the effect of the pump modification and the relation to the time of treatment, whether prophylaxis or during productive infection. Impairment of the pump is thus best suited for prophylaxis, while over-expression, if found to also modulate M2, would seem best after infection has begun. The next step towards therapies involving pump modulation is to perform knockdown and over-expression of these subunits in host cell systems.

In addition to affecting the V-ATPase pumps directly, there are indirect means of influencing pump activity. Many regulators exist which lead to dissociation or

reassembly of pumps within the membranes of intracellular compartments, one of which is glucose levels (Forgac, 1999; Nishi and Forgac, 2002; Beyenbach and Wieczorek, 2006). A decrease in the number of assembled pumps occurs as glucose levels decrease (Kane, 1995). Parra and Kane (1998) report that the detection of glucose levels is a function of the catalytic subunits, and that in order to maintain assembled pumps glycolysis must proceed beyond that of glucose conversion to glucose 6-phosphate. These findings led to the suggestion that the disassembly in the absence of glucose is an effect of reduced ATP levels (Parra and Kane, 1998). Interestingly, one of the candidate genes (*Gapdh1*) identified in this screen was the *Drosophila* homolog to GAPDH, the glycolytic enzyme that converts glyceraldehye-3-phosphate to 1,3-bisphosphoglycerate (Champe, et al., 2005). The P element that is inserted near this gene is located on the opposite strand and runs antiparallel, implying that its effects are via an antisense molecule. This would result in reduced levels of GAPDH, thereby allowing glycolysis to proceed to the sixth step before backing up. Based on these results, the slightly suppressed rough eye phenotype observed (Figure 10C and 10D), may be due to slight increases in glucose, or other intermediates, as glycolysis proceeds, yet slowly due to decreased GAPDH. Due to the bottleneck at step 6, the increase in glucose levels, or other intermediates such as glyceraldehyde 3-phosphate, may be sufficient to increase pump levels thereby offsetting the activity of M2.

Although affecting glucose levels provides a possible mechanism by which this reduction modifies M2, another possibility is that the enzyme interacts directly to help with pump assembly. In support of this idea, it was found that the glycolytic enzyme

aldolase binds the B subunit of the V_1 domain to assist in pump assembly (Lu, et al., 2007). In this study Lu and colleagues (2007) used both human and yeast aldolase mutants to show that removing this binding not only results in disassembly, but may also reduce the activity. In a similar manner GAPDH may also bind to subunits of the pump to regulate assembly and disassembly. If GAPDH is a negative regulator of pump assembly, its reduction could result in increased numbers of assembled pumps, thereby allowing the countering of M2. While these studies suggest regulation of pump activity and assembly, it cannot be ruled out that GAPDH interacts to affect M2 by another mechanism. GAPDH has also been shown to be involved in cell death via accumulation of malformed GAPDH induced by oxidative stress (Nakajima, et al., 2007). Additionally, GAPDH has also been shown to relocate from the cytoplasm to protect the DNA repair enzyme APE1 from oxidative stress (Azam, et al., 2008). How these functions of GAPDH might be involved in M2 modulation is unclear.

The gene PIK3R3 is the human homolog of the *Drosophila* gene *Pi3K21B*, and upon P element disruption, functioned as an enhancer of M2 (Figure 10I and 10J). This gene encodes the p55 gamma regulatory subunit of the enzyme PI3K, phosphatidylinositol 3-kinase. The enzyme functions via kinase cascade signaling, affecting various processes within the cell such as metabolism (Mothe, et al., 1997), cell cycle (Xia, et al., 2003) among others (Cantley, 2002). The enzyme is comprised of a catalytic subunit, p110, and a regulatory subunit; and, various forms of each are known to exist (Vanhaesebroeck, et al., 1997). The p55γ subunit has been shown to bind with IGF-1R, insulin-like growth factor 1-receptor (Mothe, et al., 1997), and more recently

with the tumor suppressor protein, retinoblastoma (Rb) (Xia, et al., 2003). While this particular subunit has not been implicated by studies in viral interactions, PI3K containing isoforms of the p85 regulatory subunit have been shown to play multiple roles in Influenza A viral infections. Ehrhardt and coworkers (2006) found a dual role for PIK3 in both anti- and pro-viral processes. While PI3K aids in interferon regulatory factor-3 activation, an antiviral mechanism, the need for PI3K in viral entry was also demonstrated (Ehrhardt, et al., 2006; Ehrhardt and Ludwig, 2009). An additional proviral function of the enzyme in apoptosis prevention was found to be mediated by binding of the influenza viral protein NS1 to the p85β subunit. This presumably activates the signaling cascade by relieving the catalytic subunit from regulation (Hale, et al., 2008; Ehrhardt and Ludwig, 2009). Furthermore, reduced numbers of virus particles produced upon PI3K inhibition supported the role of the enzyme in assisting in the viral replication cycle (Ehrhardt, et al., 2006; Ehrhardt and Ludwig, 2009). Therefore, PIK3, particularly its regulatory subunits seem to have a role at different time points during viral infection. This poses the question of whether the viral protein M2 can also interact with a regulatory subunit. In keeping with the findings presented here, a potential binding of M2 to p55γ would function to keep M2 in check, since the loss of the subunit led to increased activity. It is also possible that PI3K aids in M2 function indirectly via downstream signaling; and, that the loss of p55γ allows uninhibited signaling, encouraging M2 activity. An interesting note is that PI3K is also implied as a mediator of V-ATPase assembly via glucose levels (Sautin, et al., 2005). Using renal epithelial cells, Sautin and colleagues were able to show that inhibiting PIK3 interferes with

assembly in response to glucose; and, that expression of the p110 catalytic unit alone could initiate assembly in cells with low glucose (2005). These studies support the findings here that PIK3, and therefore possibly its regulatory subunits, function to affect M2 activity, though the mechanism is unknown.

A common theme that has appeared in the results from the screen is the interaction of proteins with the tumor-suppressor retinoblastoma. As with PIK3, a second candidate gene, RBBP6 (human homolog of *snama*) is an Rb binding protein (Sakai, et al., 1995). This protein, named retinoblastoma binding protein 6, additionally interacts with p53, also a tumor suppressor, and has a role in protein degradation via ubiquitination (Chibi, et al. 2008). Although there is no apparent connection to how it might affect M2, there are other proteins which bind both Rb and influenza proteins. Ebp1 is an ErbB-3 binding protein which has been shown to also interact with Rb (Xia, et al., 2001). Ebp1 has been shown to bind the influenza protein PB1, which is part of the viral polymerase complex (Honda, et al., 2007). This binding inhibits viral polymerase *in vitro*, and the binding site shares an overlap region to that of the Rb binding site (Honda, et al., 2007). The possibility exists that RBBP6 binds M2 in a similar fashion to the binding of Ebp1 and PB1. However, the findings here are in contrast with the above resulting scenario as M2 was suppressed in the absence of *snama* (P element insertion within the gene) (Figure 10E and 10F). Thus, there exist proteins that function to both regulate modulators of cell cycle, and are players in influenza viral infections; RBBP6 could also be such a protein. Conversely, the modification to M2 could also be an indirect effect of changes in Rb regulation and not due to a direct binding.

The candidate gene *CG30122*, or human gene HNRNPUL1 (also known as E1B-AP5) was shown to enhance M2 (Figure 10K and 10L). The P element insertion was antiparallel, presumably leading to the transcription of an antisense molecule, thus reducing protein levels. This protein has been shown to be involved in both adenoviral and influenza infections (Gabler, et al., 1998; Satterly, et al., 2007; Blackford, et al., 2008). This protein functions normally in cells as a heterogeneous nuclear ribonucleoprotein (hnRNP), but upon adenoviral infections is recruited by the viral proteins to assist in viral mRNA transport from the nucleus. E1B-AP5 is bound by the viral protein EB1-55kDa to mediate this process (Gabler, et al., 1998). During influenza infections E1B-AP5 forms a complex with the viral protein NS1, and other cellular mRNA export proteins to inhibit some cellular mRNA export, primarily those involved in antiviral pathways (Satterly, et al., 2007). There is an additional role for E1B-AP5 in adenovirus infections and the signaling pathways responding to DNA damage; these pathways are regulated by E1B-AP5 for viral replication (Blackford, et al, 2008). As this candidate has been shown to interact with viral proteins, it seems plausible that it may interact with M2. Since the *Drosophila* system does not mimic a true viral infection in that the entire viral protein assemblage is not present, the effect of E1B-AP5 reduction cannot be said to be a result of an interaction with NS1 having an effect on M2 mRNA export. Although, based on the role of E1B-AP5 in assisting in viral mRNA transport, its absence would be more likely to suppress M2 than enhance. Therefore, no logical hypotheses aptly explain the proposed interaction between M2 and E1B-AP5.

The candidate gene TBC1D24 (homolog to *CG9339*) was disrupted by the insertion of the P element, and produced an enhanced M2 phenotype (Figure 10Q and 10R). Little is understood about this particular protein; it contains a TBC (Tre-2/Bub2/Cdc16) domain that may function as a Rab-GAP domain (Ishibashi, et al., 2008). GTPase-activating proteins (GAPs) are thought to be regulators of the Rab proteins which are involved in vesicular membrane trafficking, particularly in the secretory pathways (Schwartz, et al., 2007; Grosshans, et al, 2009). The GAP proteins function to inactivate Rabs after they perform their various trafficking functions (Grosshans, et al., 2009). Inhibiting the inactivation of Rab hydrolysis by mutation of a TBC domain could affect the movement or possibly the size of the compartments. The M2 protein utilizes these pathways post synthesis to reach the plasma membrane for viral packaging (Palese and Shaw, 2007). These changes could allow more M2 to accumulate, or more activity, in the vesicular membranes. Additional pump activity, due to the inability of vesicles to separate and travel through the pathway to the plasma membrane, would increase the pH thereby enhancing the effects of M2. The possibility of a direct interaction of this protein with M2 still exists, as much of its function has yet to be elucidated.

The SENP8 human gene is the homolog of *Den1* in *Drosophila*, and encodes the SENP8 (or DEN1) human protein. The P element insertion for this gene caused an enhanced phenotype (Figure 10A and 10B). SENP8, also known as DEN1, is a protein involved in the NEDD8 pathway and functions in the processing of NEDD8, as well as removal of NEDDylation from cullin proteins, like CUL1 (Wu, et al., 2003). Furthermore, the extent of deNEDDylation was found to be dependent on the

concentration of the SENP8 (DEN1) (Wu, et al., 2003). The NEDD8 protein binds to cullins, which form a complex with other proteins including ubiquitin ligases, and functions in protein degradation (Pan, et al., 2004). These protein targets include those involved in processes such as cell cycle and signal transduction (Pan, et al., 2004). The phenotype expressed by disruption of SENP8 may be attributed to less M2 targeting by a degradation mechanism. However, it has been reported that misregulation of this process can result in tumorigenesis (Pan, et al., 2004), so the usefulness of modulating this protein to reduce viral infections must be weighed against the risk of tumor development. It must also be kept in mind that cullins are not the only target of NEDDylation. It was recently shown that proteins comprising ribosomes are also NEDDylated, which provides stability (Xirodimas, et al., 2007). Therefore, if SENP8 were involved in the NEDDylation of the ribosomal proteins, less processed (functional) NEDD8 may result in unstable ribosomes. Xirodimas and coworkers observed that cells lacking NEDDylation capabilities did in fact produce this instability (2007). This effect on ribosomes could potentially affect levels of translated proteins, including M2. Since SENP8 (DEN1) was shown to both assist NEDD8 in its processing into a functional molecule, as well as regulates binding to cullins, the method by which removal of this protein enhances M2 remains unclear (Wu, et al., 2003).

A final candidate gene is *Trap1*. This gene seems likely to be over-expressed due to the P element insertion, and produced a suppressed M2 phenotype (Figure 10M and 10N). The human homolog is the gene TRAP1, which encodes heat shock protein 75 (also known as TRAP1), and is part of the hsp90 family. This protein acts as a molecular

chaperone for retinoblastoma protein and has been found to associate with Rb both during mitosis and after heat shock (Chen, et al., 1996). In addition to this role as a possible regulator of Rb, Hua and colleagues (2007) found that hsp75 (TRAP1) functions in the mitochondria in an anti-apoptotic manner by regulating ROS levels. While these functions do not point to an obvious mechanism by which hsp75 affects M2, it is worth noting that other members of the hsp90 family interact with influenza A viral proteins to aid in viral replication. Momose et al. (2002) found that upon viral infection Hsp90 could be found in the nucleus, and interacts with the viral polymerase protein PB2 to assist synthesis activity. This raises the possibility that hsp75, although as yet thought to function primarily in the mitochondria, may function elsewhere and interact with M2. In keeping with the results obtained in this study, that over-expression suppresses M2 activity, TRAP1 would negatively regulate M2 viral functions in the host; excess of TRAP1, therefore, would further reduce activity.

As with any preliminary findings such as those presented here, future research is vital to confirming these results and to elucidating the mechanisms behind them. The next step for this study will be to confirm the enhancement or suppression of the M2 phenotype by these cellular modifications using additional alleles of the mutant protein. If the observed phenotypes are indeed due to an interaction whether direct or indirect with M2, allelic variations will support these findings. Furthermore, the proposed effects of P element insertion (abolishment, reduction, and over-expression) should be verified via RT-PCR in reference to transcript levels; work has already begun in our lab in this regard. Table 6 (Appendix B) lists suggested primers for regions of approximately 500

base pairs for the mRNA transcript analysis. Subsequent studies should then be initiated in vertebrate cell culture to show that these results are reproducible in host systems.

The antivirals currently available face the problem of resistance by the ever-evolving viral proteins. Therefore, cellular proteins may present a more stable option for therapeutics. This idea has also been suggested by others in the field of influenza research (Honda, et al. 2007; Hoffman, et al., 2008). The cellular effects of altering endogenous proteins, although only temporarily, must be taken into account when developing new antiviral treatments. As several of the proteins identified by this candidate screen are involved in binding to tumor suppressors, regulating cell cycle or ROS levels, modification poses the possibility of cancer formation. Thus, future studies should focus not only on the immediate benefits or problems with modulating these proteins, but the long term effects they may have on the cell as well.

In conclusion, the problems of acquired viral resistance, and for some drugs low efficacy, remain barriers that new antiviral developers seek to overcome**.** There are currently several new antivirals undergoing clinical trials (Biegel and Bray, 2008; Moss, et al., 2010). However, how they fair over the course of time with regards to these issues remains to be seen. The goal of this study was to employ the second-site modifier screening technique in *Drosophila*, commonly used in other areas of research, to identify host cellular proteins that may be modulated to reduce or prevent influenza infection. The first aim was to show that *Drosophila*, while not a natural host for influenza, can reliably be used to test for these interactions. The studies presented here using amantadine to target M2, show that M2 functions as an ion channel in the fly, and that

this activity can be altered. These findings support *Drosophila* as a model system for studying influenza, and by utilizing this tool several possible cellular targets were found. Future studies will reveal what role these proteins play in infection, and provide insight into how they may be used as therapies.

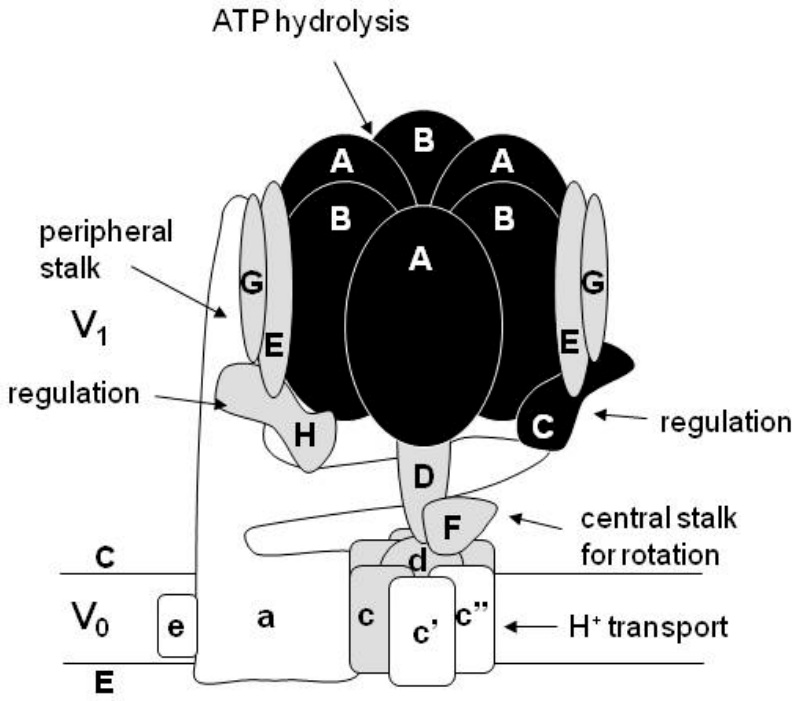

Figure 11. The vacuolar ATPase proton pump. Both domains V_1 and V_o are shown. The domains are separated by a peripheral stalk. All subunits were investigated previously for modification of M2. In those studies, subunits in gray showed slight enhancement of the M2 phenotype, while subunits in black showed the strongest enhancement.

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APPENDIX A. FLY SCREEN DATA

Table 3. M2 second-site modifiers.

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
14798	*	*Sdc*	transmembrane receptor	visual perception, axon guidance	E	N/A
14798	*	*NaCP60E*	sodium channel activity	response to chemical signals, olfactory behavior	E	N/A
14798	*	*RpL41*	ribosome structure	Translation	E	N/A
14810	*	*CG18854*	inositol trisphosphate 3-kinase	Unknown	E	N/A
14823	**	*CG10337*	Unknown	Unknown	S	N/A
14824		*α4GT1*	acetylgalactosaminyl-transferase activity	glycolipid biosynthetic process	E	N/A
14853	**	*Grp*	Kinase	female meiosis chromosomal segregation, cell cycle arrest, cell cycle, spindle assembly	E	Yes
15056	**	*RN-tre*	Rab GTPase activator activity	regulation of Rab GTPase activity	E	None
15283	**	*CG31782*	nucleic acid binding	Unknown	E	N/A
15317	**	*CG8187*	unknown	Unknown	E	N/A
15317	**	*CG30467*	unknown	acute-phase response	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
15348	*	*Den1*	NEDD-8 specific protease activity	protein deneddylation	E	N/A
15357		*Vri*	protein dimerization	circadian rhythm, bristle morphogenesis, wing hair organization	E	Yes
15366	*	*Egfr*	epidermal growth factor receptor	various organ development and morphogenesis, regulation of development, negative regulation of cell death	E	Yes
15483	*	*CG9084*	unknown	Unknown	E	N/A
15495	*	*lethal(2)37Cb*	RNA helicase activity	nuclear RNA splicing, via spliceosome	S	None
15508	**	*Mapmodulin*	phosphatase inhibitor, microtubule binding	nucleocytoplasmic transport, microtubule-based process	E	N/A
15530	*	*CG8080*	NAD^+ kinase	metabolic process	E	N/A
15549	*	*sns*	unknown	myoblast fusion, larval muscle development, cell adhesion, garland cell differentiation	S	None
15557	*	*CG14478*	DNA binding	intermale aggressive behavior	E	N/A
15565		*CG6370*	glycotransferase activity	glycosylation via asparagine	S	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
15568	**	*bchs*	zinc ion binding	bristle development, regulation of ubiquitination, regulation of synapse organization, determination of lifespan, axonogenesis, compound eye development	E	Yes
15587		*Sgt*	binding	neuromuscular synaptic transmission	E	N/A
15666		*CG4259*	endopeptidase activity	proteolysis	E	N/A
15714	*	*Orc6*	DNA binding	DNA replication initiation, mitosis (M phase)	E	N/A
15781	**	*CG13177*	unknown	Unknown	E	N/A
15794	*	*Phax*	unknown	nervous system development, snRNA export from nucleus	E	N/A
15804	**	*Atf-2*	DNA binding, transcription activator, protein homodimerization activity	positive regulation from RNA polymerase II promoter, salt stress response, stress-activated MAPK cascade, mucosal immune response	E	None
15804	**	*CG16896*	Rab GTPase activator activity	regulation of Rab GTPase activity	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
15833	*	*ab*	RNA polymerase II transcription factor	border follicle cell migration, lysosome organization, dendrite morphogenesis, neuron development, muscle organ development, negative regulation of autophagy	S	None
15838	*	*yellow-b*	unknown	Unknown	E	N/A
15892	*	*Gapdh1*	dehydrogenase activity	Glycolysis	S	N/A
15907	**	*snama*	protein binding	Unknown	S	None
15923	*	*CG1868*	transcription repressor	Unknown	E	N/A
15930		*luna*	transcription factor activator, DNA binding	regulation of transcription (DNA-dependent)	S	Yes
15936	*	*TepIV*	peptidase inhibitor	antibacterial humoral response	E	N/A
15943	*	*Gr32a*	taste receptor	sensory perception of taste	E	N/A
15949	*	*mip120*	protein and DNA binding	determination of lifespan, sperm motility, oogenesis, negative regulation of transcription from RNA Polymerase II promoter	E	Yes
15957	**	*Cp1*	endopeptidase activity	autophagic cell death, salivary gland cell death	S	None
15976		*Hydr2*	lipase activity	lipid metabolic process	S	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
16360	**	*CG3065*	zinc ion binding, nucleic acid binding	Unknown	E	N/A
16372	**	*cnn*	microtubule binding	CNS and PNS development, mitotic spindle organization, female meiotic chromosome segregation, midgut development	E	None
16386		*CG17324*	UDP-glycosyltransferase activity	metabolic process	E	N/A
16543	**	*Vha68-1*	ATP-ase (H+)	proton transport	E	N/A
16587	*	*CG9247*	exonuclease activity, nucleic acid binding	nucleic acid metabolic processes (nucleoside, nucleotide)	E	N/A
16632	*	*CG5757*	thymidylate kinase	dTDP biosynthetic process	E	N/A
16647		*ssp4*	microtubule binding	mitotic spindle elongation, microtubule severing	S	N/A
16682	*	*CG42336*	unknown	Unknown	E	N/A
16707	*	*CG30077*	unknown	Unknown	E	N/A
16711	*	*CG8486*	unknown	Unknown	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
16720	*	*Pi3K21B*	kinase binding	phosphoinositide phosphorylation, amino acid phosphorylation, regulation of cell proliferation, regulation of cell size, lipid phosphorylation	E	None
16722		*CG5591*	transcriptional regulatory activity	phagocytosis, engulfment	S	N/A
16729	*	*CG1667*	unknown	Unknown	E	N/A
16736	*	*mRpL48*	structure of ribosome	Translation	E	N/A
16737	*	*betaTub60D*	structure of cytoskeleton	response to light stimulus, larval behavior, axonogenesis	E	Yes
16741	*	*CG9526*	unknown	Unknown	S	N/A
16744	*	*Socs36E*	protein binding	haltere development, wing vein morphogenesis, notum morphogenesis, negative regulation of JAK-STAT cascade, compound eye pigmentation	S	Yes
16822		*CG18190*	microtubule binding	Unknown	S	N/A
16975	**	*CG8389*	monocarboxylate transmembrane transporter	transmembrane transport	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
17307	*	*Nop60B*	pseudouridylate synthase	ribosome biogenesis, rRNA processing, germ cell development, pseudouridine synthesis	E	None
17311	**	*CG31638*	unknown	Unknown	E	N/A
17316	*	*CG17739*	endopeptidase inhibitor activity	Unknown	E	N/A
17321	*	*CG2839*	binding	Unknown	S	N/A
17352		*CG15172*	unknown	Unknown	E	N/A
17372	*	*CG40169*	unknown	Unknown	E	N/A
17434	**	*sns*	unknown	myoblast fusion, larval muscle development, cell adhesion, garland cell differentiation	E	None
17454	**	*CG30122*	mRNA binding	unknown	E	N/A
17483	*	*CG15117*	beta-glucuronidase activity	carbohydrate metabolic process	S	N/A
17533		*CG16854*	catalytic activity	metabolic process	E	N/A
17538	***	*olf186-F*	calcium channel activity	positive regulation of calcium transport, nervous system development	E	Yes

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
17547		*StIP*	proteasome activator activity	proteasome assembly, RNA elongation from RNA Polymerase II promoter	S	N/A
17553		*Cyp4e2*	electron carrier activity	oxidation reduction	S	N/A
17581	**	*CG7337*	unknown	unknown	E	N/A
17611	*	*ms(2)34Fe*	acyl carrier	unknown	S	N/A
17634	*	*CG8920*	nucleic acid binding	unknown	E	N/A
17634	*	*CG13868*	unknown	unknown	E	N/A
17654	*	*Hr46*	protein binding, ligand-dependent nuclear receptor	mushroom body development	S	None
19807	*	*cmet*	microtubule motor activity	mitotic spindle organization, cell cycle, metaphase plate congression	S	None
19821	**	*vlc*	unknown	leg morphogenesis	E	None
19860	*	*I(2)44DEa*	LC-fatty acid-CoA ligase activity	metabolic process	S	N/A
19889	*	*DLP*	protein binding	regulation of apoptosis, determination of lifespan, positive regulation of transcription	E	N/A
19899	*	*Eno*	phosphopyruvate hydratase activity	glycolysis	S	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
19944		*Mmp2*	metalloendopeptidase	basement membrane assembly, oogenesis, imaginal disc fusion, thorax closure	E	Yes
19946	*	*crol*	RNA Polymerase II transcription factor activity	negative regulation of transcription, negative regulation of Wnt, cell adhesion, positive regulation of mitotic cycle	S	None
19970	*	*slmo*	unknown	peristalsis, larval behavior, spermatogenesis	E	None
19974		*Trap1*	unfolded protein binding, ATP binding	protein folding, stress response	S	N/A
19978	**	*ex*	protein binding	regulation of development including compound eye, transport, regulation of cell cycle, regulation of signal transduction	E	Yes
19984		*poe*	calmodulin binding	sperm development, perineurial glial growth	S	None
19987	*	*Aats-glupro*	SUMO binding	tRNA aminoacylation	S	N/A
20019	***	*CG3363*	unknown	unknown	E	N/A
20036		*Pen*	SUMO binding	cytoplasmic transport, sperm individualization, gravitaxis, lymph gland development	E	None

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
20043	*	*bin3*	protein binding	olfactory behavior, transcriptional regulation	E	N/A
20050		*CG8920*	nucleic acid binding	unknown	S	N/A
20054	*	*opa1-like*	GTP binding	mitochondrial fusion, pupariation	E	None
20058	*	*ytr*	mRNA binding	hemocyte differentiation	E	None
20059	*	*lola*	protein binding	brain morphogenesis, behavior, antimicrobial humoral response, axonogenesis, locomotion, regulation of metabolic process	E	None
20073		*gem*	transcription factor activity	regulation of transcription	E	N/A
20104	*	*Df31*	histone binding	chromatin organization, nucleosome assembly	S	None
20111		*CG13101*	unknown	unknown	S	None
20120	*	*GlcAT-S*	multiple transferase activities	biosynthetic processes (glycoprotein, proteoglycan)	E	N/A
20121	*	*CG15439*	protein and zinc ion binding	unknown	E	N/A
20123	*	*Gpdh*	glycerol-3-phosphate dehydrogenase (NAD+) activity	triglyceride metabolic process, flight behavior	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
20140	**	*Vha44*	ATP-ase (H+)	proton transport	E	N/A
20148		*MESR4*	protein and zinc ion binding	unknown	S	None
20159	**	*fs(2)ltoPP43*	Unknown	eggshell formation	E	Yes
20160	*	*cnk*	protein binding	Ras signaling, photoreceptor cell differentiation, tracheal outgrowth, wing morphogenesis, signal transduction	E	Yes
20162	*	*Wnt4*	signal transducer, receptor binding	female gonad develop, cell migration, establishment of oomatidia polarity, salivary gland morphogenesis	E	Yes
20175		*CG8677*	transcriptional repressor	negative regulation of transcription (DNA-dependent)	S	N/A
20190	**	*esg*	RNA Polymerase II transcription factor	CNS development, germ line stem cell maintenance, eye pigmentation, olfactory behavior	E	Yes
20202		*sli*	protein binding	cell motility, neuron differentiation, regulation of cell adhesion, organ development and morphogenesis, locomotory behavior	S	None
20208		*Kdm4b*	H3-K36, H3-K9 demethylase	histone demethylation (H3-K36 and H3-K9)	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
20215		*Plap*	phospholipase A2 activator	unknown	E	N/A
20247	**	*B4*	Unknown	circadian rhythm, imaginal disc development	E	Yes
20306		*S2P*	metalloendopeptidase	regulation of protein cleavage	E	N/A
20709	*	*stil*	Unknown	female sex determination and differentiation, oogenesis	E	None
20719	*	*lilli*	transcription factor activity, DNA binding	regulation of cell size, photoreceptor development, olfactory behavior, wing pattern	E	None
20756	*	*spen*	transcription regulation	organ development, gliogenesis, cell motion, cell morphogenesis, cell communication, segment specification, regulation of metabolic process	S	Yes
20758	**	*Hrb27C*	protein, DNA, mRNA, SUMO binding	regulation of mRNA splicing, positive regulation of translation, border follicle cell migration	E	None
20810		*CG9339*	Rab GTPase activator activity	regulation of Rab GTPase activity	E	N/A
20910		*Hr38*	nuclear receptor activity	phagocytosis	E	None

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
20912	*	*spi*	epidermal growth factor receptor binding	negative regulation of apoptosis, nervous system development, border follicle cell migration, wing morphogenesis	E	Yes
20921		*CG18604*	ATP binding, kinase activity	protein amino acid phosphorylation	E	N/A
21125	*	*CG3975*	Unknown	unknown	E	N/A
21204	**	*EcR*	hormone receptor, protein binding	anatomical structural development, organ and muscle development, CNS development, regulation of metabolic process	E	Yes
21234	*	*CG7830*	Unknown	unknown	E	N/A
21413	**	*Egfr*	epidermal growth factor receptor	organ development, negative regulation of cell death	E	Yes
21418		*CdGAPr*	GTPase activator activity	retinal ganglion cell axon guidance	E	Yes
22366	*	*Tsp39D*	Unknown	unknown	E	N/A
22386		*GstE8*	Unknown	unknown	E	N/A
22476	**	*CG5325*	Unknown	nervous system development	E	N/A
22514		*Trap1*	unfolded protein binding, ATP binding	protein folding, stress response	E	N/A

STOCK	SIGNIFICANCE[+]	GENE[§]	PRODUCT FUNCTION[§]	CELLULAR PROCESSES[§]	PHENOTYPE CHANGE[++]	STOCK EYE PHENOTYPE[§]
22584	**	*bin3*	protein binding	olfactory behavior, transcriptional regulation	E	N/A
22610		*Eno*	phosphopyruvate hydratase activity	glycolysis	E	N/A
22617	*	*lola*	protein binding	brain morphogenesis, behavior, antimicrobial humoral response, axonogenesis, locomotion, regulation of metabolic process	E	None
23098	**	*fbl6*	Unknown	unknown	E	N/A

[+] Phenotype was rated based on slight to severe change, and percentage of total flies expressing a change: no star means slight or few, one star is intermediate, two stars is severe or many.

[++] Enhancer (E), Suppressor (S)

§ Data was obtained from www.FlyBase.org

Table 4. Modifiers of BRLF1 and M2.

STOCK	SIGNIFICANCE[+]	GENE[§]	FUNCTION[§]	PHENOTYPE[++]
14853	**	*Grp*	Kinase	E
15056	**	*RN-tre*	GTPase activator activity	E
15357		*Vri*	protein dimerization	E
15366	*	*Egfr*	epidermal growth factor receptor	E
15557	*	*CG14478*	DNA binding	E
15568	**	*Bchs*	zinc ion binding	E
17321	*	*CG2839*	Binding	S
17533		*CG16854*	catalytic activity	E
19946	*	*Crol*	RNA Polymerase II transcription factor activity	S
19978	**	*Ex*	protein binding	E
20019	***	*CG3363*	Unknown	E
20036		*Pen*	SUMO binding	E
20190	**	*Esg*	RNA Polymerase II transcription factor	E
20208		*Kdm4b (Histone demethylase 4B)*	H3-K36, H3-K9 demethylase	E

STOCK	SIGNIFICANCE[+]	GENE[§]	FUNCTION[§]	PHENOTYPE[++]
20758	**	*Hrb27C*	protein, DNA, mRNA, SUMO binding	E
20910		*Hr38*	nuclear receptor activity	E

[+] Phenotype was rated based on slight to severe change, and percentage of total flies expressing a change: no star means slight or few, one star is intermediate, two stars is severe or many.

[++] Enhancer (E), Suppressor (S)

§ Data was obtained from www.FlyBase.org

Table 5. Candidate genes.

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
14823	**	*CG10337*	unknown; unknown	S		
15317	**	*CG8187*	unknown; unknown	E		
15317	**	*CG30467*	unknown; acute-phase response	E		
15348	*	*Den1*	NEDD-8 specific protease activity; protein deneddylation	E	SENP8	Functions in the NEDD8 pathway to remove NEDD8 from the cullin subgroup (deneddylation) of ubiquitin ligases thereby reducing activity (Wu, et al., 2003; Pan, et al., 2004)
15483	*	*CG9084*	unknown; unknown	E		
15530	*	*CG8080*	NAD^+ kinase; metabolic process	S		
15565		*CG6370*	glycotransferase activity; glycosylation via asparagines	S	RPN2	Ribophorin (also helps binding of ribosome to ER) membrane protein that functions to link mannose to asparagine residues (Crimaudo, et al., 1987)
15666		*CG4259*	endopeptidase activity; proteolysis	E		

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
15714	*	*Orc6*	DNA binding; DNA replication initiation, mitosis (M phase)	E	ORC6L	Possible roles in replication and segregation of chromosomes (Prasanth, et al., 2002)
15781	**	*CG13177*	unknown; unknown	E		
15838	*	*yellow-b*	unknown; unknown	E		
15892	*	*Gapdh1*	dehydrogenase activity; glycolysis	S	GAPDH	The dehydrogenase that catalyzes the 6th step in glycolysis, with additional roles in DNA repair (Azam, et al., 2008) and cell death (Nakajima, et al., 2007)
15907	**	*snama*	protein binding; unknown	S	RBBP6	Binds to hypophosphorylated pRB (phosphorylation of pRB is a regulatory mechanism of this tumor suppressor) (Sakai, et al., 1997)
15923	*	*CG1868*	transcription repressor; unknown	E		
15936	*	*TepIV*	peptidase inhibitor; antibacterial humoral response	E		

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
15957	**	*Cp1*	endopeptidase activity; autophagic cell death, salivary gland cell death	S	CTSL1	Proteinase found in the lysosome functioning in the catabolism of proteins such as elastins (Novinec, et al., 2007), and fibronectins (Yang, et al., 2007)
16360	**	*CG3065*	zinc ion binding, nucleic acid binding; unknown	E		
16386		*CG17324*	UDP-glycosyltransferase activity; metabolic process	E		
16543	**	*Vha68-1*	ATP-ase (H+); proton transport	E	ATP6V1A	Catalytic and nucleotide binding subunit of the V1V0 ATPase proton pump, vesicle pH regulation (Nishi and Forgac, 2002)
16587	*	*CG9247*	exonuclease activity, nucleic acid binding; nucleic acid metabolic processes (nucleoside, nucleotide)	E		
16632	*	*CG5757*	thymidylate kinase; dTDP biosynthetic process	E	DTYMK	Thymidylate kinase forms dTDP from dTMP (Huang, et al., 1994)
16682	*	*CG42336*	unknown; unknown	E		
16707	*	*CG30077*	unknown; unknown	E		

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
16711	*	*CG8486*	unknown; unknown	E		
16720	*	*Pi3K21B*	kinase binding; phosphoinositide phosphorylation, amino acid phosphorylation, regulation of cell proliferation, regulation of cell size, lipid phosphorylation	E	PIK3R3	Regulatory subunit gamma of phosphatidylinositol-3-kinase which interacts with proteins such as Rb (Xia, et al., 2003) and IGF1R (Mothe, et al., 1997)
16729	**	*CG1667*	unknown; unknown	E		
16741	*	*CG9526*	unknown; unknown	S		
16975	**	*CG8389*	monocarboxylate transmembrane transporter; transmembrane transport	E		
17311	**	*CG31638*	unknown; unknown	E		
17372	*	*CG40169*	unknown; unknown	E		
17454	**	*CG30122*	mRNA binding; unknown	E	HNRNPUL1	Nuclear RNA binding protein (hnRNP family) involved in kinase cascades during adenovirus infections. Binds to a viral oncoprotein. (Gabler, et al., 1998; Blackford, et al., 2008)

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
17483	*	*CG15117*	beta-glucuronidase activity; carbohydrate metabolic process	S	GUSB	Homotetramer found in lysosome that hydrolyzes glycosaminoglycans (Storch, et al., 2003; Tomatsu, et al. 2009)
17581	**	*CG7337*	unknown; unknown	E		
17611	*	*ms(2)34 Fe*	acyl carrier; unknown	S		
17634	*	*CG8920*	nucleic acid binding; unknown	E		
17634	*	*CG13868*	unknown; unknown	E		
19807	*	*cmet*	microtubule motor activity; mitotic spindle organization, cell cycle, metaphase plate congression	S	CENPE	During cell cycle, functions as a motor protein to assist in chromosome movement (Tanudji, et al., 2004)
19821	**	*Vlc*	unknown; leg morphogenesis	E	DLGAP1	Encodes GKAP which functions in neuronal postsynapses (Takeuchi, et al., 1997)

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
19974		*Trap1*	unfolded protein binding, ATP binding; protein folding, stress response	S	TRAP1	Tumor necrosis factor receptor-associated protein 1 (aka heat shock protein 75): Works in the mitochondria to reduce apoptosis from ROS (Hua, et al, 2007); other evidence for an interaction with Rb (Chen, et al., 1996)
19987	*	*Aats-glupro*	SUMO binding; tRNA aminoacylation	S	EPRS	Protein is an aminoacyl-tRNA synthetase that aminoacylates glutamic acid and proline tRNAs (Rho, et al., 1998)
20050		*CG8920*	nucleic acid binding; unknown	S		
20054	*	*opa1-like*	GTP binding; mitochondrial fusion, pupariation	E	OPA1	Dynamin-like protein (GTPase) in the mitochondria with roles in fusion and remodeling (Ishihara, et al., 2006)
20111		*CG13101*	unknown; unknown	S		
20120	*	*GlcAT-S*	multiple transferase activities; biosynthetic processes (glycoprotein, proteoglycan)	E		

STOCK	SIGNIFICANCE[+]	GENE[§]	GENE FUNCTION; PROCESSES[§]	M2 SCREEN RESULT[++]	HUMAN HOMOLOG[§]	HOMOLOG FUNCTION
20121	*	*CG15439*	protein and zinc ion binding; unknown	E	PHF14	PHD finger protein 14; binds ions in unknown processes (Olsen, et al., 2006)
20140	**	*Vha44*	ATP-ase (H+); proton transport	E	ATP6V1C1	Subunit of the V1V0 ATPase proton pump; vesicle pH regulation (Nishi and Forgac, 2002)
20148		*MESR4*	protein and zinc ion binding; unknown	S		
20810		*CG9339*	Rab GTPase activator activity; regulation of Rab GTPase activity	E	TBC1D24 (TBC domain family, member 24)	May have involvement in membrane trafficking by way of a Rab-GAP (GTPase activating protein) domain (Ishibashi, et al., 2009).

[+] Phenotype was rated based on slight to severe change, and percentage of total flies expressing a change: no star means slight or few, one star is intermediate, two stars is severe or many.

[++] Enhancer (E), Suppressor (S)

§ Data was obtained from www.FlyBase.org

APPENDIX B. FUTURE DIRECTIONS

Table 6. Suggested RT-PCR primers for expression analysis.

***Drosophila* Gene**	**Primers**	
Den1	Forward	5' ATGTTGCCCCACGATAGG 3'
	Reverse	5' GTTGCCCGTGTTGTTGTTCCC 3'
Gapdh	Forward	5' TCGAAGTTGTCATTGATGACC 3'
	Reverse	5' ATTTGGCCGCATCGGCCGC 3'
Snama	Forward	5' TGCAGCAGAAGCGACTGGGC 3'
	Reverse	5' GCTGATTCGTTCTCAGCCGC 3'
Vha68-1	Forward	5' AGCAGTTCGCTTCATAATGG 3'
	Reverse	5' GAGTGGTACACGAGAACACGC 3'
Pi3K21B	Forward	5' GGGATCTTTGTCTGCTGAGC 3'
	Reverse	5' TCAATGTACTTGTCCTTGCGC 3'
CG30122	Forward	5' CGGCACCCGCATTGGACTGCG 3'
	Reverse	5' CAGCTGGTCAATCGCTACCC 3'
Trap1	Forward	5' CCGCTGCGTGCAATGGTGCC 3'
	Reverse	5' CTGTACGAGCGATGGGAGTG 3'
Vha44	Forward	5' TCATCAGCTTGGTCATCTCG 3'
	Reverse	5' GGGACATGGCCAAGTATCCG 3'
CG9339	Forward	5' GCATTTCAGTCTTGATAAA 3'
	Reverse	5' CCGGTCTGGTCGGTAGCAAGG 3'