

Genome-wide RNA-interference Screen for Human Host  
Factors Vital to Influenza A Virus-induced Cell Death and  
Viral Replication

By

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## ABSTRACT

Influenza virus is a globally significant infectious agent with the potential to cause catastrophic pandemic outbreaks. Present treatment of influenza infections is restricted to only four anti-viral drugs, but there are increasing global reports of anti-viral resistance in several seasonal strains and also the 2009 pandemic swine-origin influenza virus H1N1. Possible future pandemic outbreaks, emerging new strains and drug resistance underscore the need to understand this complex virus and its pathogenicity with the goal that novel targets can be uncovered for future therapeutic development.

Extensive lung tissue damage during influenza virus infection is proposed to contribute to the development of aberrant host immune responses. Strong evidence now demonstrates the significance of the cellular death pathway in promoting efficient influenza virus replication and disease progression. Viruses rely heavily on the machinery of their host for productive replication, which is also an Achilles' heel that could be targeted for treatment. In pursuit of unraveling the complex nature of influenza virus replication, I carried out a global shRNA screen to identify specific host factors and signaling pathways that are involved in influenza-induced cell death and replication. In this study I identified 138 genes required for influenza viruses to induce infected host cell death. These genes were found to be involved in Protein Kinase A, NF- $\kappa$ B and PI3K signaling cascades. These signaling pathways are well known regulators of cell death and survival, which suggests influenza viruses may carefully regulate these pathways to reach a balance that suit their requirements for efficient proliferation, eventually at the cost of the host cell. I chose five candidate genes—BAD, MxB, TNFSF12-13, TNFSF13, and USP47—that were associated with apoptosis and the major signaling pathways

determined in my network analysis to further verify the genome-wide screen as well as elucidate the role of these potentially novel host factors in influenza virus replication.

I show in my study that influenza virus-induced cytopathology and cell death are considerably inhibited in BAD knockdown cells and both virus replication and viral protein production also are dramatically reduced. I also report here that MxB depletion protected cells from virus-mediated cytopathology and resulted in significant inhibition of influenza virus replication for H1N1 and H3N2 subtypes. Additionally, I report that TNFSF12-13, TNFSF13, and USP47, similarly, are required for efficient influenza virus replication and induction of cell death. Depletion of these proteins resulted in significant inhibition of viral propagation and conferred protection of host cells to virus killing.

Overall, my study has provided a list of novel host factors that play significant roles during influenza virus infection. Further studies on these potential genes and their encoded protein products may uncover possible new targets for drug development for future therapeutic treatment. In addition to providing greater understanding of influenza virus infection, these studies will also highlight important fundamentals of cellular processes that may be broadly applicable to other fields of research.

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## **DEDICATION**

To the three people who mean the world to me:  
my parents and sister.

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# COPYRIGHT MATERIAL

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# ABBREVIATIONS

Ab	Antibody
APRIL	A proliferation-inducing ligand
ASA	Acetylsalicylic acid
ANT	Adenine nucleotide translocator
APAF-1	Apoptosis protease activating factor 1
AIF	Apoptosis-inducing factor
Ago2	Argonaut 2 protein
BAD	Bcl-2 antagonist of death
CID	Central interactive domain
Co-IP	Co-immunoprecipitation
cRNA	Complementary RNA
CTL	Cytotoxic T-lymphocyte
Cyt c	Cytochrome c
CMV	Cytomegalovirus
CPE	Cytopathologic effect
DISC	Death-inducing signaling complex
DUB	Deubiquitylation
DMEM	Dulbecco's modified Eagle's medium
EM	Electron microscopy
ECL	Enhanced chemiluminescence
eIF2a	Eukaryotic translation initiation factor 2 alpha
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain-containing protein
GED	GTPase effector domain
FRET	Fluorescence resonance energy transfer
HA	Hemagglutinin
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HFL	Human fetal liver
HIV	Human immunodeficiency virus
IKK	I $\kappa$ B kinase
IF	Immunofluorescence
IAP	Inhibitor-of-apoptosis
IFN	Interferon
INV	Inactivated vaccines
KD	Knockdown
KO	Knockout
LAV	Live-attenuated vaccines
LZ	Leucine zipper motif
MDCK	Madin Darby canine kidney
MEK	MAPK kinase/ERK kinase
mRNA	Messenger RNA
MSGSC	Michael Smith Genome Sciences Centre
miRNA	microRNA
MMP	Mitochondrial membrane permeabilization
MAPK	Mitogen-activated protein kinase
NA	Neuraminidase

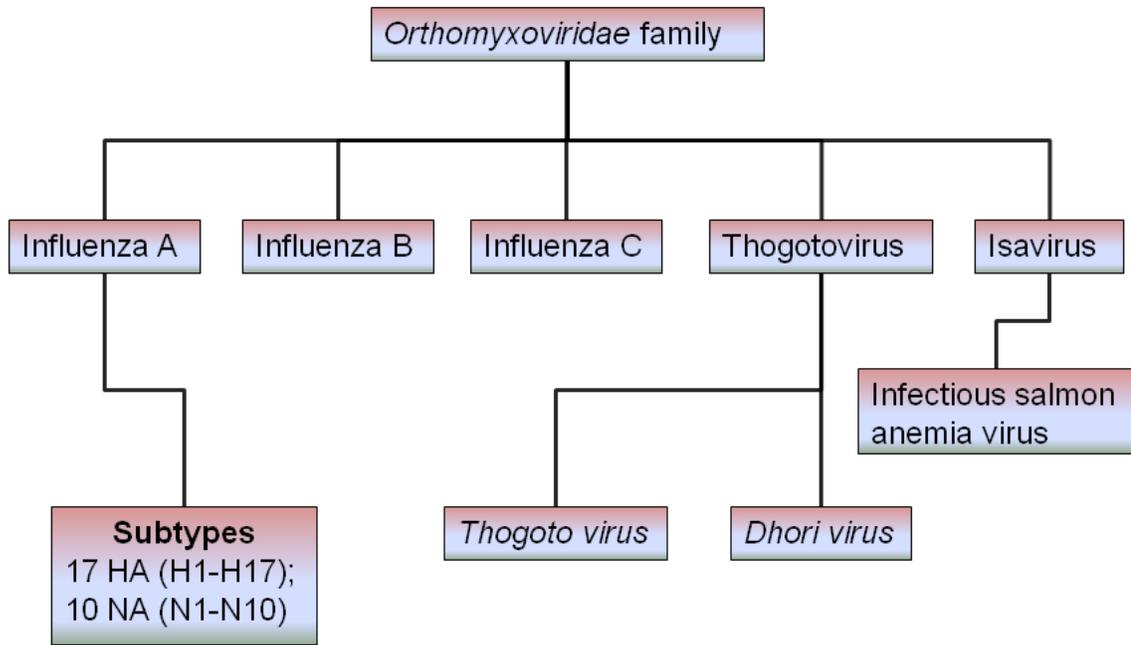
NSi	Non-targeting control (either siRNA or shRNA)
NEP/NS2	Nuclear export protein/nonstructural protein 2
NES	Nuclear export signal
NLS	Nuclear localization signal
NP	Nucleoprotein
OM	Outer membrane
PBL	Peripheral blood lymphocytes
PTPC	Permeability transition pore complex
PBS	Phosphate-buffer saline
PFU	Plaque-forming unit
PH	Pleckstrin homology domain
PABII	Poly (A)-binding protein II
PA	Polymerase acid protein
PB1	Polymerase basic 1 protein
PB2	Polymerase basic protein 2
PRD	Proline/arginine rich domain
PKR	Protein kinase R
RIG-I	Retinoic acid-inducible gene-I
RNAi	RNA interference
(v)RNP	(viral) Ribonucleoprotein
RISC	RNA-induced silencing complex
shRNA	Short hairpin RNA
SCF <sup>b-Trep</sup>	Skp1/Cull1/F-box protein b-transducin repeat-containing protein
siRNA	Small interfering RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOIV	Swine-origin influenza virus
TRAIL	TNF-related apoptosis inducing ligand
TLR	Toll-like receptor
TMD	Transmembrane domain
TRIM	Tripartite motif
TNF	Tumour necrosis factor
FDA	U.S. Food and Drug Administration
UBC	Ubiquitin c
UCH	Ubiquitin C-terminal hydrolases
Ub	Ubiquitin/Ubiquitylation
USP	Ubiquitin-specific proteases
UTR	Untranslated region
VV	Vaccinia virus
vRNA	Viral RNA
VDAC	Voltage-dependent ion channel
WNV	West Nile virus
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis

# Part I | Introduction

## 1.1 A Perspective on Influenza Viruses

Human infection by influenza was not extensively studied until 1933 when the first human influenza viruses were initially isolated (293). Since then extraordinary progress has been made in elucidating the components of the virus and in understanding the medical consequences of an influenza virus infection. The impact of many of these discoveries extended far beyond the influenza virus field and have sparked new developments in disciplines such as immunology and protein structure, as well as furthering our basic understanding of viruses in general. For example, the discovery of interferon was as a result of studying infection with heat-inactivated influenza virus (111).

Influenza viruses belong to the family Orthomyxoviridae, which are enveloped, single-stranded, negative-sense, 8-segmented RNA viruses (Figure 1). Of the 3 known serotypes of influenza (A, B, C), only types A and B cause frequent and occasionally severe diseases in humans. Unlike influenza B, which has only 1 type, influenza A viruses are categorized into multiple subtypes based on a combination of the 17 known hemagglutinin (HA) and 10 neuraminidase (NA) genes that code for these viral envelope or surface proteins (321, 329). Of these 17 HA subtypes, 6 have been found in human infections (H1, H2, H3, H5, H7, and H9). So far, only 3 subtypes of HA (H1, H2, H3) and 2 subtypes of NA (N1, N2) have caused pandemics in humans. Given the significance of influenza A virus in its potential to develop pandemics, and its use in my research, this group of viruses will be the main focus hereafter.



**Figure 1. Classification of Orthomyxoviridae family members.**

The intensity of influenza virus research has increased over the years as a result of heightened concern of a future pandemic threat. Presently, many advanced techniques have emerged that allow for greater progression of this area of research. For example, the advent of technologies such as reverse genetics (reviewed in (225)), which allows the artificial generation of influenza viruses, had helped decipher the certain viral factors that control interspecies transmission and pathogenicity (355). Reverse genetics had also led to the resurrection of the 1918 pandemic influenza virus from RNA fragments, which permitted the study of its pathogenicity in an animal model (325).

Despite recent advances, much remains to be discovered about the molecular determinants of interspecies transmission and pathogenicity, the complex interaction between virus and the host cell, and the epidemiology and immunology of influenza viruses. With a continual threat of another pandemic influenza outbreak, a detailed molecular understanding of virus-host interactions is sorely needed in order to know how best to disable the virus. It will be important to learn more about the cell's signaling pathways and how they are modulated during influenza virus replication. Further research efforts will open the door to the development of more reliable diagnostic tests, safe and broadly effective antivirals, and more effective vaccines that allow for worldwide coverage. The availability of such new armaments will be imperative during a pandemic outbreak.

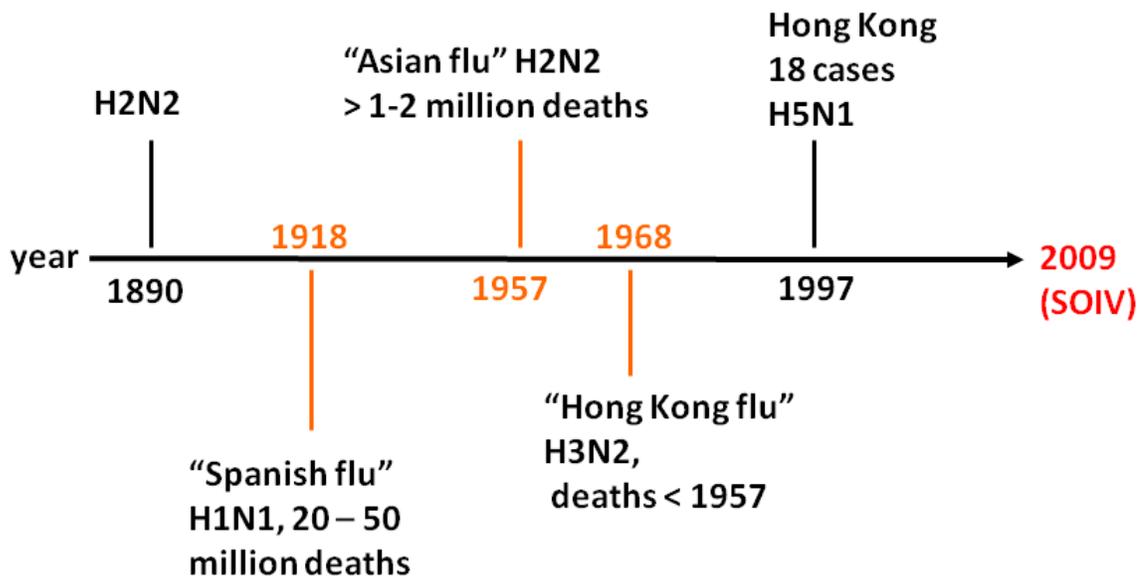
## 1.2 Notable Influenza Outbreaks

### 1.2.1 Historical Pandemics of the 1900s

Before the emergence of the 2009 swine-origin influenza virus pandemic strain (H1N1) (SOIV), history had already witnessed the outbreak of 3 major influenza pandemics during the twentieth century: in 1918 (the Spanish flu), 1957 (the Asian flu), and 1968 (the Hong Kong flu) (Figure 2). These incidents have been widely studied with the help of available epidemiologic records and any preserved, archived viral isolates or infected tissue specimens.

The first of these pandemics coincided with World War I and has infamously become known as the 1918 Spanish flu. The 1918 pandemic infected a record estimate of one-third of the world's population (approximately 500 million people), with an estimated mortality of 50 to 56 million deaths (323). Although the subsequent 1957 and 1968 pandemics (now shown to have originated in Asia) resulted in lower morbidity and mortality relative to the 1918 pandemic, there was still recorded an estimated 1-2 million deaths worldwide (100). The pattern of reoccurrence of subsequent pandemics gave rise to the concept that similar, future influenza pandemics could and would occur (360).

Pandemic influenza viruses are thought to arise when there is frequent human contact with certain animal species that can be infected with their own specific influenza viruses and when these viruses develop the ability to jump the species barrier to infect humans. This interspecies transmission is believed possible either in the presence of certain gene mutations or the acquisition of genes from human-adapted strains that permit the binding of such animal influenza viruses to surface proteins on human respiratory epithelial cell receptors (77).



**Figure 2. Historical pandemic influenza A virus outbreaks.** Timeline of significant influenza virus pandemic outbreaks. Important pandemic outbreaks with major death tolls are indicated in orange. The most recent pandemic outbreak is indicated in red. The year the outbreak occurred are shown near the central line.

Birds are the natural reservoir for influenza A viruses, although other animals such as pigs and horses have also acquired and maintained their own separate genetic lineages of influenza (71, 82). The origin of the 1918 (H1N1) pandemic influenza virus has become more controversial recently, and there is a debate over whether it was derived from a human influenza strain existing before 1918 (210) or directly from a purely avian influenza strain from around 1918 (69) or whether it was generated by the reassortment or recombination between human and avian influenza viruses co-circulating around that time (132). The uncertainty about the 1918 virus origin stems from the fact that there are very few viral isolates available for analysis from before 1918; therefore, the complete diversity of avian influenza viruses circulating then cannot be known.

On the other hand, greater understanding has been achieved in regards to the origins of the 1957 and 1968 influenza pandemics. The 1957 pandemic was caused by an influenza H2N2 reassortant whose HA, NA, and polymerase basic protein 1 (PB1) gene segments originated from avian influenza strains and the remaining gene segments from an H1N1 human pandemic influenza virus subtype that had been circulating since its emergence in 1918 (122). Over time this H2N2 strain was replaced by an H1N1 subtype, which itself was replaced by another reassortant, the 1968 H3N2 pandemic subtype. The 1968 H3N2 virus had acquired its gene segments HA and PB1 from an avian influenza strain and a circulating H2N2 virus (122). Since 1977, this H3N2 reassortant virus has been co-circulating with an H1N1 strain similar to the 1918 H1N1 pandemic virus that was accidentally released from a laboratory (124). These 2 viruses now make up the current seasonal influenza A virus subtypes for more than 30 years. Analyses of the viruses that caused the 1957 and 1968 influenza pandemics proved that zoonotic

transmissions of influenza viruses (ie, from animals to human) with gene reassortment were capable of generating antigenically new influenza strains that are novel to human immunity and with significant effects on the public health.

### **1.2.2 H5N1**

In contrast to seasonal epidemics, pandemics arise when antigenically novel viruses emerge and are readily transmissible within the naïve human population. Such events are rare and occur after zoonotic transmission of viruses through recombination events between established human and avian strains or possibly through direct adaptation of avian strains for efficient human to human transmission (32, 86). Concerns over an avian influenza pandemic have risen since 1997 with the recognition of H5N1 as a cause of fulminant disease in humans (139, 242, 348). Pathologically fatal H5N1 causes diffuse alveolar damage and progression to multiple organ dysfunction with a case fatality rate estimated at more than 50% (29, 224, 242, 348). Although H5N1 disease is severe, transmission rates have been low, and viral adaptation to efficient human to human transmission has not yet occurred (333). Understanding viral and host barriers that prevent transmission may be critical in establishing rational control measures as well as predicting and stratifying risk for individual strains of influenza.

### **1.2.3 Swine-Origin Influenza Virus (H1N1) 2009 Pandemic**

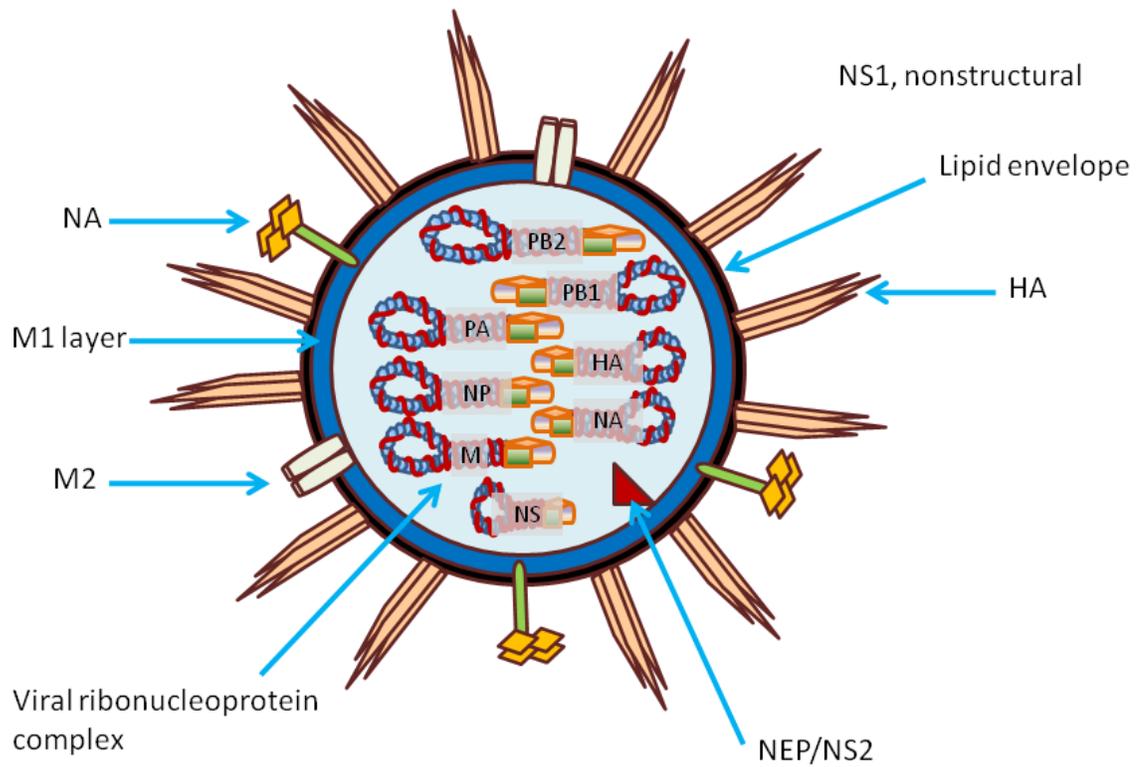
April 2009 saw the emergence of the first influenza pandemic virus in more than 40 years. It was predicted that the next influenza A virus pandemic would be of avian origin and arise from Asia; however, the zoonotic origin of the novel influenza A H1N1 2009 virus (SOIV) was swine rather than avian and the geographic origin was in the Americas

rather than Southeast Asia (274, 373), specifically the first outbreak occurred in Mexico with a total of 854 cases of pneumonia and 59 deaths (236, 317). Evolutionary studies of the SOIV genomics indicated that it had been circulating in pigs for several years before its transmission to humans (317). SOIV was initially susceptible to zanamavir and oseltamavir but determined to be resistant to amantidine and rimantadine, a clear distinction from circulating seasonal H1N1 which was widely resistant to oseltamivir (236).

### **1.3 Influenza Virus Structure**

Influenza A viruses have a complex structure and possess a lipid membrane derived from the host cell (Figure 3). The lipid envelope harbors the HA, the NA, and the M2 proteins that project from the surface of the virus. The matrix protein (M1) lies just beneath the envelope. The nuclear export protein/nonstructural protein 2 (NEP/NS2) protein is also part of the virion (114). Influenza A virus particles are characterized by distinctive spikes that are observable via electron microscopy (EM) (74). HA and NA proteins together make up the spikes with approximate ratio of four HA to one NA. Morphologically, influenza viruses are pleomorphic (74). The spherical particles have a diameter of ~ 100 nm, but filamentous particles with elongated viral structures (> 300 nm) have also been observed (74).

The internal structures of influenza remains for the large part unknown. However, the use of EM has allowed for the visualization of the underlying M1 layer in damaged particles; this has revealed a helical superstructure (270). The ribonucleoprotein (RNP) complexes consist of a strand that is folded back on itself to form a double-helical arrangement (230), and mounting evidence supports the proposal that individual virus



**Figure 3. Influenza virion structure.** Schematic diagram of a cross-sectional cut-out showing structural components of an influenza virion. NS1 nonstructural protein is not packaged with the virion, and is therefore indicated in the background.

particles preferentially package only eight essential RNA segments (36, 227, 229).

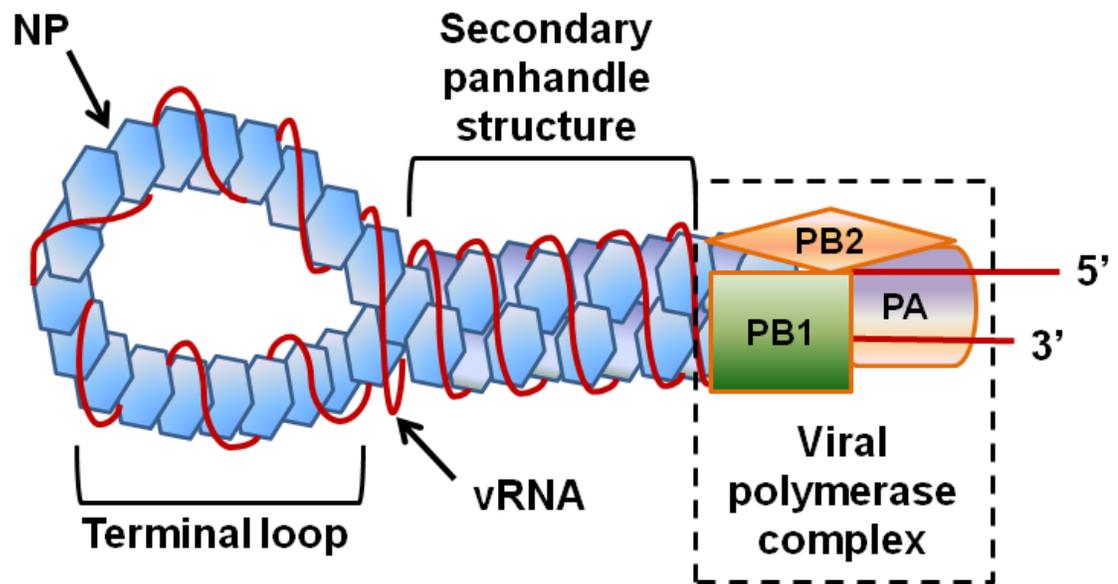
## **1.4 Viral Proteins**

### **1.4.1 PB1, PB2, and PA**

Influenza virus-encoded RNA-dependent RNA polymerase is a complex of three proteins: PB1, PB2, and PA (228) (Figure 4). The PB1 protein catalyzes the sequential addition of nucleotides during RNA chain elongation. This protein is also responsible for binding to the terminal ends of both vRNA and complementary RNA (cRNA) for initiation of transcription and replication (228). The interaction with the 3' end of the vRNA activates the endonuclease activity of PB1, which generates the capped primer required for mRNA synthesis (228). The PB2 protein plays a critical role in the initiation of transcription because it is responsible for binding the cap on host pre-mRNA molecules. No specific function has been ascribed to the PA protein, but mutations affecting both transcription and replication have been described, indicating its role in both processes (106).

### **1.4.2 The M1 Protein**

M1 is the most abundant virion protein and lies just beneath the lipid envelope. M1 associates with the cytoplasmic tails of the glycoproteins and with the RNPs, thereby forming a bridge between the inner core components and the membrane proteins (275). The M1 protein consists of two globular helical domains that are linked by a protease-sensitive region (5). M1 is proposed to play a vital role in assembly by recruiting the viral components to the site of assembly at the plasma membrane. Additionally, M1, along with the nuclear export protein, NEP/NS2, are involved in directing the nuclear



**Figure 4. Schematic depiction of viral RNP structure.** Influenza virus genome consists of ribonucleoprotein complexes. RNP complexes are comprised of single-stranded, negative-sense RNA wrapped around NP protein in a pinloop structure with the viral polymerases associated at one end.

export of RNPs (45). M1 has also been shown to be necessary and sufficient for the formation of virus-like particles, providing evidence for its essential role in the budding process (220).

### **1.4.3 M2 Protein**

The M2 protein of influenza A viruses is a tetrameric integral membrane protein (268). M2 has been shown to possess ion channel activity. Its major role is to conduct protons from the acidified endosomes into the interior of the virus. The low pH will dissociate the RNP complex from the rest of the viral components (268), thus completing the uncoating process. The M2 protein ion channel is acid gated (not voltage gated) and highly selective for H<sup>+</sup> ions (268).

### **1.4.4 Hemagglutinin**

The major functions of HA are receptor-binding and fusion activities. The HA is a trimeric rod-shaped molecule with the carboxy terminus inserted into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface. Full activity of the HA protein requires cleavage of the HA0 precursor into HA1 and HA2 subunits (77, 299).

The receptor-binding pocket of an avian HA preferentially accommodates the 2,3-linked sialic acid, whereas human HAs preferentially accommodate the 2,6-linked sialic acid (299). Another major function of the HA is acidic pH-triggered fusion required for the uncoating process (77, 299). The acidic pH induces a conformational change in HA that aligns the fusion peptide in an anti-parallel fashion to the membrane anchor of the HA2 (299); this brings the endosomal membrane into juxtaposition with the viral

membrane, leading to the formation of a fusion pore through which the RNP can enter the cytoplasm.

The influenza virus HA is a major determinant recognized by the adaptive immune system of the host. Following infection and replication, a vigorous immune response is induced that usually results in the formation of neutralizing antibodies (298). The amino acids undergoing change are almost exclusively on the HA1 (298). Many of these changes get fixed (accumulate over time), defining the antigenic drift (mutations) of influenza viruses.

### **1.4.5 Neuraminidase**

The NA is a type II integral membrane protein and the second major glycoprotein of influenza A viruses (77). The 9 subtypes of the A virus NA fall into two major groups (N1, N4, N5, N8 and N2, N3, N6, N7, N9) based on sequence comparisons. However, the newest N10, isolated from bats, showed a highly divergent sequence from both influenza A and B NA genes, suggesting an older ancestral relationship to present influenza viruses (321). The influenza A virus NAs have a highly conserved short cytoplasmic tail and a hydrophobic transmembrane region. The transmembrane domain provides the anchor for the stalk and the head domains (77, 321). The viral NA removes the sialic/neuraminic acid receptor from the surface of the cell and from the virus particles to prevent recognition by the HA of the virus (77, 299). The NA thus has a role in releasing the virus from the infected cell and in cleansing the environment (e.g., mucus and cell surfaces) of sialic acid receptors to allow for virus spread (77). Like the HA, NA molecules are antigenic and variants are selected in nature. Antibodies directed against the NA are usually not neutralizing (77).

### 1.4.6 NS1

NS1 is a nuclear, dimeric protein with dsRNA-binding activity (76, 145). The NS1 RNA-binding domain forms a symmetric homodimer with a six-helical fold; epitopes on the second helix of each monomer constitute an arginine-rich binding site for nucleic acid (89). The remaining portion of NS1 has been termed the effector domain and includes binding sites for poly (A)-binding protein II (PABII) and the 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF) (89). NS1 has also been reported to interact with several other host factors, including the eukaryotic translation initiation factor 4GI, poly (A)-binding protein I, staufer, NS1-I and NS1-BP (89, 145).

Studies have found that wild-type (WT) influenza virus induces far less interferon (IFN) than does delNS1 virus (89). Expression of NS1 alone inhibits the activation of the IFN- $\beta$  promoter in response to infection with a heterologous virus or even delNS1 virus (341). The precise mechanism for how NS1 suppresses IFN synthesis remains unclear, but many studies strongly suggest that the IFN antagonist properties of NS1 depend on its ability to bind dsRNA (145, 209, 341). Intracellular dsRNA serves as the signal for virus infection and triggers IFN synthesis; therefore, a simplified model for how NS1 antagonizes IFN induction would be that it sequesters dsRNA and thus prevents the downstream signaling required to activate IRF-3, NF- $\kappa$ B, and AP-1 (89, 209, 341). On the other hand, other studies provide further evidence to suggest that the ability of NS1 to inhibit IFN synthesis is not solely dependent on dsRNA-binding and that additional mechanisms are involved (76, 89, 163, 195, 209).

### **1.4.7 PB1-F2**

PB1-F2 protein is encoded by the alternative ORF in the PB1 gene of influenza A viruses (41). This protein has a number of unique features: (1) not all influenza A viruses encode for this protein, (2) variable expression in individual infected cells, (3) rapid proteasome-dependent degradation, (4) mitochondrial localization, and (5) apoptotic or pro-apoptotic properties (41). PB1-F2 affects apoptosis and may contribute to the pathogenicity and lethality of influenza A viruses (41). Sequence analysis showed that, in addition to the strains with an ORF for full-length PB1-F2, there are some with an ORF for different truncated forms of PB1-F2 (41).

### **1.4.8 NEP/NS2**

Viral nuclear export protein (NEP), previously known as nonstructural protein 2 (NS2), is responsible for recruiting the export machinery and directing export of the RNP complex (17). NEP/NS2 has been found to interact with the export receptor, Crm1, and several nucleoporins. NEP/NS2 also associates with M1 (17). A methionine/leucine-rich nuclear export signal (NES), identified in the N-terminus of NEP/NS2, is shown to be critical for RNP export (17).

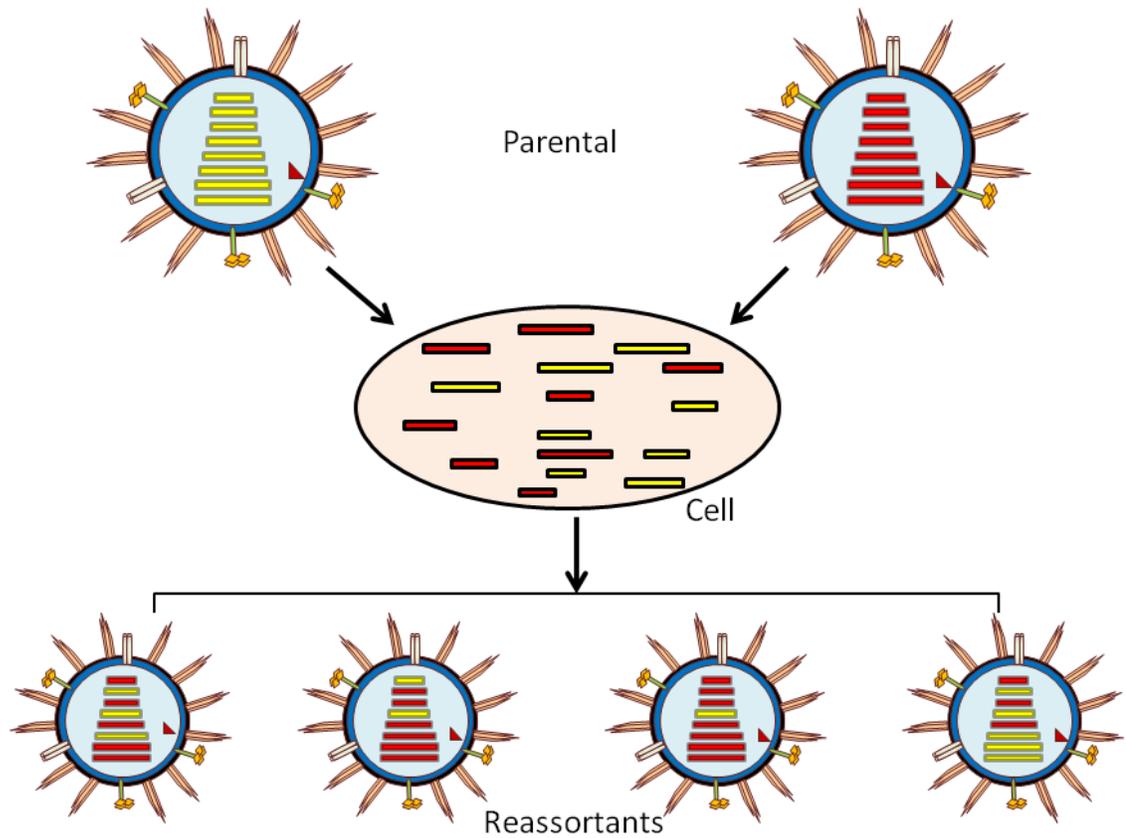
## **1.5 Influenza Virus Genome Structure**

Influenza virus genome segments exist only as a complex with ribonucleoproteins and never as naked RNA. The virus genome consists of eight negative-sense, single-stranded RNA segments that exist as individual complexes called the RNP complex (228) (Figure 4). The RNP complex consists of the viral RNA (vRNA) segments, PB1, polymerase basic 2 protein (PB2), polymerase acid protein (PA), and nucleoprotein (NP)

(228). The NP coats the RNA, and the three polymerase proteins bind to the partially complementary ends of the vRNA (228). Genes are flanked by non-coding regions, with high sequence conservation at the extreme 5' and 3' ends (228).

The eight segments of influenza A viruses are numbered in the order of decreasing length. Segments 1, 3, 4, 5, 6 encode just one protein per segment: the PB2, PA, HA, NP and NA proteins, respectively (18). Segment 2 encodes the polymerase subunit PB1; in most influenza A viruses, this segment also codes for the accessory protein PB1-F2, a small, 87-amino acid protein with pro-apoptotic activity (146), from an alternate reading frame near the 5' end of the PB1 gene. (146). Segments 7 and 8 encode for the M1 and NS1 proteins, respectively. The remaining influenza virus proteins, M2 and NEP/NS2, arise from splice variants of the M and NS mRNA, respectively (151).

A significant feature for having a segmented genome is antigenic shift, in which reassortment can occur in cells co-infected with different human and animal viruses (or different virus subtypes) that give rise to progeny virions harbouring a heterogeneous genome from both parental subtypes (Figure 5). These progeny virions may encode completely novel antigenic proteins to which the human population has no pre-existing immunity. Pandemic influenza arises when antigenic shift generates a virus to which humans are susceptible but immunologically naïve. Antigenic shift is presently the more well accepted mechanism to give rise to the influenza A (H1N1) virus that was the causative agent of the 1918–1919 “Spanish flu,” and is certainly the mechanism that gave rise to the recent SOIV (H1N1) pandemic of 2009 (131, 324). Characterization of the reconstructed 1918 influenza virus revealed that its extreme virulence was due to its unique constellation of genes, especially the HA, NA, and PB1 genes being significant



**Figure 5. Production of reassortants through assortment.** Schematic diagram illustrating co-infection of different influenza virus strains that result in the production of progeny virions (reassortants) with mixed genomic segments that originated from both parents. This process is called assortment.

factors that contributed to its high pathogenicity (243). It is now generally accepted that the global spread of the pandemic was almost certainly enabled by the acquisition of antigenically novel surface proteins, to which much of the world's population was immunologically naïve (323).

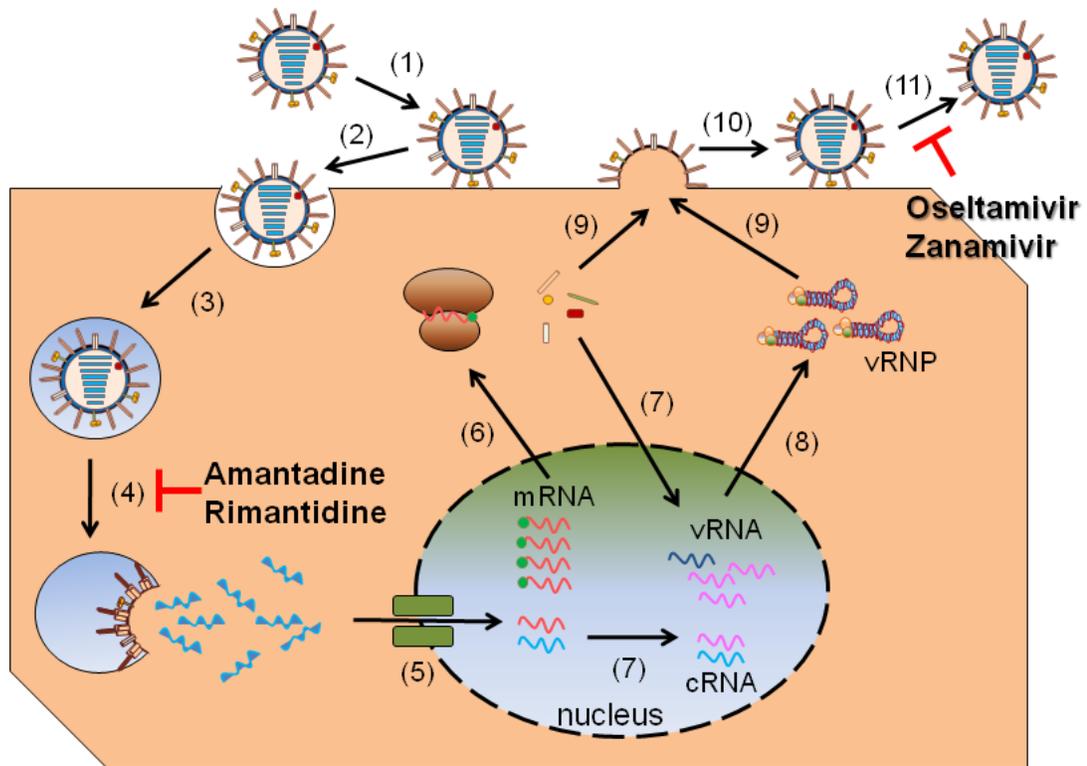
## **1.6 Stages of Influenza Virus Replication**

### **1.6.1 Influenza Virus Attachment**

Influenza viruses bind to neuraminic acids (sialic acids) on the surface of cells to initiate infection and replication (299) (Figure 6). Human influenza viruses preferentially bind to N-acetylneuraminic acid attached to the penultimate galactose sugar by an  $\alpha 2,6$  linkage (SA $\alpha 2,6$ Gal), whereas avian influenza viruses mostly bind to sialic acid with an  $\alpha 2,3$  linkage (299). Human tracheal epithelial cells contain mostly SA $\alpha 2,6$ Gal, while the gut epithelium from ducks possesses mostly SA $\alpha 2,3$ Gal sugar moieties (299). However, this viral specificity is not absolute; avian and human cells can contain both neuraminic acid linkages ( $\alpha 2,3$  and  $\alpha 2,6$ ) (193). Ciliated cells in the human airway epithelium are known to be infected with avian influenza viruses because these cells have sialylated proteins with  $\alpha 2,3$  linkages (193). Moreover, adaptive mutations in the receptor-binding site of the viral HA will allow for interspecies transmission and infection.

### **1.6.2 Entry, Fusion, and Uncoating**

There are four cellular internalization mechanisms: (a) clathrin-coated pits; (b) caveolae; (c) non-clathrin, non-caveolae pathways, and (d) macropinocytosis (150). Clathrin-mediated endocytosis has been the accepted model for influenza virus entry (150). However, a non-clathrin, non-caveolae-mediated internalization mechanism has



**Figure 6. Influenza virus lifecycle.** (1) Virus attachment to sialic acid specific moieties. (2) Virus enters via receptor-mediated endocytosis. (3 and 4) Fusion of viral and endosome membrane to release viral RNP genome to cytosol. (5) viral RNP gets transported through nuclear pores into the nucleus. (6) Transcription of viral mRNA and protein synthesis occurs. (7) Specific viral proteins are imported back into the nucleus to initiate viral genomic RNA production. (8) Progeny vRNP are exported out of the nucleus. (9) Viral assembly and genomic packaging occurs at cell membrane. (10 and 11) Immature progeny virions get released by neuraminidase activity. Anti-viral drug blockage of specific stages in the viral lifecycle is indicated in red.

also been described for influenza viruses (150). The latter pathway depends on low pH and trafficking to late endosomes because it requires protein kinase C, Rab5, and Rab7 functions (150).

Influenza viruses require low pH to fuse with endosomal membranes (Figure 6). The low pH of the endosome activates fusion of the viral membrane with that of the endosome. Fusion occurs in three general steps: (1) the HA0 precursor is first cleaved into HA1 and HA2 subunits; (2) the low pH environment in the endosome induces a conformational change in the HA subunits to expose the fusion peptide at the N-terminus of HA2; (3) the transmembrane domain of the HA2 (in the viral membrane) and the fusion peptide (inserted into the host endosomal membrane) are in juxtaposition in the low pH-induced HA structure (77, 299). The concerted structural change of several hemagglutinin molecules then opens up a pore that releases the vRNP into the cytoplasm of the cell. Effective uncoating also depends on the M2 protein ion channel activity, which allows the influx of H<sup>+</sup> ions from the endosome into the virus particle; this disrupts protein-protein interactions and releases the RNP from the M1 protein matrix, thus completing the uncoating process (268). The time frame for the uncoating process revealed that the majority of virus particles showed penetration occurs ~ 25 minutes after adsorption, then ~ 10 minutes later (~34 minutes after adsorption), RNP complexes are found in the nucleus (189). RNP molecules are taken up through active translocation across the nuclear membrane involving the nucleocytoplasmic trafficking machinery of the host cell (189).

## 1.6.2 Influenza Virus Transcription

One of the characteristics of the influenza virus life cycle that is unusual for an RNA virus is its dependence on nuclear functions (Figure 6). All influenza viral RNA synthesis occurs in the nucleus. The trafficking of RNPs rely on an active cellular nuclear import machinery (238, 347). All proteins in the viral RNP complex possess nuclear localization signals (NLSs) that mediate their interaction with the nuclear import machinery (17). However, the signals on NP have been shown to be both sufficient and necessary for the import of vRNA (17, 44).

Once in the nucleus, the incoming negative-sense vRNA is transcribed into messenger RNA (mRNA) by a primer-dependent mechanism. These mRNA products are incomplete copies of the vRNA template. Viral mRNAs are capped and polyadenylated, unlike vRNA. Replication occurs via a two-step process. First, a full-length, positive-sense copy of the vRNA is made that is referred to as cRNA. Second, cRNA is used as a template to produce more vRNA. All of these reactions, ~~vRNA~~, vRNA→cRNA, and cRNA→vRNA are catalyzed by the same viral polymerase complex (PB1, PB2, and PA).

Influenza virus mRNA synthesis requires a 5' capped primer, which it steals from host pre-mRNA transcripts to initiate its own mRNA synthesis (207). This process is known as cap-snatching and involves the cap-binding function of the PB2 protein and endonuclease function of the PB1 protein (207). The initiation of transcription commences with binding of the 5' end of the vRNA to the PB1 subunit (207). This induces an allosteric change in the polymerase that allows the PB2 protein to recognize and bind the cap structure on host pre-mRNAs (207), leading to cleavage of the bound

pre-mRNA 5' cap. RNA chain elongation is catalyzed by the polymerase function of PB1 and continues until a stretch of uridine residues signals for polyadenylation (207); this occurs before the 5' end of the vRNA.

Unlike host cells, which use a specific poly(A) polymerase for generating the poly(A) tail on mRNA transcripts, polyadenylation of influenza virus mRNAs is catalyzed by the same polymerase that is used for transcription. This activity is dependent on an uninterrupted stretch of five to seven "U" residues and the adjacent double-stranded region of the vRNA promoter (207). The current model proposes that the 5' end of the vRNA remains bound to the polymerase during elongation, while the template is threaded through in a 3' to 5' direction (207). When the polymerase nears the 5' end, it is blocked by steric hindrance and, consequently, it stutters on the preceding stretch of uridines, which it repeatedly copies to produce a poly(A) tail. In support of this model, mutations introduced into the end of the vRNA that prevent or weaken polymerase binding have been shown to also inhibit polyadenylation (207). The polyadenylation signal is vital for proper nuclear export of the viral mRNA (207).

All members of the Orthomyxovirus family extend the coding capacity of their genomes by producing two proteins from one gene via an alternative splicing mechanism. Studies have demonstrated that splicing can occur in the absence of any viral proteins, which suggest the virus is using the cellular splicing machinery (207).

### **1.6.3 The Switch from Transcription to Replication**

The vRNA serves as a template for both mRNA and cRNA synthesis, and yet the means of initiation and termination for the generation of these two molecules are quite different (Figure 6). In contrast to mRNA synthesis, initiation of cRNA synthesis occurs

without a capped primer. cRNA molecules are full-length complementary copies of vRNA, and newly synthesized vRNAs are encapsidated with NP (207). It has been proposed that NP encapsidation controls the switch between mRNA and cRNA synthesis (207). In support of this hypothesis are observations that replication depends on de novo protein synthesis, and that free NP has been shown to be required for production of full-length cRNA (207). A new model has recently been proposed that disputes the existence of a switch, instead suggesting a stabilization role for NP and the polymerase (207). In contrast to earlier reports, this study claims that the incoming polymerase is able to synthesize both mRNA and cRNA, but newly synthesized cRNAs get degraded. cRNA degradation is only inhibited when there is a sufficient pool of polymerase and NP to encapsidate the cRNA and protect it; therefore at early times post infection there is a bias toward mRNA accumulation (207). Thus, exactly how the viral polymerases switch between transcription to replication is still under debate and the molecular details remain to be elucidated.

In the second stage of replication, the positive-sense cRNA serves as a template for the synthesis of negative-sense genomic vRNA (Figure 6). As with cRNA synthesis, this reaction also occurs via a primer-independent mechanism and generates full-length products (207).

#### **1.6.4 Influenza Virus Nuclear Export of Ribonucleoproteins**

The current model for RNP nuclear export involves the formation of an RNP-M1-NEP/NS2 complex, which is formed in the nucleus (17). Present understanding of this process indicates that M1 associates with RNPs in the nucleus and may actually promote the formation of RNP complexes (17). M1 makes contact with both the vRNA and NP

(17). Significant evidence has shown that nuclear import of M1 is required for subsequent export of RNP complexes (17).

NEP/NS2 is responsible for recruiting the export machinery and directing export of the RNP complex (17). NEP/NS2 has been found to interact with the export receptor, Crm1, and several nucleoporins. NEP/NS2 also associates with M1 (17). A methionine/leucine-rich nuclear export signal located in the N-terminus of NEP/NS2 is critical for the protein's ability to export vRNP from the nucleus (17).

### **1.6.5 Influenza Virus Assembly and Release**

Correct assembly and packaging of a full complement of RNA genome segments is a requirement for a fully infectious virion (Figure 6). The precise mechanism for packaging the eight viral RNA segments is not well understood, but two different models have been proposed. The first model, the random incorporation model, assumes that a common structural feature is present on all vRNAs (vRNPs), which enables random incorporation of the segments into budding virions. In support of this model, some studies have shown evidence that virions may possess more than eight vRNPs, which would assure the presence of a full complement of eight vRNPs in a significant percentage of virus particles (7, 64).

In contrast, the second model, the selective incorporation model, suggests that each vRNA segment acts independently, allowing each segment to be packaged selectively. This model suggests that each vRNA segment contains a unique "packaging signal", which permits every virion to possess a full complement of the eight vRNP segments. The packaging to specific segments is hypothesized to occur via specific RNA-RNA or protein-RNA interactions. There is increasing evidence to support the existence of a

packaging signal within the coding regions at both the 5' and 3' ends of the genomic RNA (73). For example, the coding regions of the NA (74), HA (333), NS (73), PB2, PB1, and PA (188) segments have all been demonstrated to increase incorporation within assembling virions. Segment-specific packaging is hypothesized to occur via specific RNA-RNA or protein-RNA interactions (228). This model is also supported by data showing specific interference of deleted RNA segments with packaging of the corresponding WT RNA segment but not with any other genome segment (228).

Influenza viruses assemble and bud from the apical plasma membrane of polarized cells (275) (e.g., lung epithelial cells of the infected host). Individual viral envelope proteins are seen to accumulate at the same polar surface where virus budding occurs, suggesting that they determine the maturation site. Neither HA nor NA are in fact essential for virus budding (220). The only viral product shown to be absolutely required for assembly of virus particles is the matrix protein, M1 (220).

HA, NA, and M2 are all directed to the virus assembly site on the apical plasma membrane via their apical sorting signals (275). The signals for HA and NA have been described to reside in their transmembrane domains (TMD), while the sorting signal for M2 has not yet been characterized (275). The TMD of HA and NA also contain the determinants for association with lipid rafts (220, 275). Lipid rafts are non-ionic, detergent-resistant lipid microdomains within the plasma membrane that are rich in sphingolipids and cholesterol. Examination of the lipid content of purified virus particles indicates that influenza virus buds preferentially from these domains (275). Raft association of HA has been shown to be essential for efficient virus replication (275). This is believed to be due to a requirement for concentrated “patches” of HA at the

plasma membrane, which governs the level of HA incorporation into budding particles. A similar explanation holds for raft association of NA because an optimal amount of NA must be incorporated to allow for efficient virus release. In contrast to HA and NA, the majority of M2 protein is excluded from lipid rafts, which may reflect its low abundance in virus particles (275). In comparison to the integral membrane proteins, relatively little is known about how the remaining viral components reach the assembly site.

Initiation of bud formation requires outward curvature of the plasma membrane, which is proposed to be stimulated by the accumulation of M1 at the inner leaflet of the lipid bilayer (220). The budding process is completed when the membranes fuse at the base of the bud and the enveloped virus particle is released following fission from the cell membrane. M1 is the driving force behind this process because budding cannot occur in the absence of M1. It is reported that M1 alone can induce the formation of virus-like particles (220). The extent to which the membrane is extruded before pinching off occurs affects the size and shape of the virus particle. Generally, influenza virus particles are either spherical or filamentous, and this characteristic morphology is genetically linked to the M segment (63). Host factors such as polarization and an intact actin cytoskeleton also play a critical role in determining the shape of filamentous particles.

Influenza virus particles have to be actively released after the viral envelope has separated from the cell membrane during the completion of budding. This is because the HA anchors the virus to the cell by binding to sialic acid-containing receptors on the cell surface (299). The enzymatic activity of the NA protein is required to remove the sialic acid, and thereby releases the virus from its host cell (77). NA activity is also required to

remove sialic acid from the carbohydrates present on the viral glycoproteins themselves so the individual virus particles do not aggregate.

## 1.7 Reverse Genetics

Since influenza viruses are negative-sense RNA viruses, introduction of the genomic RNAs into cells does not result in the formation of infectious virus (as it does in the case of positive-strand RNA viruses). In 1999, a decade after the initial influenza reverse genetics system was described, two research groups reported the generation of influenza viruses entirely from cloned cDNAs (225). In one reported system, cDNA from each of the eight genome segments was cloned in negative orientation between a truncated human RNA polymerase I promoter and the hepatitis delta virus ribozyme (225). Transfection of the eight vRNA-encoding plasmids into Vero cells along with four polII-driven plasmids expressing NP and the polymerase complex (PB1, PB2, PA) resulted in recovery of infectious virus (225). Improvements to this system now include the transfection of co-cultured 293T cells (human cell line necessary due to the human RNA polymerase I promoter) and Madin Darby Canine Kidney (MDCK) cells, which support high levels of virus replication (225).

Further improvements to these systems were reported in which only eight plasmids were required. The plasmids contained cDNAs of genomic segments cloned in negative orientation with a human RNA polymerase I promoter at the 5' end and the mouse RNA polymerase I terminator at the 3' end (225). The cellular RNA polymerase I was responsible for the copying of the cDNA into vRNA (225). Downstream of the RNA polymerase I terminator was a cytomegalovirus (CMV) immediate-early promoter (225). A polyadenylation sequence was inserted at the other end, giving rise to a polyII-driven

mRNA transcript from the opposite DNA strand (225). Expressed viral proteins and vRNAs then assembled in the transfected cells and resulted in the formation of infectious virus derived entirely from only eight plasmids. More recently, a single plasmid containing the cDNAs of all eight RNAs resulted in the generation of infectious virus when transfected into cells (225). These proteins allowed rescue of fully infectious virus with full-length vRNA segments (also transcribed from the plasmid).

Influenza viruses expressing foreign genes have also been generated, demonstrating the use of influenza virus as a vector to deliver foreign antigens to the immune system. Numerous approaches have been successful for the expression of foreign antigens by influenza viruses. These include: (a) replacement of the antigenic domains of either the influenza HA or NA glycoproteins with epitopes from foreign proteins; (b) modification of existing viral genomic segments to express influenza viral proteins fused to foreign proteins; (c) replacement of ectodomains of surface glycoproteins with those of foreign glycoproteins; (d) preparation of viruses with foreign antigens encoded by a ninth RNA segment.

Advantages of influenza viruses over other viruses for the expression of foreign proteins include the fact that influenza virus is extremely safe as a non-integrating, non-oncogenic virus. Infection with influenza viruses also elicits a strong and long-lasting immune response (298), and thus recombinant influenza viruses may be useful vaccine vectors in the future. The limitations related to the use of influenza viruses for expression of foreign antigens include the limited capacity of influenza viruses to express foreign sequences and the requirement of packaging signals on both the 5' and 3' ends of the vRNA, which may interfere with the expression of foreign genes.

The advances of reverse genetics techniques have been of great benefit to the study of structure/function of different influenza virus genes, their proteins, and their roles in influenza replication. Reverse genetics has also been used to rescue an influenza virus expressing all eight genes of the “extinct” 1918 pandemic virus, which has allowed its extraordinary virulence to be studied. In the future, reverse genetics may play a more important role in designing novel and improved influenza viruses vaccines, in either killed or live attenuated virus vaccine types.

## **1.8 Influenza Pathogenesis**

Influenza A viruses can cause local epidemics or worldwide pandemics with significant infection rates. Despite effective vaccines, more than 200,000 infections and hospitalizations are reported in the United States and Canada annually; moreover, mortality can range in the millions during serious pandemic outbreaks (292, 350).

Influenza viruses cause highly contagious respiratory disease with potentially fatal outcomes. Symptoms include fever, headache, cough, sore throat, nasal congestion, sneezing, and body aches (8). These symptoms are self-limiting and improve after several days; however, the 2009 pandemic H1N1 influenza virus has proven to be more pathogenic than seasonal influenza viruses, and the pathogenicity of highly pathogenic H5N1 viruses is even greater (323).

Of the four pandemic outbreaks that occurred in the 20th century, the 1918 virus was the most devastating, causing massive acute pulmonary hemorrhage and edema (172, 323). A study with cynomolgus macaques has shown that infection with the 1918 virus resulted in disruption of the macaques’ antiviral immune responses, high virus titers and severe lung damage (132). The study demonstrated that the 1918 virus possesses high

virulence to cause fatal pulmonary disease. It has been observed that genetically similar influenza viruses can cause a broad range of severity of diseases in humans, which indicate that host conditions play an important role in determining the pathogenesis of influenza infection (75). Animal models such as mice, guinea pigs, ferrets, and non-human primates, are employed to analyze the involvement of host factors in influenza virus infections, and gene-targeted mouse models are used for testing the function of individual host genes in vivo. Type I interferon is induced by viral infection that result in the production of antiviral factors; studies with IFN- $\beta$  knockout mice have shown these mice to be highly susceptible to influenza virus infection (136). This indicates that type I IFN is a key host factor in the innate immune responses to infection with influenza virus; that the magnitude of type I IFN response influences the severity of virus pathogenicity. Thus, the pathogenesis of influenza virus infection in humans depends on a combination of virus and host factors.

### **1.8.1 Virulence Factors**

The influenza viral proteins play significant roles in the lung pathology of infected humans. Among these proteins, HA is responsible for the specificity of infection (38, 55, 169, 326, 368). The HA epitope is a major target of the human immune response, which is why the seasonal influenza vaccine is mainly characterized by its HA (rather than its NA) composition for influenza A virus (316). The HA of seasonal influenza virus binds to  $\alpha$ 2-6 sialylated glycans, which are expressed on the surface of the epithelial cells of the upper respiratory tract in humans (335). Inflammation caused by seasonal influenza virus infection is mainly limited to the upper respiratory tract and the disease is often mild. Influenza viruses can easily be transmitted among the human population by nasal

discharges that contain high titers of infectious virus. Unlike seasonal influenza viruses, highly pathogenic avian H5N1 influenza viruses preferentially recognize  $\alpha$ 2-3 sialylated glycans and primarily infect type 2 pneumocytes in the human lung (287, 329). HPAIV infection often results in severe pneumonia in humans (139, 287, 319). However, due to the location of its primary target cells deep in the lower respiratory tract, it is difficult for HPAIV to cause widespread infection among humans. It has been shown that specific mutations in the HAs of H5N1 viruses granted these viruses the ability to bind to  $\alpha$ 2-6 as well as  $\alpha$ 2-3 sialylated glycans (108, 181, 345). In the case of SOIV, a D222G substitution in HA, which was observed in severe and fatal cases, changes the receptor binding specificity of the virus from  $\alpha$ 2-6 to  $\alpha$ 2-3 sialylated glycans (38, 169). A study using cultures of human tracheobronchial epithelial cells showed that SOIV with the D222G substitution in its HA could infect ciliated bronchial cells (169). This cell tropism alteration mediated by an HA mutation may increase the severity of pneumonia. Therefore, influenza virus HAs are an important virulent factor that must be carefully monitored for amino acid mutations (or antigenic drift) that may alter their pandemic potential and/or mutations that confer higher pathogenicity to existing strains.

HA also influences pathogenicity via its susceptibility to host proteases. Influenza viruses require their HAs to be cleaved into two subunits, HA1 and HA2 in order to become infectious (299). The HA proteins of seasonal influenza viruses possess a single arginine at the cleavage sites and are cleaved by trypsin-like proteases that are produced by respiratory and gastrointestinal cells (104). In contrast, the HA of HPAIV possesses multiple basic amino acids at the cleavage site and is susceptible to ubiquitous furin and

PC6, which reside in the trans-Golgi network (104). This is one reason why HPAIV cause severe systemic infection leading to multiple organ failure and death.

The viral RNA polymerase complex consists of PA, PB1, and PB2. This complex is responsible for the transcription and replication of the viral genome. When the viral RNA polymerase genes of a highly lethal A/Vietnam/1203/04 (VN1203) H5N1 were replaced with those of a low pathogenic H5N1 virus, the pathogenicity of VN1203 was dramatically reduced in these animals (271). Additionally, specific mutations in PA and PB2 enhanced avian virus replication in mammalian cells (25, 294). Watanabe et al. also demonstrated that the RNA polymerase complex and NP played a role in the pathogenicity of the 1918 pandemic virus (344). Thus, the viral RNA polymerase complex also contributes to influenza virus pathogenicity.

Certain influenza viruses have been determined to encode for an extra protein in the PB1 segment called PB1-F2, which is a 90-amino acid protein that preferentially localizes to the mitochondria of infected cells (34). PB1-F2 induces apoptosis and is a known virulence factor (364). The high virulence of both the 1918 pandemic and H5N1 viruses was due to the amino acid change N665S in PB1-F2 (42). This mutation increases the secretion of proinflammatory cytokines, such as TNF- $\alpha$  and virus titers in the lungs. Other viral proteins, such as NA and NS1, are also implicated in the virulence of influenza virus. NA is important for efficient viral replication (243), while NS1 functions as an interferon antagonist in virus-infected cells (89).

## **1.8.2 Host factors**

The pathogenesis of influenza virus is an outcome that depends on the function of the immune system and how well the virus can evade it. When influenza viruses infect

respiratory epithelial cells or alveolar macrophages, the single-stranded RNA of the influenza virus is recognized by toll-like receptor (TLR) 7 (58) and retinoic acid-inducible gene-I (RIG-I) (251). The signaling pathways of TLR7 and RIG-I induce the production of type I IFNs and activate antiviral host responses (13, 58, 120). However, viral NS1 protein can interfere with the RIG-I signaling pathway and allow influenza viruses to escape the innate immune response (76, 209). A recent study revealed that NS1 inhibits the function of tripartite motif (TRIM) 25 in the ubiquitination of RIG-I, which is an essential step in the type I IFN response (76). NS1 also binds to protein kinase R (PKR), a well-known antiviral protein (163, 209). The binding of NS1 and PKR inhibits the host protein from downregulating the translation of the viral mRNA, which is mediated by phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2a) (163).

RIG-I and TLR7 also induce the production of inflammatory proteins mediated by NF- $\kappa$ B activation (58, 110, 192). Several inflammatory cytokines and chemokines are upregulated during influenza virus infection such as IL-1b, IL-6, IL-8, TNF $\alpha$ , CCL2 (MCP-1), CCL3 (MIP-1a), CCL5 (RANTES), and CXCL10 (IP-10) (97, 248, 290). Although the innate immune response is indispensable for the protection of the host against influenza virus infection, the unregulated response of proinflammatory cytokines and chemokines can harm rather than protect respiratory organs. Studies with the 1918 pandemic virus reported aberrant cytokine response that resulted in significant lung damage to the infected host (132). As it turns out, the innate immunity is like a two-edged sword with two distinct roles in the pathogenesis of influenza virus infection.

In contrast, adaptive immunity, which involves viral antigen-specific antibodies and cytotoxic T lymphocyte activity, has been shown to efficiently eliminate virus-infected cells, enabling the host to overcome viral infectious diseases. Cytokines produced by antigen presenting cells activate Th1-type and Th2-type cells, which arise from CD4<sup>+</sup> helper T cell precursors, and CD8<sup>+</sup> cytotoxic T-lymphocytes (CTL). Activated Th1 cells enhance IgG2a antibody (Ab) production and proliferation of CD8<sup>+</sup> CTL, whereas Th2-type cells enhance IgA, IgG1, and IgE Ab production (314). These responses result in the production of neutralizing antibodies, which bind to viral antigens. Th1 cells also secrete IFN- $\gamma$ , which also inhibits virus replication (314). CD8<sup>+</sup> CTLs, on the other hand, recognize MHC class I-antigenic peptide complexes on virus-infected epithelial cells and eliminates the infected host cell by exocytosis of cytotoxic granules containing perforin and granzymes (116). Thus, individuals with immunodeficiency of B cells or T cells are highly susceptible to influenza virus infection (16, 79). Therefore, adaptive immunity provides essential protection from influenza virus infection and effective prevention of re-infections.

## **1.9 Clinical Presentations**

### **1.9.1 Seasonal Influenza**

Seasonal influenza is usually a self-limiting disease. After an average incubation period of around 1 to 2 days, onset of illness is characterized by an abrupt onset of fever and chills accompanied by headache, generalized myalgia, rhinorrhea, sore throat, and cough (8). Gastrointestinal symptoms such as vomiting, abdominal pain, and diarrhea are also reported (8). Lower respiratory tract infection, including croup, bronchitis,

bronchiolitis, and pneumonia, is often the most common cause for hospitalization (8). The central nervous system may also present clinical symptoms that include encephalopathy, postinfluenza encephalitis, transverse myelitis, Guillain-Barré syndrome, and acute necrotizing encephalitis (8). Myositis often occurs 3 days (range, 0–18 days) after onset of illness. In young infants, influenza can mimic generalized sepsis (8). Myocarditis can occur as a rare complication. Epidemiologically, most deaths occur in infants and the elderly (>65 years old) during the annual influenza epidemics as a result of decreased immunity against influenza virus infection (8). The mortality curve typically presents with a U shape when age-specific excess mortality caused by pneumonia and seasonal influenza is plotted (212).

### **1.9.2 Pandemic Influenza A (H1N1) 2009**

Studies on the pandemic influenza A (H1N1) 2009 has shown that most cases of SOIV infection are mild and self-limiting and present in a manner that is indistinguishable from seasonal influenza. Those with pre-existing medical conditions such as the traditional chronic diseases (eg, diabetes, asthma, renal or cardiac failure, and any form of immunosuppression) are at a greater risk of developing a more severe disease and even possibly death from the viral infection.

Similar to the 1918 virus, the age distribution of infection with SOIV also differs from seasonal influenza. For example, the older age groups (>65 years) have always been considered to be vulnerable to seasonal influenza infection, but they seem to be less frequently infected by the 2009 pandemic virus. This trend is now thought to be caused by some pre-existing cross-reacting immunity to this virus as a result of past exposure to the older circulating seasonal influenza A (H1N1) strains that are more similar to the

current pandemic A (H1N1) 2009 virus (93). The current circulating seasonal influenza A (H1N1) virus and its corresponding seasonal influenza vaccine antigen components seem to not provide any cross-immunity to the pandemic strain (93). In the more frequently targeted younger adult age groups, an unusual feature has been observed; more patients in this group progress to more serious respiratory disease, whereas there is also a significant gastrointestinal component (nausea, vomiting, and diarrhea in 10%–50% of cases) involved (317).

## **1.10 Antivirals of Influenza Virus**

Given that influenza virus remains a constant threat to public health, major efforts have been directed at discovering effective antivirals and novel vaccines over the past several decades, and this endeavor further heightened after the 2009 pandemic outbreak. Presently there are only four approved drugs available for use in humans: amantadine, rimantadine, oseltamivir, and zanamivir. Past and current approaches to antiviral therapy are briefly discussed below.

### **1.10.1 Amantadine and Rimantadine**

Amantadine was first approved in the United States for use against influenza A viruses in 1966 (10). The closely related rimantadine was approved for use in the United States in 1993 (10). Both these drugs target the viral M2 ion channel protein of influenza A virus, which is required for proper uncoating of viral RNPs during the viral entry process (50) (Figure 6). Like the neuraminidase inhibitors (described below), amantadine and rimantadine are effective for prophylaxis and for treatment of influenza A virus infection (10). However, a major setback with the use of these drugs is that they require

administration early after symptoms appear to be effective. Also, the major difficulties associated with these drugs are the rapid development of resistant mutants and toxicity (50, 216, 328). For example, the recent SOIV 2009 pandemic strain has already been determined to be resistant to both amantadine and rimantadine (291). Resistant mutants possess mutations within the transmembrane domains of the M2 protein (113), and resistance mutations to amantadine confer resistance to rimantadine and vice versa (252).

Several studies have demonstrated the significance of drug resistance development with use of amantadine and rimantadine. For example, one study showed that among 7000 human influenza A viruses isolated between 1994 and 2005, a dramatic increase in the frequency of amantadine-resistant H3N2 viruses was found, with 0.4 percent of isolates from 1994-1995 being resistant and 12.3 percent being resistant in 2004-2005 (22). Another study from the United States found that 193 of the 209 H3N2 isolates had a S31N mutation known to confer amantadine resistance (23). These observations prompted the retraction of amantadine and rimantadine for use as treatment or prophylaxis of influenza in the United States until susceptibility to these specific antivirals is reestablished among circulating influenza A isolates (23). These observations also highlight the potential difficulties that might be associated with widespread use of amantadine or rimantadine during an influenza pandemic. One potential means of addressing these difficulties would be the development of new M2 inhibitors.

### **1.10.2 Neuraminidase Inhibitors**

It has been shown the enzymatic function of the viral NA is significant to release progeny virions from the cell surface (241); that absence of NA activity significantly

impairs virus replication (77). The active site of the enzyme is well conserved between influenza A and B virus strains, thus making the enzyme a viable target for antiviral development (77). It was later shown that neuraminic acid analogs inhibit influenza virus replication in tissue culture and that aggregates of virus are formed at the cell surface in the presence of these drugs (237, 239) (Figure 6). Based on the three-dimensional x-ray structure of NA, von Itzstein et al. (337) designed a derivative of neuraminic acid, which replaced the OH group of the previously studied neuraminidase inhibitor, DANA (201) with a guanidino group at C-atom 4. This compound, zanamivir (commercially known as Relenza), is pharmacologically ineffective (unstable) unless administered by inhalation or by nasal spray (127). But when properly administered, this compound can be a potent anti-influenza drug, both prophylactically and therapeutically (reviewed in (198)).

A search for compounds that are orally active led to oseltamivir (commercially known as Tamiflu) (164). Its pro-drug is an ethyl ester of a compound that has a hydrophobic side chain, making the drug pass through the gut into the bloodstream (164). The pro-drug gets hydrolyzed in vivo to the active form oseltamivir carboxylate, (GS4014). Both zanamivir and oseltamivir are reversible competitive inhibitors of the viral NA (50, 113). Oseltamivir has been shown to be highly effective against all influenza A virus tested (reviewed in (113, 198, 291)), although the concentrations required for inhibition can vary significantly from strain to strain (291). In animal models, including mice and ferrets, oseltamivir carboxylate and zanamivir are effective as therapeutic and prophylactic agents (53). Although in human patients both drugs reduce the time to recovery following influenza virus infection, a major limitation with these drugs is the requirement to administer beginning 1-2 days following onset of

symptoms to be effective (291). Although resistant variants have been described with escape mutations in the HA and NA (113, 291), resistant strains selected in the laboratory against zanamivir show markedly reduced infectivity (312).

Changes in HA or NA have been described as 2 mechanisms of viral resistance to NA inhibitors. Resistance mutations in the HA reduce the protein's affinity for sialic acids and facilitate virus release from the infected cell under conditions where NA activity is inhibited (113). More importantly, mutations at specific residues in the NA active site that directly interact with neuraminidase inhibitors confer drug resistance. The extent to which neuraminidase inhibitor-resistance may be problematic remains uncertain. A study analyzing over 1000 clinical influenza virus isolates from 1996 to 1999 was unable to find evidence of naturally occurring resistance (198). However, more recently, studies found neuraminidase inhibitor-resistant viruses in children treated for influenza with oseltamivir (301). Similarly, oseltamivir-resistant viruses have been isolated from children infected with highly pathogenic avian H5N1 influenza viruses and treated with oseltamivir (154). Oseltamivir is considered by many the current front line drug available for use in an influenza pandemic, in part because neuraminidase inhibitor resistance arises less frequently than adamantane resistance (113). However, the last two mentioned studies have demonstrated that resistance does develop during active treatment with the NA inhibitors.

### **1.10.3 Ribavirin**

Ribavirin, a U.S. Food and Drug Administration (FDA)-approved antiviral, is known to inhibit influenza in humans, but toxicity remains a serious problem. Ribavirin is a nucleoside with antiviral activity against many viruses, and it inhibits the replication

of both influenza A and B viruses (10). However, ribavirin administration is limited by toxicity and the need to deliver the drug intravenously or by inhalation, since oral ribavirin was lacking in efficacy in clinical trials (10). Viramidine, a ribavirin prodrug has also been found to inhibit influenza virus replication in vitro and in vivo and may have fewer toxic effects than ribavirin (288).

#### **1.10.4 Inhibition of Hemagglutinin**

Although the x-ray crystallographic structures of the HA and of HA-ligand complexes have been known for more than two decades (240), continuing attempts to develop synthetic drugs that interfere with the HA-sialic acid interaction to block virus to receptor attachment remain unsuccessful. Theoretically, this approach could work but the strategy of using sialic acid analogs has so far failed to advance to human trials (240).

Another approach concerns the inhibition of the posttranslational cleavage of the HA, which results in a molecule unable to undergo the conformational change (or activation) required for fusion/uncoating. For example, quinone derivatives that prevent the first stage of the conformational change of the HA and thus inhibit infection were discovered in 1993 (14), and other compounds have also been identified that force the HA into an inactive state (103). However, none of these influenza virus HA inhibitors have blocked all HA types or subtypes, and none has acceptable  $IC_{50}$  values in the submicromolar range (240).

#### **1.10.5 Antisense Oligonucleotides and siRNAs**

More recently, small interfering (si)RNA inhibition has become a major area of interest as an alternate route to interfere with influenza virus replication in tissue culture

and in animals. RNA interference (RNAi) is a process by which small molecules of double-stranded RNA direct the sequence-specific degradation of mRNA molecules. In mammalian cells, the 21-nucleotide siRNA duplexes directly induce RNA interference by bypassing the need for Dicer-mediated processing of dsRNA (85). Theoretically, viral mRNA, as well as viral cRNA and viral genomic RNA (vRNA), could be targets for an siRNA approach. Several siRNA molecules have been developed that are directed against specific influenza virus genes and were shown to successfully inhibit influenza virus replication in tissue culture and mice (320). However, many challenges remain for this approach. For example, the effective delivery of these molecules defies standard approaches, and contradictory results have been reported regarding the induction of interferons and cytokines by siRNAs. These challenges represent an obstacle to the use of siRNA molecules as specific and nontoxic inhibitors to influenza infection (56). Additionally, influenza viruses may be able to overcome the effects of RNA silencing by expressing a viral RNAi antagonist (54).

### **1.10.6 Host Responses as Therapeutic Targets in Influenza Virus Infection**

Infection with influenza viruses results in the activation of a variety of intracellular signaling responses (43, 343). Although most of the cellular responses are initiated as processes to defend against the invading pathogen, viruses have acquired the capability to exploit some of these activities to ensure efficient replication (178, 336, 356, 357). Taking advantage of cellular signaling factors to support viral replication is very effective but also creates dependencies that might be used to develop novel anti-viral drugs that disrupt signal transmission. A few inhibitors of different signaling pathways have been

shown to suppress virus replication in cell culture models, but so far only two specific virus-supportive signaling pathways are proven as suitable targets for an anti-viral approach in vivo in mouse models: the Raf/MEK/ERK mitogenic kinase cascade (176) and the IKK/NF- $\kappa$ B module (177).

The Raf/MEK/ERK signaling pathway belongs to the family of mitogen-activated protein kinase (MAPK) cascades (255); the pathway is activated via receptor tyrosine kinases or G-protein-coupled receptors, leading to the stepwise phosphorylation and activation of the serine threonine kinase Raf, the dual specificity kinase MEK (MAPK kinase/ERK kinase) and the MAPK ERK (extracellular signal regulated kinase). Activated ERK phosphorylates a variety of substrates that regulate many different functions in the cell (349). Studies have determined that influenza A virus infection (and influenza B virus, too) activates this kinase (255). Notably, the inhibition of Raf/MEK/ERK signaling pathway resulted in strongly impaired growth of avian and human influenza A as well as human influenza B viruses (253), which indicates that activation of the Raf/MEK/ERK kinase cascade is required for efficient virus growth. Conversely, virus titers were enhanced in cells in which the pathway was pre-activated by expression of constitutively active mutants of Raf or MEK (Ludwig et al., 2004; Olschlager et al. , 2004 ). It was shown that the inhibition of the signaling cascade led to nuclear retention of the viral RNP complexes in late stages of the replication cycle (256), which suggested the pathway controls the active nuclear export of RNPs (256).

The requirement of Raf/MEK/ERK activation for efficient influenza virus replication suggests that this pathway could be a promising cellular target for anti-influenza approaches. In support for this, studies have shown inhibitors of the cascade

exhibited a strong anti-viral activity with little toxicity in cell culture (253, 256). Clinical investigation with several inhibitors of the Raf/MEK/ERK cascade demonstrated that the cascade can be effectively inhibited in humans (176). What remains to be determined is whether these inhibitors would also block influenza replication during human infection.

The NF- $\kappa$ B signaling cascade is another important influenza virus induced signaling process now being considered as a possible target for drug development. NF- $\kappa$ B regulates expression of a variety of antiviral cytokines including IFN- $\beta$ , that is the initiator of a strong type I IFN defense program (9, 15). Due to this role in antiviral gene expression, NF- $\kappa$ B and the upstream activator kinase I $\kappa$ B kinase (IKK) 2 were regarded as bona-fide components of the innate immune response to virus infections (15). However, two independent studies made a surprising observation that replication of influenza viruses is much more efficient in cells with pre-activated NF- $\kappa$ B (226, 356). Conversely, virus titers were significantly reduced in host cells in which NF- $\kappa$ B signaling was impaired by inhibitors such as BAY11-7085, BAY11-7082, the use of dominant-negative mutants of IKK2, or the inhibitor of  $\kappa$ B, I $\kappa$ B $\alpha$  (226, 356). These studies indicate that influenza viruses have acquired the capability to usurp the antiviral activity of NF- $\kappa$ B into a virus-supportive action.

The viral dependence on NF- $\kappa$ B function was demonstrated at least in part due to the NF- $\kappa$ B dependent expression of factors such as tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) or FasL (356), which are known activators of a cell death program executed by a family of apoptosis regulating proteases termed caspases (59, 260). For that reason, treatment with caspase inhibitors or by using siRNA to suppress expression of a major effector caspase-3 strongly impaired influenza virus

propagation (357). Caspases specifically cleave cellular proteins including those of the nuclear pores resulting in an enhanced diffusion limit for protein transport in and out of the nucleus (68, 125, 140). This function appears to be relevant for viral replication because in the presence of both caspase inhibitors and NF- $\kappa$ B inhibitors, a nuclear retention of viral RNP complexes could be observed (4, 357), which most likely prevented formation of progeny virus particles. Thus, the typical anti-apoptotic and antiviral function of NF- $\kappa$ B is converted into a pro-apoptotic and pro-viral function during influenza virus infection (59).

Additionally, NF- $\kappa$ B may affect more than just specific stages in the virus life cycle because this cellular factor also regulates the majority of cytokine and chemokine signaling (219). Consequently, endothelial cells or infiltrating monocytes might strongly influence the outcome of the cytokine burst due to activation of NF- $\kappa$ B in airway epithelia after influenza virus infection. Findings from genome-wide gene expression array assays provided evidence to support this, in that they demonstrated a major role of NF- $\kappa$ B for cytokine responses induced by the H5N1 virus (332).

As a result of these findings, NF- $\kappa$ B activity is now considered to be a suitable target for anti-viral intervention. The first proof-of-concept study was performed with acetylsalicylic acid (ASA), also known as aspirin, which is an efficient and selective inhibitor of IKK2 in low millimolar concentration ranges (361). ASA has now been shown to efficiently block influenza virus replication, including H5N1 strains, in cell culture by several orders of magnitude in a concentration range that was not toxic for the host cell (197).

The increasing frequency of resistance to the current drugs that exclusively target viral factors raises concern new drugs that target viral structures will sooner or later share the fate of M2 and NA inhibitors. Thus, a paradigm change in drug development is urgently needed. Targeting cellular rather than viral factors could be the most promising approach to prevent the problem of resistance because the pathogen simply cannot replace the missing cellular function. According to aforementioned studies, it is certainly possible to target cellular factors with little to no harmful side effects to the host, and the prospect for these drugs to select for resistant virus variants is much lower.

## **1.11 Influenza Vaccine**

Antiviral drugs are important in the treatment of influenza infection; however, annual epidemics are primarily controlled through comprehensive vaccination programs, and vaccination during an pandemic outbreak may reduce spread and overall morbidity. Influenza vaccine is comprised of the three most common influenza virus types in circulation: influenza B viruses, influenza A (H1N1), and influenza A (H3N2). Due to the dynamic nature of influenza virus genetic variance as it circulates within the population, the composition of the vaccine (the specific strains to be included) is determined annually by the World Health Organization in conjunction with other global institutions. Influenza vaccine is currently available in two forms: inactivated vaccines (INV) and live-attenuated (LAV) vaccines (279). INV consists of specific virus subtype propagated in embryonated chicken eggs, purified, and then inactivated. INV is given intramuscularly, and comes in three forms: whole-virus, detergent split-virus, and subunit-virus vaccines. Split-virus vaccines involved treating the killed viral particles with detergent to disrupt protein-protein interaction. Split-virus vaccines contain

essentially all viral structural proteins and disrupted portions of the viral membrane. Subunit vaccines, on the other hand, contain only the purified HA and NA proteins from the inactivated virus (279). INV is the flu shot commonly given to most patients. INV elicits both systemic and localized Ab response. Neutralizing IgG against HA and NA viral surface antigens is the predominant response observed, which correlates with the inhibition of disease progression and severity. However, INV is less effective in inducing an IgA response. The vaccine also does not provide effective heterosubtypic immunity (279).

LAV has the same subtypical viral composition as INV, but the difference is that LAV contains live, cold-adapted viruses with its replicative ability inhibited at temperatures exceeding 25°C. This restrains viral replication to the upper respiratory tract (279). LAV is available in Canada and U.S.A. under the commercial name FluMist®. LAV is delivered intranasally and induces IgA response. LAV is not recommended for high risk groups such as pregnant women, elderly and immunocompromised individuals since the vaccine consists of live viruses (279).

There are several drawbacks to current influenza vaccine strategies. Vaccine efficacy can be significantly reduced if the vaccine components are not well-matched to the circulating strain. Vaccine production, including the time when the vaccine composition is decided to actual pharmaceutical production, is a long process that takes from 6 to 8 months, during which time the circulating strain can undergo antigenic drift that can also reduce vaccine efficacy. There are also possibilities of supply shortages due to contamination of embryonated eggs, reduction in vaccine manufacturers, and unexpected early outbreaks before production can begin. The dependency on eggs for

vaccine production will always be limited by the availability of number of eggs, as well as the ability to propagate vaccine strains in these eggs. For example, SOIV 2009 pandemic strain replicated very poorly in eggs; this reduced early vaccine production and resulted in limited vaccine supply (279). Therefore, present vaccine production methodology and reagents have pitfalls that pose serious challenges during large epidemics and especially during severe pandemic outbreaks. Thus, an alternate approach may be to target host proteins required by the virus but dispensable by the cell; this is the underlying rationale for the work described in this thesis.

## **1.12 RNA Interference**

The ability of functional genomics to provide a global view makes it one of the most useful approaches for studying virus–host interactions. The sequencing of the human genome ushered a new era into the field of modern biology; it is now possible to elucidate molecular pathways relevant to development and disease with a breadth never before seen. Large-scale RNAi screens have the capability of producing genome-wide loss-of-function phenotypes that can place previously uncharacterized viral-host interactions into the context of a specific pathway within the cell, and ultimately lead to a systems level understanding of a process as well as the comprehensive identification of the molecular components underlying influenza infection, or cellular functions in general.

RNAi regulates gene expression through sequence-specific targeting of mRNA, which is achieved with use of either short hairpin (sh)RNA or siRNA species (250, 263). shRNA molecules are produced ectopically and siRNAs are delivered exogenously to the cell (Figure 7). Production and processing of shRNA molecules are similar to the

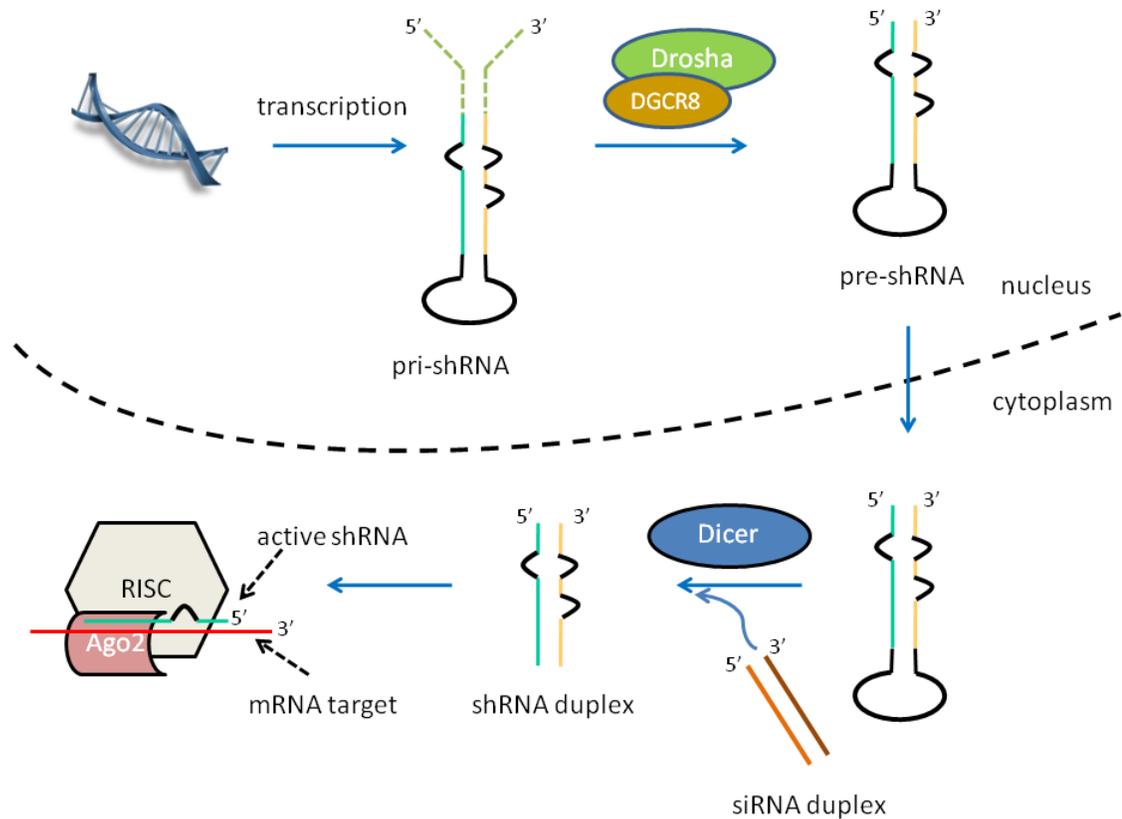
production of endogenous host micro (mi)RNA. shRNA are transcribed by RNA polymerases into primary shRNA molecules with significant secondary structure (250). This secondary structure is recognized by the Drosha–DGCR8 complex, which cleaves the shRNA into its second immature form, a 70 nucleotide hairpin containing a 2 bp overhang on its 3' end (250). This 'pre-shRNA' is then exported from the nucleus to the cytoplasm, where it is processed by Dicer into its fully active form (250).

The processing of siRNA duplexes and shRNA converge into a conserved RNAi-modulating pathway at the point of cleavage by Dicer, as siRNA and miRNA moieties utilize the same cellular machinery to target mRNA transcripts (263). After processing by Dicer, mature siRNAs and shRNAs associate with proteins to create an RNA-induced silencing complex (RISC) (250, 263). The proteins present in RISC vary between species, but the core proteins include Dicer and the Argonaute protein family (250, 263). Mature siRNAs and shRNAs are subsequently assembled into RISC, where the guide strand is then able to target its exact complementary mRNA sequence within the cell, allowing it to be cleaved by Ago2 (263). Although the mechanism for this selection is not yet well understood, it is thought that once a strand is selected and loaded into RISC, the other strand is destroyed (250). The remaining RNA then guides RISC to the 3' untranslated regions (UTRs) of various mRNA transcripts, leading to the repression of protein expression (250).

Viral plasmids containing shRNA motifs have been engineered for experiments requiring the use of cultured cells that are especially difficult to transfect (Figure 8). Retroviral-, adenoviral-, and lentiviral-based systems have been successfully employed by many groups (11, 184, 306). Lentivirus and adenovirus are capable of infecting both

dividing and nondividing cells, providing the most feasible way to achieve knockdown in extremely slow-growing and nondividing cell types.

Viral plasmids also allow the generation of a stable knockdown, as their shRNA-containing genomes integrate into the host cell and thus replicate with each division of the cell, which is a necessary factor for experiments that require longer than the 5–7 day window of knockdown that the transient siRNAs provide (Figure 8). To improve on the siRNA and shRNA technologies, a few groups have optimized shRNA sequences with miRNA-like properties to make what is now known as “shRNA-mirs”; this inclusion of miRNA properties has been shown to increase their potency and/or specificity (30). shRNA-mirs, like previous shRNAs, contain unique hairpin loops of complementary sense and antisense strands but use miRNA precursors as the backbone for the delivery of hairpins (30, 70). These hairpins are flanked by the stem sequences found within miRNAs, providing extensive secondary structure beyond the early-designed shRNA molecules (30). With the incorporation of miRNA precursors as the backbone, shRNA-mirs have been shown to efficiently and specifically target and inhibit gene expression in both transient (365) and stable (57) cell lines, producing a more potent gene silencing effect than traditionally designed shRNAs (289). One notable advantage with shRNA-mirs is that gene silencing is still effective when shRNA-mir cassettes integrate into the genome as a low-number or even single copy (57). This efficiency is especially important for lowering the potential for off-target effects and mediating sufficient knockdown to observe phenotypes. shRNA-mirs are a viable alternate option to the traditionally designed shRNAs on account of their differences in efficiency and specificity.



**Figure 7. shRNA and siRNA processing.** shRNAmir is integrated into the host genome as discussed in Section 1.11. Transcription produces (pri)mary-shRNA which gets processed by Drosha and DGCR8 to produce the (pre)cursor-shRNA. Pre-shRNA gets transported into the cytoplasm where the loop structure gets cleaved by Dicer to produce the shRNA duplex. The anti-sense strand of the shRNA duplex (referred to as the active shRNA) is loaded onto RISC. This mature shRNA guides the RISC complex to the mRNA target, which gets degraded by Argonaut 2 (Ago2). siRNA duplexes enter the RNAi process at post-Dicer stage.

The availability of the genome-wide lentiviral-based shRNAmir libraries, developed by the RNA Consortium and Cold Spring Harbor Laboratories (289) and commercially available through ThermoScientific Open Biosystems®, and vast siRNA microarray libraries now provide the foundation to identify, on a grand scale, host genes that are necessary for viral replication. This powerful tool is employed by some groups, including us, to identify novel cellular genes whose knockdown attenuates influenza replication.

### **1.13 Cell Death**

Processes such as apoptosis, necrosis, and autophagy are well studied processes associated with cell death in a eukaryotic system. Necrosis and autophagy will only be briefly discussed here in comparison to apoptosis. Apoptosis and necrosis are two distinct forms of cell death, whereas autophagy is one of the main protein degradation pathways; however, taken to extremes, autophagy can also lead to cell death (372). Necrosis is observed under diseased conditions, whereas apoptosis can occur under both physiological and pathological states (327). Cells undergoing any one of these processes show characteristic phenotypes. For example, necrosis causes excessive fluid influx that lead to cell swelling and eventual lysis. Phenotypic changes to chromatin, often observed in cells undergoing apoptosis as discussed in Section 1.13.1, do not occur in cells undergoing necrosis, and intracellular content is released into the external environment due to a loss of membrane integrity (327); this does not happen in apoptosis. Autophagy is distinctly different from apoptosis, and involves fundamentally different sets of regulatory and executioner molecules (372). Autophagy has been associated with

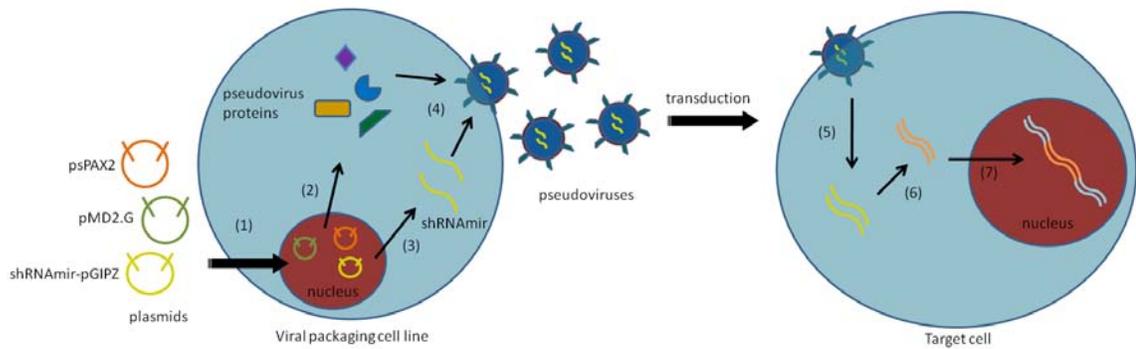
influenza infection (268) but little is known about the occurrence of necrosis during viral infection. Of the cell death pathways, I will focus on apoptosis.

### **1.13.1 Apoptosis**

Apoptosis is a cellular death mechanism that may be elicited by several molecular pathways. The best characterized and the most prominent ones are called the extrinsic and intrinsic pathways (Figure 9). The extrinsic pathway (also known as “death receptor pathway”) is triggered by a ligand-induced activation of death receptors such as TNF receptor-1, CD95/Fas (the receptor of CD95L/FasL) (121), and TRAIL receptors-1 and -2 (339). Apoptosis activation via the intrinsic pathway (also called “mitochondrial pathway”) occurs from an intracellular cascade of events in which mitochondrial permeabilization plays a crucial role (66, 142).

Both routes to apoptotic death can be divided to three distinct phases: initiation, integration/decision, and execution/degradation (144). The initiation phase is highly heterogeneous and depends on the nature of the death-inducing signal. The integration/decision phase involves the near-to-simultaneous activation of caspases and mitochondrial death effectors in a complex molecular interplay, where the decision to die has reached an irreversible stage. The execution/degradation phase of apoptosis ultimately lead to cell shrinkage, chromatin condensation, nuclear fragmentation (which is frequently accompanied by internucleosomal DNA fragmentation), blebbing, and phosphatidylserine exposure on the surface of the plasma membrane (308).

The activation of caspases (“cysteine protease cleaving after Asp”), a specific class of proteases, is required for the rapid and full-blown manifestation of these apoptotic characteristics (257). However, not all caspases are required for apoptosis and the



**Figure 8. shRNAmir packaging and lentivirus transduction.** Pseudo-lentivirus containing shRNAmir are produced by (1) co-transfection of plasmids psPAX2 (encodes for lentiviral structural proteins), pMD2.G (encodes for VV G proteins), and shRNAmir-pGIPZ (encodes for shRNAmir) in HEK-293T cells at a 1:2:2 ratio. (2) Transcription and translation of psPAX2 and pMD2.G occur. (3) single-stranded (ss), positive sense shRNAmir genomic RNA is replicated. (4) Assembly and packaging into virions. (5) Target cell is transduced, lentivirus attachment and uncoating of genomic RNA. (6) Reverse transcription of ssRNA to DNA. (7) shRNAmir DNA translocates to the nucleus and integrates into host genome.

process generally results from the activation of a limited subset of caspases, in particular, caspases-3, -6, and -7 (257). These are the “executioner” caspases, and they mediate their effects by the cleavage of specific substrates in the cell. The activation of the executioner caspases-3 and -7 by initiator caspases-8, -9, and -10 are the best characterized apoptotic pathways (21).

In the extrinsic pathway, ligation of death receptors causes the recruitment and oligomerization of the adapter molecule FADD (Fas-associating death domain-containing protein) within the death-inducing signaling complex (DISC). Oligomerized FADD binds the initiator caspases-8 and -10, causing their dimerization and activation (142).

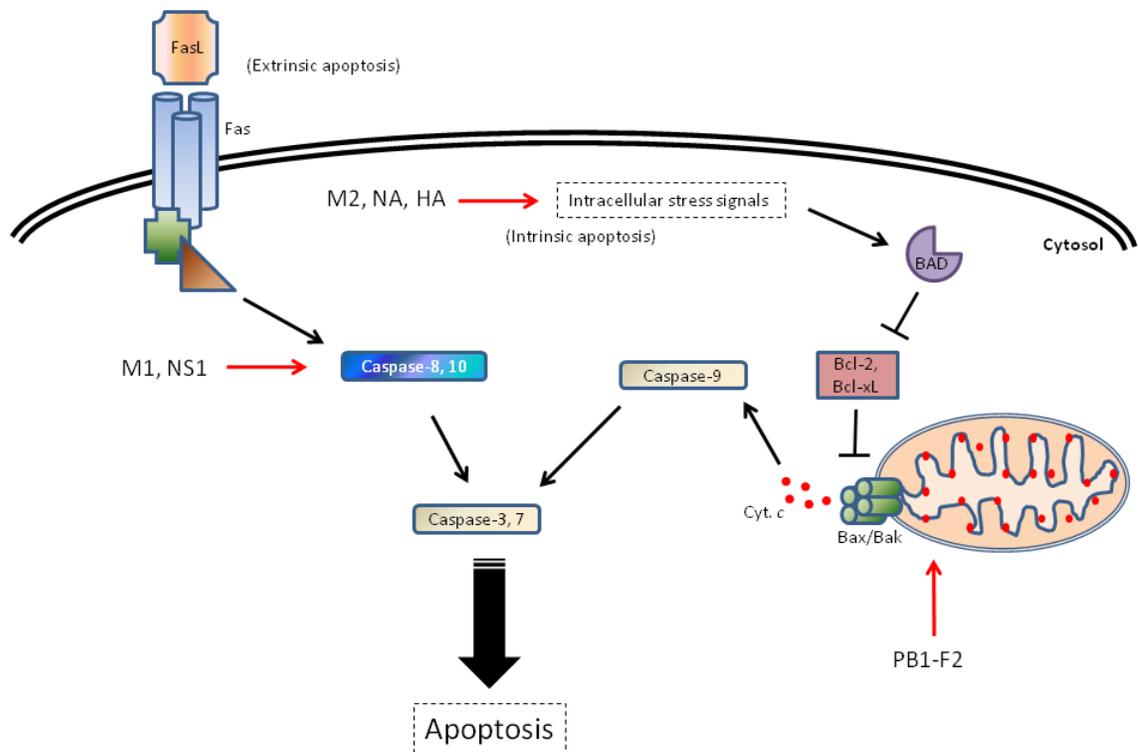
Despite the availability of the extrinsic pathway, most cell death in vertebrates proceeds via the intrinsic or mitochondrial pathway of apoptosis (142). In this case, the executioner caspases are cleaved and activated by the initiator caspase-9, which itself is activated by multimerization on the adapter molecule apoptosis protease activating factor 1 (APAF-1) within a multiprotein complex called “apoptosome.” APAF-1 is present in the cytosol as a monomer, and its activation depends on the presence of cytochrome c (Cyt c) and ATP/dATP (27). Cyt c normally resides only in the intermembrane space of the mitochondria where it functions as an electron shuttle in the respiratory chain (142), and its release is required for the generation of the apoptosome. Thus, mitochondrial membrane permeabilization (MMP) is the critical event responsible for caspase activation in the intrinsic pathway. The importance of MMP is demonstrated by data that showed MMP can even commit a cell to die when caspases are not activated. Caspase-independent death (35, 143) can occur because of an irreversible loss of mitochondrial

function as well as the mitochondrial release of caspase-independent death effectors including apoptosis-inducing factor (AIF) (309) and endonuclease G (EndoG) (161).

### **1.13.2 Bcl-2 Family**

The Bcl-2 family of proteins contain at least one Bcl-2 homology (BH) region. The family may be divided into 3 different groups: (1) antiapoptotic multi-domain proteins (prototypes: Bcl-2, Bcl-XL), which contain four BH domains (BH1234); (2) proapoptotic multidomain proteins (prototypes: Bax, Bak), which contain three BH domains (BH123); and (3) proapoptotic BH3-only proteins (prototypes: Bid, Bad) (80). Additional COOH-terminal transmembrane domain are found in some members of each subgroup; this mediates the proteins' insertion into the outer membrane (OM) of the mitochondria. The main site of action of Bcl-2-like proteins is the mitochondrial membrane (6). Generally, BH1234 proteins mainly reside in OM, where they protect against MMP, binding to and neutralizing other proapoptotic proteins from the Bcl-2 family, which on the contrary induce MMP (6).

Under non-apoptotic conditions, the BH123 protein Bak is associated with the OM, whereas the other BH123 protein Bax resides in the cytosol (66). However, upon apoptosis induction, Bax inserts into the OM (66), where it forms supramolecular pores (121). This pore formation results from Bax forming either homo-oligomeric channels or in association with other proapoptotic members such as Bak or tBid (truncated Bid) that cause transient discontinuities within the OM (66) to allow the release of apoptogenic proteins within the mitochondria into the cytosol. The pore theory emerged from the realization that the 3D structures of Bcl-xL (and later, of Bax) had significant similarity with the pore-forming translocation domain of the diptheria toxin (310). The pore



**Figure 9. Apoptosis signaling.** Pro-apoptotic ligands (i.e. FasL) bind to their receptors (i.e. Fas) to activate the extrinsic apoptosis signaling pathway, which results in the activation of caspase-8 and caspase-10. Intracellular signals such as cellular stress or anti-viral signals activate the intrinsic apoptosis signaling pathway via the mitochondria. Both signaling cascades converge at effector caspases-3 and -7, which eventually lead to apoptosis or cell death. Refer to Section 1.12 for more details on apoptosis signaling.

formation of Bax and/or Bak permits the release of Cyt c out of the mitochondria; however, the precise mechanism for which Bax/Bak achieves this is still not completely understood. It is also an ongoing conundrum how Bax and Bak translocate to the mitochondria and whether they are attracted through specific properties of the lipid or protein composition of the OM. Reportedly, large Bax oligomers organize into clusters near the mitochondria (66), which result in Bak colocalizing in these apoptotic clusters. Formation of these complexes has been reported to occur in a caspase-independent fashion and to be inhibited completely and specifically by Bcl-xL (221).

The third group of family members, the BH3-only proteins, can exert their proapoptotic action by two different mechanisms. Some BH3-only proteins (the “facilitators,” prototype: BAD) preferentially interact with BH1234 proteins to promote MMP; this characteristic interaction dissociates them from other BH3-only or from BH123 proteins. Other BH3-only proteins (the “activators,” prototype: tBid) directly activate BH123 proteins to initiate MMP, either by stimulating the translocation of Bax to mitochondrial membranes or by local effects on Bak (46).

Two different types of “BH3 mimetics,” have been generated, which are a class of pharmacological agents that bind to multidomain Bcl-2 family proteins or so-called “BH3 receptors.” One type of BH3 mimetics (prototype: ABT-737) only binds to BH1234 proteins—and hence facilitates apoptosis induction by neutralizing the antiapoptotic proteins of the Bcl-2 family (234)—while a second type of BH3 mimetics also binds to BH123 proteins and directly induces apoptosis in a Bax/Bak-dependent manner (340).

The specific mechanism by which the antiapoptotic members of the Bcl-2 family inhibit MMP remains a matter of debate. The present consensus is that antiapoptotic

members of the Bcl-2 family would simply act as inhibitors of their proapoptotic counterparts without any independent effects on other mitochondrial proteins. MMP suppression, in this case, could be either achieved by direct interaction with the pore-forming members of the Bcl-2 family, or indirectly by neutralizing BH3-only proteins (232). However, there are evidence to suggest that Bcl-2 and Bcl-xL carry out two functions on the mitochondria, namely, the inhibition of pore formation by Bax and Bak as well as the inhibition of pores formed by proteins from the permeability transition pore complex (PTPC), such as the voltage-dependent ion channel (VDAC) and adenine nucleotide translocator (ANT) (190, 208, 284, 285).

Evidence are emerging to strongly suggest that Bcl-2 family proteins have cellular functions beyond apoptosis regulation. For example, BAD regulates glucose-driven mitochondrial respiration, insulin secretion and glucose homeostasis *in vivo* (47, 48); whereas several Bcl-2 proteins regulate the  $\text{Ca}^{2+}$  stores at the endoplasmic reticulum and affect  $\text{Ca}^{2+}$  homeostasis (254). It is also possible these proteins may influence other homeostatic pathways, such as autophagy (159). These observations revealed that Bcl-2 family members are integral components of distinct cellular homeostatic pathways with functionalities separate from their capacity to regulate apoptosis (117, 118, 375). By being embedded in these processes, they serve as critical checkpoints for death when cellular homeostasis is violated.

### **1.13.2.1 The Pro-Survival Proteins**

The pro-survival proteins of the Bcl-2 family include Bcl-2, Bcl-w, Bcl-xL, Mcl-1 and A1. They possess up to four BH domains and all have similar 3D structures (303). The BH1, BH2 and BH3 domains interact to form a hydrophobic groove on the surface,

which is critical for the pro-survival function of these proteins by accommodating the BH3 domain of its pro-apoptotic partners. Although overexpression of any of the pro-survival Bcl-2 family members prevents the death induced by many apoptotic stimuli, suggesting significant functional redundancy, it is rare that a single pro-survival protein ensures the survival of a cell population under physiological conditions. T-cells, as an example, successively use Bcl-2 and Mcl-1, then A1 and Bcl-xL, then Mcl-1 and Bcl-xL, and finally Bcl-2 again to survive the different stages of their development (304). This may suggest either a selective expression of these genes in response to different survival signals, or that apoptotic signals come in different forms at each of these stages. Although it has been known for a long time that overexpressed pro-survival proteins prevent initiator caspase activation by prohibiting the release of Cyt c to the cytosol, the molecular mechanisms that these proteins use to protect mitochondrial integrity remain unclear. Pro-survival proteins, such as Bcl-xL, are proposed to sequester the activators and also neutralize the membrane-bound Bax to prevent them from homo-oligomerizing (12). A recent study has found Bcl-xL actually ‘retrotranslocate’ Bax from the mitochondrial membrane back to the cytosol as a mechanism to inhibit Bax mediated dysregulation of the mitochondria (61).

Genetic studies have helped to identify the physiological role of the prosurvival proteins in mice. The various phenotypes illustrate the different requirements for these proteins in different cell types and at various stages of development. Bcl-2-deficient mice develop polycystic kidney disease and profound lymphopenia and turn gray as a result of the loss of melanocyte stem cells (218, 331). These mice fail to thrive and die

early of renal failure. Loss of Bcl-xL causes massive apoptosis in the nervous and hematopoietic systems and embryos die at mid-gestation (217).

### **1.13.2.2 The BH3-Only Proteins**

The BH3-only proteins are pro-apoptotic and the only region that these proteins have in common with each other and their relatives of the Bcl-2 family is the BH3 domain (352). The mammalian BH3-only proteins currently known include Bad, Bid, Bik/NBK, Bim/Bod, Bmf, Hrk/DP5, Noxa and Puma/BBC3. Overexpression of BH3-only proteins promotes apoptotic death in many cell types, but requires the presence of either Bax or Bak for the death to occur (376).

All BH3-only proteins bind strongly to at least some pro-survival members of the family. The hydrophobic groove formed by the BH1, BH2 and BH3 domains at the surface of the pro-survival molecules serve as sites for interaction with the BH3 domain of BH3-only proteins. Some BH3-only proteins (Bim, Puma and Bid) bind to all the pro-survival molecules, whereas others have a more limited repertoire (33). Bad, for example, binds Bcl-2, Bcl-xL and Bcl-w, but not Mcl-1 or A1. Noxa, by contrast, binds Mcl-1 and A1, but not the others. The ability to bind its target protein and promote apoptosis is entirely dependent on the BH3 domain (28, 186, 353).

### **1.13.3 Bcl-Associated Death Promoter Protein**

Structural studies and mutational analysis have shown that direct activation of BAX and BAK through the neutralization of anti-apoptotic molecules are necessary for the induction of mitochondrial outer membrane permeabilization (155). Some BH3-only

proteins can interact with both anti- and pro-apoptotic molecules, but others such as BAD only bind and neutralize anti-apoptotic Bcl-2 partners (12, 33, 78, 128, 148, 158).

BAD promotes apoptosis by binding and neutralizing the pro-survival signal of Bcl-2, Bcl-xL and Bcl-W. Characterization of BAD-deficient and phosphorylation knock-in mouse models showed that inhibition of BAD's pro-apoptotic activity protected cells from apoptosis (255). The apoptotic activity of BAD is inhibited on phosphorylation by survival kinases (46). There are 3 evolutionarily conserved BAD phosphorylation sites at serine residues: S112, S136 and S155 (52, 171, 315, 334, 367, 372). The S155 site is positioned within the BAD BH3 domain, which binds the hydrophobic groove of anti-apoptotic partners (235, 366). The negative charge phosphorylation bestows on S155 at this position renders the interaction between the BAD BH3 helix and the hydrophobic groove of anti-apoptotic Bcl-2 energetically unfavorable.

A tiered phosphorylation model has been proposed based on evidence from combinatorial mutation studies, which showed that modifications at S136 and/or S112 are required prior to phosphorylation at S155 (52). Notably, S136 phosphorylation appears to be an apical event that is independent of the phosphorylation status of S112 or S155. Although both phosphorylated S112 and S136 can bind 14-3-3 proteins, phosphorylation at S136 appeared to be the more predominant 14-3-3-binding site (191). Mutational studies showed that substitution of S136 with alanine interfered with S155 phosphorylation, which suggests that interaction between 14-3-3 with phosphorylated S136 may induce conformational changes that allow access of S155 kinases to the BAD BH3 domain (52). The tiered phosphorylation model proposes that at least two serine

residues must be phosphorylated to completely neutralize the apoptotic activity of BAD. In corroboration of this theory, inhibition of either S136 or S155 phosphorylation impairs the growth-factor mediated protection of cerebellar granule neurons from apoptosis (52). However, constitutively phosphorylated BAD mutants at S155D or S155E confer protection from apoptosis regardless of the S112 and S136 phosphorylation status, suggesting that S155 phosphorylation is a major requirement for neutralizing the apoptotic function of BAD (52, 315).

BAD phosphorylation not only alters its association with anti-apoptotic proteins, but also influences its subcellular localization. Initial studies produced evidence to suggest that the association of phosphorylated BAD with 14-3-3 proteins caused its localization to shift from the mitochondria to the cytosol (366); on the other hand, dephosphorylated BAD translocates to the mitochondria and associates with anti-apoptotic Bcl-2 family partners. However, the regulation of BAD subcellular localization appears to be more complex. For example, a portion of phosphorylated BAD can reside at the mitochondria in healthy cells (47).

The pro-apoptotic activity of BAD is further modulated by caspase cleavage (40, 126, 281), including caspase-2, -3, -7, -8, -10 and granzyme B (40). N-terminally truncated BAD has a higher affinity for BCL-xL and the mitochondrial membrane compared to uncleaved protein. Moreover, the N-terminal truncation renders it more difficult to phosphorylate and less likely to interact with 14-3-3; thus, caspase-cleaved BAD is a more potent inducer of Cyt c release and apoptosis (40, 281). It remains to be determined whether or not BAD cleavage occurs selectively at the mitochondrial membrane. Truncated BAD is reported to enhance FAS-induced apoptosis analogous to

the effects of BID, which on caspase cleavage initiates a mitochondrial amplification loop during apoptosis induced by death receptors (160).

In addition to its well-known pro-apoptotic activity, BAD also plays significant roles in a variety of metabolic processes, including glucose metabolism and insulin secretion (47), and possibly cell cycle regulation (69). The pro-apoptotic activity of BAD is independent of its function in glucose metabolism and cell cycle regulation, as phosphorylated BAD maintains its ability to activate the glucokinase protein complex that leads to changes in glucose metabolism and insulin secretion (194). BAD's involvement in cell cycle regulation was recently reported in MCF7 breast cancer cells, where it was shown that overexpression of the protein in these cells inhibited the G<sub>1</sub> to S phase transition, and this activity is independent of its proapoptotic function (69). BAD activity in cell cycle inhibition appears to be regulated by different phosphorylation sites: S75 and S99 (69).

## **1.14 Influenza Virus–Induced Apoptosis**

Apoptosis has long been considered a host anti-viral response to block virus spread from the immediate infected area. However, accumulating evidence now strongly suggest many viruses manipulate the cell death signaling pathway to promote viral replication, including influenza viruses (166, 178, 199, 200, 222, 233, 268, 356, 357), HIV-1 (342), and West Nile Virus (WNV) (244, 272). Pro-apoptotic factors such as TRAIL, and the death receptor Fas and its ligand FasL are shown to be upregulated upon influenza virus infection, reportedly by the activation of NF-κB (356). When receptors to TRAIL and Fas signaling are blocked with soluble monoclonal antibodies, influenza virus titre is significantly reduced (356). A study reported that HIV-1 virus production

was enhanced upon expression of FasL (342). Caspase-3 activation was reported necessary for WNV-induced neuron cell death (272), and WNV-induced apoptosis has been shown to associate with the up-regulation of Bax gene expression (244). Caspase-3(-/-) mice were shown to be more resistant to lethal WNV infection compared to wild-type mice (272).

Influenza A viruses have been shown to induce apoptosis in a variety of cells (101, 313) through several pathways: PKR activation (81); upregulation of Fas (338); stress activation (166); and activation by TGF- $\beta$  (278). Signal induction through these pathways lead to the activation of transcription factors such as NF- $\kappa$ B and AP-1. In the context of a viral infection, apoptosis is generally considered to be an antiviral response of the host; however, it appears that influenza viruses have managed to manipulate this mechanism for their own benefit by the induction of proapoptotic factors such as TRAIL and FasL (356). Although the specific pro- and anti-apoptotic signals generated are reported to be dependent on the cell type involved, several viral proteins have clear effects on apoptosis (Figure 9). For example, the NA can activate TGF- $\beta$  (214, 278), and the ectopic expression of M1 and M2 also induces apoptosis (215). The M1 has also been shown to interact with caspase-8, but the consequence of such an interaction remains unclear (371). Notably, both pro- and anti-apoptotic signals have been described following the expression of the NS1 protein (277, 370), which suggest that the induction of apoptosis signaling may be tightly regulated by the virus.

Several lines of evidence showed that Bcl-2 family members have both pro- and anti-viral roles during influenza infection. For example, ectopic overexpression of anti-apoptotic Bcl-2 protein impaired virus production and inhibited influenza virus-induced

apoptosis (101, 233). However, conflicting roles have been reported for Bak and Bax. Bak is reported to have an anti-viral role in influenza replication and was downregulated upon viral infection (199). Paradoxically, Bax activation was necessary for efficient influenza virus propagation (199). Thus, these observations suggest that only a specific subset of the pro-apoptotic Bcl-2 family members positively contribute to influenza virus infection, and another subset may instead have anti-viral roles. A number of influenza virus strains are known to encode a unique viral protein called PB1-F2, which appears to function specifically in the induction of apoptosis. PB1-F2 localizes to the mitochondria, leading to the permeabilization of the mitochondrial membrane and the consequential release of Cyt c (31, 34, 364). As mentioned above, not all influenza viruses express PB1-F2, but expression and reverse genetics studies suggest this protein may enhance pathogenicity (42, 146). Moreover, studies suggest these effects of PB1-F2 are strain-specific and host-specific (reviewed in (146)). Although many questions remain regarding the underlying mechanism of PB1-F2 on viral pathogenicity, GST pull-down experiments do show that the PB1-F2 protein interacts with ANT3, present in the inner mitochondrial membrane, and VDAC1, present in the outer mitochondrial membrane (364). These 2 proteins are implicated in the induction of apoptosis via mitochondrial permeability. Presently, it is proposed that PB1-F2 acts through the mitochondrial permeability transition pore complex to induce apoptosis; additional evidence are now emerging to also suggest PB1-F2 may play a possible role in the down-regulation of the host immune response to influenza virus infections (364).

## 1.15 USP47 and Deubiquitylation

Post-translational modifications of cellular proteins by ubiquitin and ubiquitin-like proteins are an essential regulatory mechanism in many important biological processes such as apoptosis, vesicular trafficking, transcriptional activation, signal transduction, and intracellular proteolysis (60, 84, 170, 262, 346). The coordinate action of multiple ubiquitin-conjugating and deubiquitylating enzymes regulate the dynamic and reversible process of these post-translational modifications. Conjugation of ubiquitin and related molecules to proteins is catalyzed by the successive actions of three types of enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-protein ligase (E3) enzymes (112).

The reverse reaction, removal of the ubiquitin (Ub) and ubiquitin-like domains, is catalyzed by proteases called deubiquitylating enzymes (DUBs) (156), which are categorized into seven protease families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), sumo-specific proteases (SENPs), autophagins, otubains, ataxin-3/josephin ubiquitin proteases, and JAMM isopeptidases (37, 262, 351). Among the diverse family members of proteases, the USPs have garnered special interest due to the broad inclusion of proteases from different eukaryotic organisms and to the different functions ascribed to these enzymes in both normal and pathological conditions.

USPs are cysteine proteases that vary greatly in size and structural complexity (37, 259, 351). The amino acid sequence of these proteases contain several conserved regions that include those surrounding the Cys, His, and Asp/Asn residues, which form part of the catalytic triad of these enzymes. Additionally, the N-terminal and C-terminal extensions of USPs are proposed to function in cellular localization and substrate

specificity. USPs may act at multiple levels in the ubiquitin pathway (37, 351). These activities include the generation of free ubiquitin from precursor fusion proteins, the regulation of protein localization and substrate activity by removal of ubiquitin from a ubiquitylated substrate, removal or editing of polyubiquitin from target proteins to regulate substrate degradation by the proteasome, and recycling ubiquitin from disassembled chains by hydrolyzing the isopeptide-linked ubiquitin units (37, 259, 351).

USP protease activity affects a variety of biological and pathological processes such as regulation of cellular growth pathways (37), stabilization of the p53 tumor suppressor (162), or inhibition of chromatin-mediated gene silencing (211). The involvement of USP proteases with such diverse processes has stimulated the search for new family members that could play additional yet uncharacterized functions.

The resultant finding in mammalian systems revealed more than 50 distinct USP proteases, which indicates that this proteolytic system has acquired a high degree of complexity during eukaryote evolution. In support of this complexity, it was discovered that USP18 and USP21 actually targets other ubiquitin-related protein modifiers; USP18 specifically removes ISG15 from conjugated proteins (182) and USP21 hydrolyzes Nedd8 derivatives (82).

Among the 50 newly identified USP proteases, USP47 is suggested to be a novel interactor of the E3 ubiquitin ligase, Skp1/Cul1/F-box protein b-transducin repeat-containing protein ( $SCF^{b-Trcp}$ ) (249). The authors found that both  $\beta$ -Trcp1 and  $\beta$ -Trcp2 bind specifically to USP47. Point mutations introduced into the  $\beta$ -Trcp WD-repeat region completely abolished USP47 binding, which indicates this to be an E3-substrate-type interaction. However, unlike canonical  $\beta$ -Trcp substrates, silencing of  $\beta$ -Trcp or

modulation in a variety of processes, such as cell-cycle progression, DNA damage checkpoint responses or the TNF pathway activation did not affect USP47 protein levels. Interestingly, siRNA-mediated depletion of USP47 in U2OS cells induced accumulation of Cdc25A and decreased cell survival. This study proposed USP47 as a novel  $\beta$ -Trcp interactor that regulates cell growth and survival (249).

On the other hand, Parsons *et. al.* identified USP47 as the major enzyme involved in deubiquitylation of the key BER DNA polymerase (Pol  $\beta$ ) (246). These authors demonstrated that USP47 is required for stability of newly synthesized cytoplasmic Pol  $\beta$  that is used as a source for nuclear Pol  $\beta$  involved in DNA repair. They further showed that knockdown of USP47 caused an increased level of ubiquitylated Pol  $\beta$ , decreased levels of Pol  $\beta$ , and a subsequent deficiency in BER, leading to accumulation of DNA strand breaks and decreased cell viability in response to DNA damage in HeLa cells (246).

Interestingly, USP47 was among the 12 DUBs identified using a siRNA library approach that are necessary for aspects of the hepatocyte growth factor (HGF)-dependent scattering response of A549 cells (26). Unlike Parsons *et. al.* (246) and Peschiaroli *et. al.* (249), this study showed with an MTS assay that cell proliferation and viability was not significantly affected following DUB knockdown with siRNA in A549 cells (26). It is known that USP47 is only produced at significant levels by one or a few tissues (259) and, from the reported studies, suggest that its specific physiological role may be cell-type dependent.

## 1.16 TNFSF13 and TNFSF12-13

### 1.16.1 TNFSF13

TNF is a family of structurally related molecules closely connected to the regulation of cell differentiation and death pathways. TNFSF13 (a proliferation-inducing ligand; APRIL) is categorized as a new member of the TNF family based on structural analyses (88). The *tnfsf13* gene is flanked within 1 kb by *tnfsf12* (also known as TWEAK), contains six exons, five introns and is mapped to human chromosome 17 (17p13.1) (Figure 10A). The *tnfsf13* gene is transcribed into 4 transcripts: (1) a 2276 bp transcript designated as  $\alpha$ -TNFSF13 with a single, continuous open reading frame predicted to encode a 250-amino acid protein; (2)  $\beta$ -TNFSF13 is a 2228 bp transcript with an open reading frame predicted to encode a protein of 234-amino acids; (3)  $\gamma$ -TNFSF13 is composed of 2095 bp predicted to encode a 257-amino-acid protein; and (4) a reported  $\Delta$ -TNFSF13 variant (940 bp) encoding for a yet to be determined protein (123).

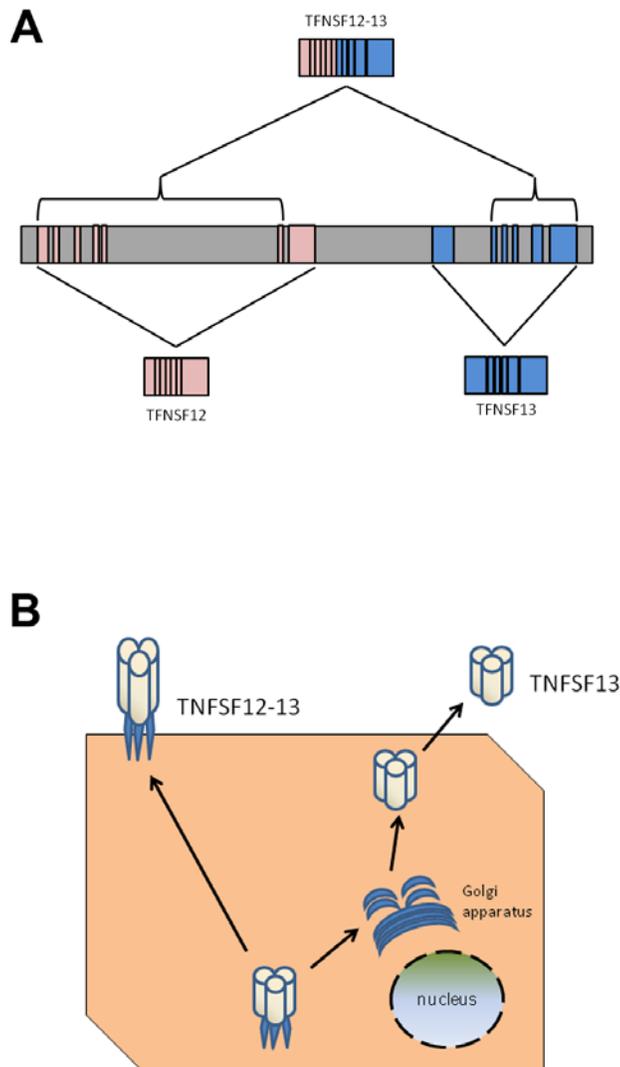
TNFSF13 transcripts are found at low levels in normal tissues with the greatest expression observed in peripheral blood lymphocytes (PBLs) (88). In contrast, leukemia cell lines and lymphoid tissues are reported to have higher mRNA levels (88). Moreover, endogenous TNFSF13 protein can readily be detected in human myeloid leukemia cell lines such as U937 and Mono Mac 1 4 (258).

The  $\alpha$ -TNFSF13 transcript translates to a type II transmembrane protein with a predicted cytoplasmic domain, a hydrophobic transmembrane region and an extracellular domain of 201 amino acids. TNFSF13 is processed and cleaved in the Golgi apparatus

by a furin-convertase enzyme into a secretory protein (sTNFSF13); therefore, it is not a membrane-anchored protein (173) (Figure 10B). However, one study has recently demonstrated that macrophages express a membrane-bound form of TNFSF13 (157). The predicted molecular weight of TNFSF13 is 27 kDa, but post-translational modification into soluble TNFSF13 reduces its molecular weight to only 17 kDa. TNFSF13 shares 50% similarity with BAFF, a B-cell activating factor also belonging to the TNF family, and can form trimers like others of the TNF ligand family members. TNFSF13 is predicted to harbour two N-glycosylation sites and a mature NH<sub>2</sub>-terminal sequence encoding for a cluster of basic amino acid residues that suggests TNFSF13 could interact with proteoglycans (109).

TNFSF13 is suggested to have a diverse, and conflicting, biological role that involves growth-stimulating activity in tumour cell lines (88), a pro-apoptotic effect (123) and a protective effect against death ligand-induced apoptosis (269). Hahne *et. al.* (88) concluded in their study that TNFSF13 induced cell proliferation, which could promote tumor growth, through a novel receptor. In a different study, TNFSF13 was shown to have a protective effect against death-induced apoptosis (269), which was associated with an upregulation of X-linked inhibitor of apoptosis (XIAP), a member of the inhibitor-of-apoptosis (IAP) family thought to block death via indirect inhibition of caspases (269).

In contrast, Kelly *et. al.* (123) found that purified, FLAG-tagged TNFSF13 caused Jurkat cell death with kinetics that paralleled FasL (124). *In vitro* binding experiments demonstrated that TNFSF13 co-precipitated Fas and HVEM and suggested TNFSF13 as an alternate ligand for these receptors. Moreover, these authors identified two TNFSF13 splice variants designated TRDL-1b and TRDL-1g that differed by two small deletions



**Figure 10. TNFSF13 and TNFSF12-13 structure.** Illustration showing (A) the portion of human chromosome 17 (17p13.1) that contains the *tnfsf12* and *tnfsf13* open reading frames in pink and blue, respectively. Transcripts of these 2 genes and the hybrid transcript of TNFSF12-13 are shown below and above the chromosome, respectively. (B) Diagram demonstrating post-translation processing of TNFSF13 and TNFSF12-13 proteins. TNFSF13 is processed in the golgi apparatus where the transmembrane domain is removed, which results in a secreted protein. TNFSF12-13 is anchored to the plasma membrane by its transmembrane domain.

within the exon (123). This led them to suggest that deletion of a significant portion of the TNFSF13 NH2 terminus could alter its activity and receptor binding specificity, and, therefore, elicit different responses. Thus, both alternative splicing and polymorphisms may affect the generation of functional TNFSF13. Further studies to determine the trimerization state and receptor binding capabilities of the full-length protein compared with various NH2-terminal truncations will help clarify the physiological role of TNFSF13.

In addition, other studies have shown that TNFSF13 can act as a costimulator of primary T and B cells (363), suggesting a role for this ligand in immune regulation. Transgenic mice expressing TNFSF13 in T-cells have been generated (300) that appear normal and do not show any B-cell hyperplasia. In contrast, T-cells expressing TNFSF13 in vitro showed enhanced survival correlated with elevated expression levels of the anti-apoptotic protein Bcl-2; this characteristic is, however, observed in activated CD4 T cells in vivo as well. Aside from direct effects on T-cells, transgenic expression of TNFSF13 affects T-cell independent humoral responses. In a study by Litinskiy *et. al.* (170), dendritic cells were shown to upregulate TNFSF13 expression upon exposure to IFN- $\alpha$ , IFN- $\gamma$ , or CD40L, which induces class-switching in B cells. Together, these results suggest that TNFSF13 is able to critically link the innate and adaptive immune responses.

Unfortunately, however, this theory was not corroborated by deletion of the TNFSF13 gene as conflicting results have been reported with TNFSF13 knockout (KO) models. For example, deletion of the TNFSF13 gene resulted in embryonic lethality due to a defect in the development of the heart (reviewed in (179)), which could implicate an important and unique function for TNFSF13 during development. On the contrary, it has

been reported that a mouse deficient for TNFSF13 was actually successfully generated by another study (330). This KO strain did not show histological abnormalities and, more importantly, no defects in T or B cell responses (330). Presently, it remains unclear how these conflicting findings may be resolved.

### **1.16.2 TNFSF12-13**

TNFSF12-13 (TWE-PRIL) was discovered during the analysis of the molecular regulation of *tnfsf13* gene expression, which identified an endogenous hybrid transcript between TNFSF12 and TNFSF13 mRNAs in human primary T-cells and monocytes (258).

TNFSF12-13 mRNA encompasses TNFSF12 exons 1-6 fused to TNFSF13 exons 2-6 via splice donor/acceptor sites of TNFSF12 and TNFSF13 mRNAs (258) (Figure 10A). This mRNA gives rise to a hybrid protein composed of TNFSF12 cytoplasmic and transmembrane domains fused to the TNFSF13 C-terminal domain (258). Both TNFSF12-13 mRNA and protein were detected in primary human T lymphocytes and monocytic cell lines. As a result of its unique composition, TNFSF12-13 protein is membrane anchored like TNFSF12 but presents the TNFSF13 receptor-binding domain at the cell surface (258). This hybrid protein has been determined to be a biologically active ligand that stimulates cycling in T- and B-lymphoma cell lines (258). TNF ligands are characterized by their C-terminal domain, which mediates their binding to the cognate receptor. TNFSF12-13 and TNFSF13 share their receptor-binding domain, which suggests both may bind the same receptors (258). Regardless, the different cellular localizations of TNFSF12-13 and TNFSF13 suggest they may also exert distinct biological roles (Figure 10B). However, aside from limited data on their role in T- and

B-lymphoma cell lines, very little else is known about the physiological and molecular activities of TNFSF12-13. As of present, there are no prior reports that associate this hybrid protein with any known pathogens.

## **1.17 MxB**

Mx proteins were initially discovered in an inbred mouse strain (A2G) that showed a high degree of resistance against infection with influenza A viruses (167, 267). Later, evidence for an Mx-related protein in human was obtained with a monoclonal antibody against mouse Mx1 that cross-reacted with an IFN-induced protein in human cells. Subsequently, two human Mx genes were identified and shown to code for proteins called MxA (76 kDa) and MxB (73 kDa) (1). Both human Mx genes are located in close proximity to each other on the distal part of the long arm of chromosome 21 (21q22.3) (105). Despite this, sequence homology showed that the antivirally active human MxA protein is more closely related to the antiviral porcine Mx1, canine Mx1, bovine Mx1 and ovine Mx proteins than to its non-antiviral human MxB counterpart, which in turn clusters with canine Mx2 (reviewed in (91)). Specifically, human MxA and mouse Mx2 protein are the most closely related with a 77% sequence identity, whereas human MxA and MxB are only 63% identical (1). Human MxB and mouse Mx1 proteins are the least closely related with only 56% sequence identity (1).

Further studies resulted in the classification of Mx proteins as a distinct subclass of the dynamin superfamily of high molecular weight GTPases. Accordingly, Mx proteins display a similar structural organization with a GTPase domain at the N-terminus, a “central interactive domain” (CID) in the middle, and an effector domain with leucine zipper motifs (LZ) at the C-terminal end (91). The CID and the effector domains are

connected by a flexible hinge that is proteinase K-sensitive, and back-folding of the LZ region on CID resulted in increased GTPase activity, implying that the C-terminal part acts as a GTPase effector domain (GED) (280). The N-terminal region is observed to be highly conserved with a tripartite GTP-binding consensus motif, whereas the C-terminal region is divergent. Like dynamin, Mx GTPases have a relatively low affinity for GTP and a high intrinsic rate of GTP hydrolysis (297). Unlike dynamin, however, Mx GTPases also have unique amino-terminal extensions of 25-90 residues that may mediate other specific functions (91). Also, unlike dynamin, Mx proteins lack a proline/arginine rich domain (PRD) and pleckstrin homology domain (PH) implicated in membrane binding of classical dynamin (280).

As with many dynamin-like large GTPases, purified Mx proteins also form high molecular weight homo-oligomers and self-assemble into ring-like, helical structures (133, 204). Homo-oligomerization occurs through the binding of the LZ region of one molecule to the CID of a second neighbouring molecule (90). Self-assembly of Mx proteins is reported to be critical for GTPase activity, protein stability, and recognition of viral target structures (115).

The three MxB mRNA variants of 2.8, 3.1 kb, and 4 kb were identified in human fetal liver (HFL) cells treated with IFN- $\alpha_2$  or IFN- $\beta$ , but were barely detectable in HFL cells treated with IFN- $\gamma$ . However, even in IFN-treated HFL cells, MxB RNAs were several-fold less abundant than MxA RNAs (1).

Human MxB differs from MxA in that MxB displays an NLS-like sequence in its amino-terminal extension and in lacking detectable antiviral activity. Furthermore, MxB

is localized to the intranuclear and/or cytoplasmic face of nuclear pores and may play a role in regulating nucleocytoplasmic transport and cell-cycle progression (129, 202, 203).

## Part II | Hypothesis and Objectives

Influenza virus, a significant etiological agent with the potential to develop into catastrophic pandemic outbreaks, has been a major focus of many recent studies. Despite its importance as human pathogens, presently approved anti-viral drugs for the treatment of influenza infection are limited to only 2 viral protein targets, but there are increasing global reports of anti-viral resistant isolates of seasonal strains and also the 2009 pandemic SOIV H1N1 (10, 291). Coupled with the continual threat posed by the highly-pathogenic avian influenza virus H5N1 and possible future pandemic outbreaks (242, 276), there is now a strong need for further investigation of this complex virus and its pathogenicity with the goal that novel targets can be uncovered for future therapeutic development. However, despite intense research in this field in recent years, significant mechanisms in most host-viral interactions involved in disease establishment and progression remain elusive.

Viruses are obligate parasites that rely heavily on the machinery of their host for productive replication, which is also an Achilles' heel that could be targeted for treatment. In addition to manipulating host factors that promote viral replication, influenza virus is known to influence host signaling pathways that lead to extensive cell death and tissue damage during the late course of an infection (98), and the induction of cell death has been suggested to be a catalyst for aberrant host immune response during disease progression (98). I hypothesize that there are specific non-essential cellular genes required for influenza virus replication, and a subset of these plays a critical role in all influenza replication and pathogenicity independent of viral subtypes. In my study, "non-essential" implies interference with the functions of these genes will not have any

apparent significant detrimental affects on normal host cell physiology. Thus, my overall objective, in this study, was to set out to identify not only host factors that contribute to the replication of influenza viruses but also to identify genes required for virus-induced cell death.

The availability of human genome-wide lentiviral-based shRNAi libraries provides a foundation to identify host genes important in virtually any process on a global scale. This approach will allow for a more rapid and comprehensive discovery of molecular components underlying influenza pathogenicity as well as lead to a systems-level understanding of the processes. With the set-up of my RNAi global screen parameters, which are discussed in further details under the Materials and Methods section, I expected to identify both populations of genes that promote and antagonize influenza virus infection, while permitting the survival of the infected host. The identification of genes involved in influenza infection may allow for the development of drugs against specific targets that not only will inhibit viral replication but also reduce virus-induced tissue damage during disease progression. Moreover, this rapid screening will lead to the identification of novel host proteins that may serve as potential drug targets to accelerate the development of new classes of antiviral drugs for much urgently needed treatment. Overall, my study is driven by the following three objectives:

- (1) Identify specific non-essential cellular genes vital for influenza virus replication and virus-induced cell death.
- (2) Determine a subset of cellular factors that plays a critical role in the replication of different influenza virus strains

(3) Delineate the molecular mechanisms of these cellular factors in the context of influenza virus replication

## Part III | Materials and Methods

### 3.1. Cells and Virus

HEK-293T cells and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1· non-essential amino acids (Gibco®, Invitrogen), 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine. Influenza virus strains A/New York/55/2004 (H3N2) (NY55), A/Puerto Rico/8/1934 (H1N1) (PR8) and A/California/07/2009 (H1N1) (SOIV) were grown in 10-day old embryonated chicken eggs and titered on MDCK cells. High titre virus stocks were obtained by growing virus in MDCK cells; viruses were concentrated at 64,000 ×g for 2 h at 4°C.

### 3.2. Influenza Virus Infection and Plaque Assay

Sets of transduced or transfected A549 cells were infected with influenza virus strains A/New York/55/2004(H3N2) at an MOI of 1 (shRNA) or 0.1 (siRNA) PFU/cell; or with A/PR/8/34(H1N1) or SOIV at an MOI of 0.1. Cell monolayers were washed twice with 1X phosphate buffered saline (PBS; 137 mM NaCl, 0.3 mM KCl, 0.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM KH<sub>2</sub>PO<sub>4</sub>) and infected with viruses diluted in gel saline (137 mM NaCl, 0.2 mM CaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 19 mM HBO<sub>3</sub>, 0.1 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 0.3%(w/v) gelatin). At 72 hpi, supernatants were harvested and virus yield was titrated by plaque assay on MDCK cells. MDCK cell monolayers were washed twice with 1× PBS, infected with 1:10 serial dilutions of viruses in gel saline, and overlaid with a mixture of 0.6% type-I agarose (Sigma Aldrich), 1× DMEM (with no fetal calf serum), and 2.5 µg/ml trypsin for 3 days. All influenza virus infections occurred at 35°C in 5% CO<sub>2</sub>

humidified environment, including the plaque assay. For western and immunofluorescence assays, cells were infected with NY55 at MOI 1 and processed at 24 hpi.

### **3.3. Human Whole-Genome Screen**

A549 cells were transduced at MOI 0.3 with each of 7 Decode RNA GIPZ Lentiviral Positive Screening Library pools according to manufacturer's protocol (Open Biosystems). After 72 h, cells were washed twice with 1x phosphate-buffered saline (PBS) and infected with NY55 at an MOI of 7 PFU per cell. At 72 hpi, cells were washed twice with 1x PBS and harvested. Genomic DNA was isolated by phenol/chloroform extraction followed by ethanol precipitation.

### **3.4. Lentivirus Packaging and Transduction**

*E. coli* clones containing individual human shRNAmir or non-targeting shRNAmir control in pGIPZ plasmids were obtained from Open Biosystems (Table 1-4). These *E. coli* clones were cultured in 2X LB broth (low salt; 2%(w/v) LB-Broth-Lennox; 1%(w/v) Peptone; 0.5%(w/v) Yeast Extract; 100 µg/ml ampicillin) at 37°C overnight with shaking at 250 rpm. Plasmids were isolated with QIAGEN Maxiprep kit following manufacturer's protocol. Individual shRNAs were packaged into lentivirus particles by co-transfection of each shRNAmir-pGIPZ with pMD2.G and psPAX2 (Addgene plasmids 12259 and 12260, respectively) in HEK-293T cells at a ratio of 2:2:1, respectively, with transfection reagent Arrest-In (Open Biosystems). Lentivirus-containing supernatants were harvested at 48 hours (h) and 72 h post-transfection. After the last harvest time point, supernatants were centrifuged at 64,000 xg for 1.5 h at 4°C,

and lentivirus-containing pellets were resuspended in serum-free 1X DMEM. Lentivirus particles were titered on A549 cells and GFP-expressing cell colonies enumerated with Axio Observer.Z1 fitted with EC Plan-NEOFLUAR 10x/0.3 Ph1 M27 objective (Carl Zeiss MicroImaging GmbH, Germany). Stable A549 knockdown cells were produced by transducing with lentivirus at an MOI of 0.5. At 72 h post-transduction, 3 ug/ml puromycin (Sigma) was added to the media. Cells were passaged over a period of 2 weeks in puromycin-supplemented completed 1× DMEM media to select for transductants and establish a stably transduced cell line.

### **3.5. siRNA Transfection and siRNA array**

A549 cells were treated with 25 nM of each of 4 ON-Targetplus siRNA (Dharmacon) targeting individual host genes or with ON-Targetplus non-targeting siRNA control (Table 1-4). siRNAs were introduced into cells with Lipofectamine RNAiMAX (Invitrogen). 1× serum-free Opti-MEM media (Invitrogen) was used to dilute the siRNAs and RNAiMAX separately; the two diluants were combined and incubated at room temperature for 20 minutes. After incubation, the transfection mixture was added to the cells. Each cell set was re-treated with the same siRNA 24 h later. After a further 24 h (48 h from the first transfection), cells were infected with virus. Reverse transfection of siRNA array (Dharmacon) was carried out according to manufacturer's directions. Briefly, each well, in replicate 96-well plates, was transfected with 0.25 ul of Dharmafect 1 (Dharmacon) and incubated at room temperature for 30 min. After the incubation,  $5 \times 10^3$  A549 cells were added to each well and incubated for 48 h and then infected with virus.

**Table 1. shRNA and siRNA sequences for BAD.**

<b>Oligo ID</b>	<b>shRNA</b>	<b>siRNA</b>
V2HS_262043	GACTTGGACTTGGATGTAA	
V2HS_243025	CAGTGACCTTCGCTCCACA	
V2HS_201511	GAGTTTGTGGACTCCTTTA	
V2HS_202976	GTGCTCACTACCAAATGTT	
V2HS_15289	CTCACTACCAAATGTTAAT	
J-003870-09		GAUCGGAACUUGGGCAGGG
J-003870-10		CAGAGUUUGAGCCGAGUGA
J-003870-11		GAGCUCCGGAGGAUGAGUG
J-003870-12		UUGUGGACUCCUUUAAGAA

**Table 2. shRNA and siRNA sequences for MxB.**

<b>Oligo ID</b>	<b>shRNA</b>	<b>siRNA</b>
V2HS_152031	GACAAGATGTTCTTTCTAA	
J-011736-05		GAGCACGAUUGAAGACAUA
J-011736-06		GGAGAAUGAGACCCGUUUA
J-011736-07		GAAUUUACCGGCUCACUCA
J-011736-08		GGGACGCCUUCACAGAAUA

**Table 3. shRNA and siRNA sequences for TNFSF12-13 and TNFSF13.**

<b>Oligo ID</b>	<b>shRNA*</b>	<b>siRNA</b>
V2HS_17313	GCCGCCCTCTGCTAGGGAA	
V2HS_17314	GATATTCTGAGTGTCATAA	
V2HS_17316	GGTGCCTTCGCAGTCAAAT	
V2HS_17317	GAGACTCTATTCCGATGTA	
V2HS_17318	CTCCAGAGATGTAGCTATT	
J-032530-05		GGGCAAGGGCGAAACUUA
J-032530-06		GCAGGUGUCUCCAUUUAC
J-032530-07		UGACAGAGGUGAUGUGGCA
J-032530-08		GGAGUUUAUCUGCUGUAUA
J-011523-05		GGGCAAGGGCGAAACUUA
J-011523-06		GCAGGUGUCUCCAUUUAC
J-011523-07		UGACAGAGGUGAUGUGGCA
J-011523-08		GGAGUUUAUCUGCUGUAUA

\* shRNA targets both TNFSF12-13 and TNFSF13

**Table 4. shRNA and siRNA sequences for USP47.**

<b>Oligo ID</b>	<b>shRNA</b>	<b>siRNA</b>
V2HS_174637	GAATCTGTCTTGAAACCAA	
V2HS_174639	CGCAATACATGCAAGATAA	
V2HS_174641	GGATTCCTTTGGATGATAT	
V2HS_174642	GATTTAGACTGGAATCCTA	
V2HS_218228	CAATGACTTGCTATTTGAA	
J-006093-05		GCAACGAUUUCUCCAUGA
J-006093-06		CAACAUGUCAGCAGGAUAA
J-006093-07		GCUGUCGCCUUGUUAAAUA
J-006093-08		CGCAAUACAUGCAAGAUAA

### **3.6. Western Blot**

Cell lysates were obtained by lysing cells in RIPA buffer (50mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% SDS) with complete protease inhibitor (Roche). 20 µg of lysate was solubilised in 1X protein sample buffer (0.3 M Tris, pH 6.8; 50% Glycerol; 0.3 M SDS; 5 mM DTT; 10 mM Bromophenol blue) and subjected to electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE), transferred to Immobilon-P PVDF membranes (Millipore), and blotted with antibodies indicated in Table 5. Cytoplasmic lysate for caspase immunoblot was obtained by lysing cells in Caspase Lysis buffer (20 mM Tris-Hcl, pH 7.5; 0.5% NP-40, 0.5 mM PMSF, 100 µM β-glycerol-3-phosphate; protease inhibitor cocktail (Roche)). 40 µg of lysate was solubilised in 1X protein sample buffer and electrophoresed on 15% SDS-PAGE and transferred to Immobilon-P<sup>sq</sup> PVDF membranes (Millipore). Blots were subjected to secondary antibodies conjugated with HRP (Table 5) and detected with in-house enhanced chemiluminescence (ECL) reagent. Blot image and protein band quantification were obtained with Alpha Innotech FluorChem® Q Imaging System and processed using Adobe® Photoshop®.

### **3.7. Caspase-Glo 3/7 Assay**

Caspase-3/7 activity was detected using Caspase-Glo® 3/7 assay (Promega), according to manufacturer's protocol. 5000 cells were seeded per well in 96-well, white-walled plates. Cells were infected with NY55 virus at MOI 1. As a positive control for caspase activity, cells were treated with 1 µM staurosporine for 24 h. Caspase substrate was added to each well at 72 hpi and 24 h post-treatment for staurosporine.

Luminescence was detected with a BioTek Synergy<sup>TM</sup> 4 plate reader and data was processed with Gen5<sup>TM</sup> software.

### **3.8. Cytochrome c Release Assay**

At specific time points post-infection, cell monolayers were washed once with 1· PBS and harvested with a cell scraper. Cells were pelleted at 180 ×g at 4°C for 5 min. Cell pellets were resuspended in digitonin lysis buffer (0.5 mM NaCl, 6.7 μM NaH<sub>2</sub>PO<sub>4</sub>, 53 μM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM sucrose, 19 μg digitonin) with complete protease inhibitor (Roche). The resuspended cell pellets were incubated on ice for 10 min, then cell lysates were centrifuged at 14,000 ×g for 10 min at 4°C. The supernatants (cytoplasmic fraction) were transferred to clean tubes. The lysate pellets were resuspended in Triton X-100 lysis buffer (0.02% Triton X-100, 5 mM Tris, pH 8.0) with complete protease inhibitor. Resuspended pellets were sonicated for 20 sec and then centrifuged for 10 min at 14,000 ×g. These supernatants (mitochondria fraction) were transferred to clean tubes. Both fractions were used for western blotting.

### **3.9. PCR and Real-Time PCR**

PCR was carried out on isolated genomic DNA using Expand High Fidelity polymerase mix (Roche) and product was purified from polyacrylamide gels. PCR primers used for Illumina sequencing are proprietary properties of Illumina® and Canada's Michael Smith Genome Sciences Centre (MSGSC; Vancouver, British Columbia). Pooled cDNA was sequenced by high-throughput Illumina® sequencing technology at MSGSC. Knockdown efficiency of target cellular mRNA was determined via real-time PCR. Total mRNA was isolated using RNeasy Mini Kit (QIAGEN)

**Table 5. List of antibodies used in my study.**

<b>Antibody</b>	<b>Clone</b>	<b>Host</b>	<b>Assay*</b>	<b>Source</b>
<i>Primary Antibodies:</i>				
Monoclonal $\alpha$ -NS1 (3F5)	3F5	Mouse	WB	(261)
Monoclonal $\alpha$ -NP	F26-9	Mouse	WB	(349), gift from Dr. Mingyi Li
Polyclonal $\alpha$ -HA		Rabbit	WB	Rockland
Monoclonal $\alpha$ -BAD	C-7	Mouse	WB, IP	Santa Cruz
Monoclonal $\alpha$ -cleaved caspase-3 (Asp175)		Mouse	WB	R&D Systems
Monoclonal $\alpha$ -phospho-BAD (Ser136)		Rabbit	WB	Cell Signaling
Monoclonal $\alpha$ -phospho-BAD (Ser112)		Mouse	WB	Cell Signaling
Polyclonal $\alpha$ -cleaved PARP (Asp214)		Rabbit	WB	Cell Signaling
Monoclonal $\alpha$ -cytochrome c		Rabbit	WB	Cell Signaling
Polyclonal $\alpha$ -cleaved caspase-7 (Asp198)		Rabbit	WB	Cell Signaling
Polyclonal $\alpha$ -human Mx2	N-17	Goat	WB	Santa Cruz
Polyclonal $\alpha$ -human Mx1		Rabbit	WB	Sigma Aldrick
Polyclonal $\alpha$ -beta-actin		Rabbit	WB	Cell Signaling
Monoclonal $\alpha$ -Bcl-2		Rabbit	IF, IP	R&D Systems
Monoclonal $\alpha$ -Bcl-xL	54H6	Rabbit	IF, IP	Cell Signaling
<i>Secondary Antibodies:</i>				
Polyclonal $\alpha$ -rabbit HRP			WB	Cell Signaling
Polyclonal $\alpha$ -mouse HRP			WB	Cell Signaling
Alexa Fluor® 546			IF	Invitrogen***
Alexa Fluor® 633			IF	Invitrogen
Alexa Fluor® 594			IF	Invitrogen
Cy2			IF	Jackson
				ImmunoResearch
DAPI**			IF	Invitrogen

\* WB, western blot; IF, immunofluorescence assay; IP, immunoprecipitation

\*\* DAPI is a stain

\*\*\* Life Technologies

according to manufacturer's protocol. 500 ng of purified mRNA was used to generate cDNA with random hexamer primers (Applied Biosystems) and SuperScript® II Reverse Transcriptase (Invitrogen) according to manufacturer's protocol. Real time PCR reaction mix (25 µl) consisted of: 12.5 µl of SYBR® Green PCR Master Mix (Invitrogen), 0.5 µl cDNA template, and 1 µl each of 100 µM forward and reverse primers (Table 6). Reactions were run in duplicate on Applied Biosystems 7300 Real-Time PCR System. The cycling conditions were as follows: 50°C for 2 min., 95°C for 2 min., and 50 cycles of (95°C for 15 sec. and 60°C for 30 sec.). C<sub>t</sub> values were normalized to 18S rRNA control and compared to non-targeting ("non-targeting") sh/siRNA negative control.

### **3.10. Cell Viability Assay**

Cell viability was determined using Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's protocol or trypan blue exclusion assay. For trypan blue exclusion assay, ~ 1x10<sup>6</sup> infected or uninfected cells were stained with 20 µl of trypan blue solution and ~ 14 µl of the stained cells were placed on a hemocytometer. A total of 200 cells were counted and the percentage of viable cells was calculated with the following formula:

$$\% \text{ viable cell} = \frac{(\text{total number of cells counted}) - (\text{total number of dead cells})}{\text{total number of cells counted}} \times 100$$

### **3.11. Immunofluorescence Assay**

At 24 hpi, cells were fixed with 4% paraformaldehyde for 15 min., permeabilized with 0.1% (v/v) Triton X-100 for 5 min., blocked with 1% BSA in PBS for 1 h and probed with primary antibody in 1% BSA/PBS at 4°C overnight (Table 5). Afterwards,

cells were incubated with appropriate secondary antibodies in 1% BSA in PBS for 1 h at room temperature (Table 5). Immunofluorescence microscopy was performed with Axio Observer.Z1 fitted with Plan-Apochromat 63x/1.40 Oil DIC M27 objective (Carl Zeiss MicroImaging GmbH, Germany), AxioCamHR3 and AxioVision imaging software. Images were collected at 1388 x 1040-pixel resolution. The images were rendered on Adobe® Photoshop®. Manders Overlap Coefficient and Weighted Co-localization Coefficients for the confocal images were determined by Zen software (Zeiss) according to previously described mathematical algorithms (183).

### **3.12. Immunoprecipitation Assay**

At 24 hpi, proteins were crosslinked *in vivo* by incubating infected cell monolayers with 0.45%(v/v) formaldehyde/1× PBS solution for 10 min, after which crosslink reaction was stopped with 100 mM glycine for 5 min. Cells were harvested and lysed in IP Lysis Buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.5% NP-40; 1 mM phenylmethylsulfonyl fluoride; protease inhibitor cocktail). IP reaction consisted of 500 µg of protein lysate, 5 µg of antibody (Table 5). 100 mM NaCl, and 50 µl of Protein G agarose bead slurry (Pierce). IP reaction without antibody was included as negative control.

### **3.13. Bioinformatics Analysis**

Sequences were analysed by an in-house computer algorithm. Genes were functionally categorized using PANTHER ontology system (206, 318). Data analysis, network and functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)). Molecules are represented as

**Table 6. Primers for quantitative real time PCR.**

Target Gene	Sequence (5' → 3')		Amplicon size (nt)
<b>18S rRNA(84)</b>	Fwd:	TGAGAAACGGCTACACATC	112
	Rev:	TTACAGGGCCTCGAAAGAGT	
<b>MxB</b>	Fwd:	CTGGAGGCACTGTCAGGAGT	60
	Rev:	CGGACACCTGGTTACGATTC	
<b>TNFSF12-13</b>	Fwd:	GCCAGATCGGGGAGTTTATAGT	71
	Rev:	CAGGGCATCGGAACTCTG	
<b>USP47</b>	Fwd:	ACCAACTGGTCCCGAAAGA	109
	Rev:	TCTTTATCTGTCAAATGCAGAAAGTT	
<b>BAD</b>	Fwd:	ACCCGGCAGACAGATGAG	66
	Rev:	CTTCCTCTCCACCGTAGC	
<b>TNFSF13</b>	Fwd:	AAGGGTATCCCTGGCAGAGT	129
	Rev:	GCAGGACAGAGTGCTGCTT	

nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes (e.g., P for phosphorylation, T for transcription).

### **3.14 Statistical Analysis**

Statistical analyses were calculated using ANOVA and Student's t-test in SigmaPlot® software.

## Part IV | Results

### 4.1. Influenza Virus shRNA Global Screen 4.1.1 Host

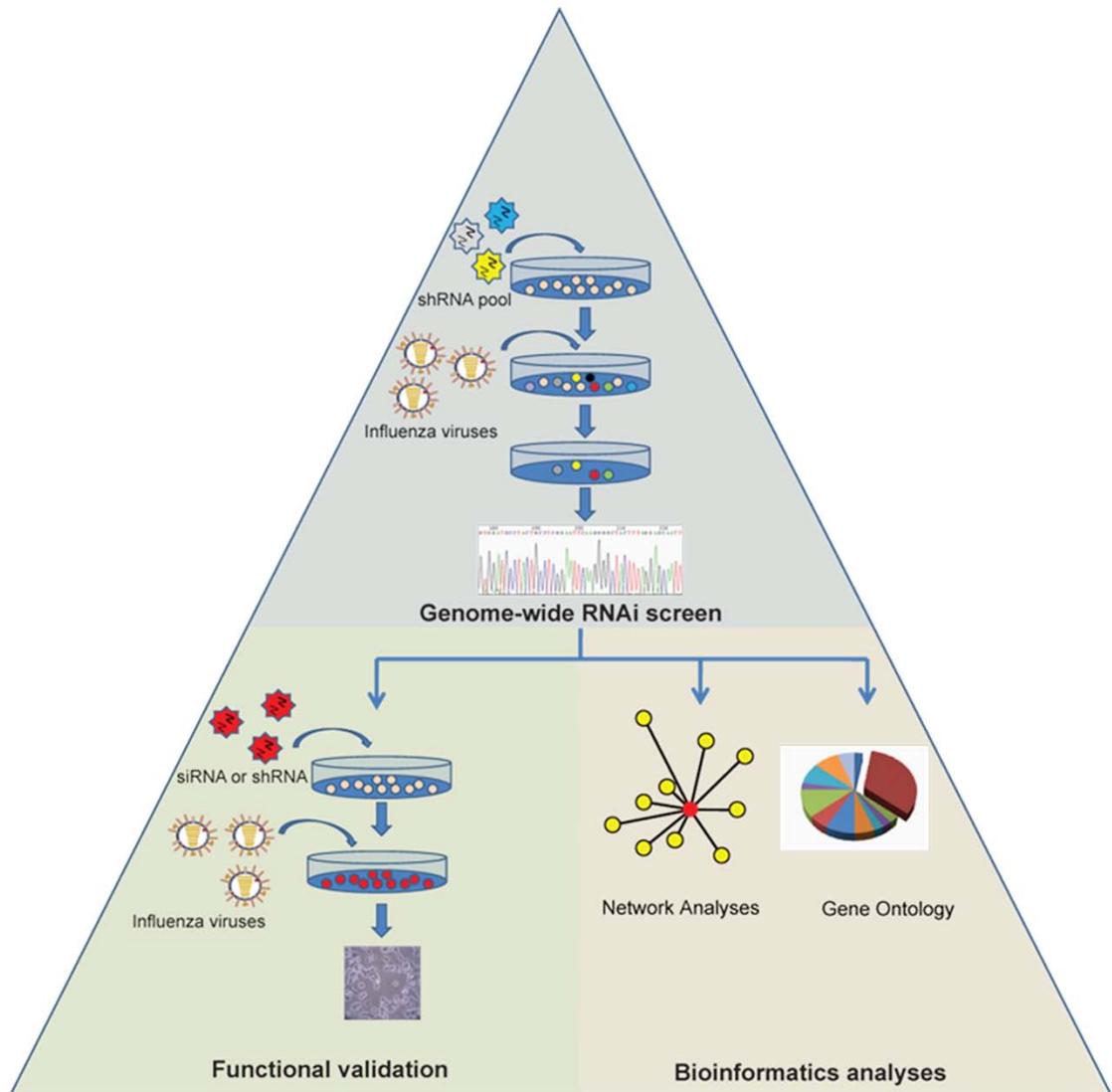
#### Factors Identified in Influenza Virus Genome-wide RNAi Screen

Virus-mediated cell death is an important cause of severe lung and tissue damage during an influenza virus infection (132, 196). Additionally, the activation of the cell death signaling pathway has been reported to be significant for influenza virus replication (322, 356, 357). In this respect, I wanted to identify host factors that are required for virus-induced cell death during influenza virus infection. My approach was a positive selection for cells that survive a high MOI influenza virus infection in the presence of a genome-wide RNAi knockdown with shRNA. My RNAi study was designated to identify both gene populations that are involved in indirect or direct induction of cell death during influenza virus infection.

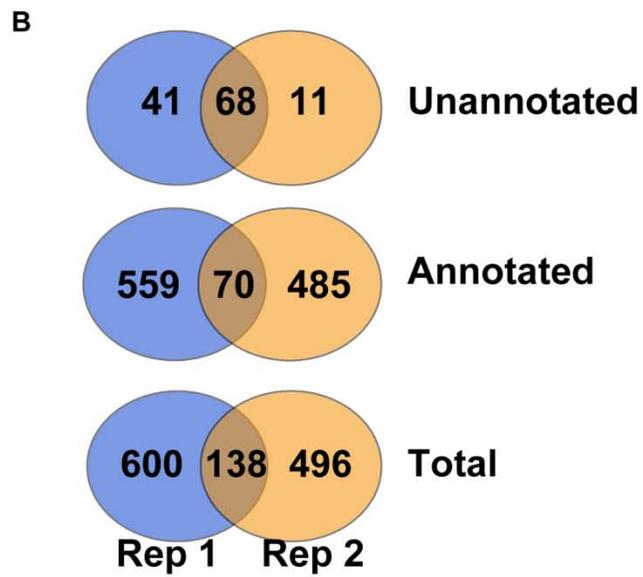
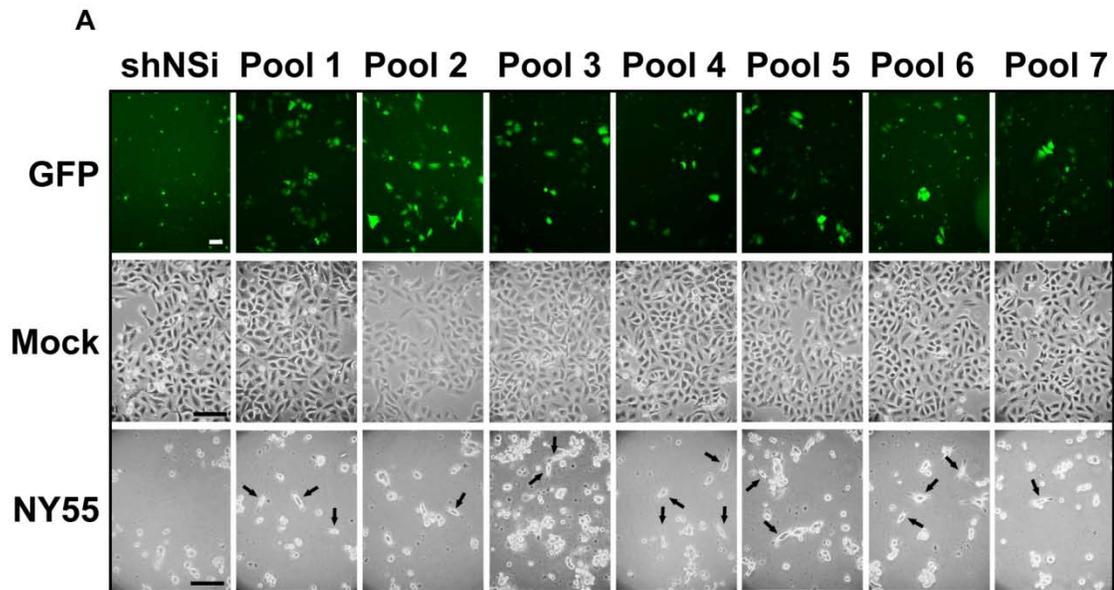
I transduced human lung adenocarcinoma A549 cells at an MOI of 0.3 with the lentiviral-based Decode™ RNAi library (Open Biosystems) (Figure 11). The library consists of 7 pools, each with ~ 10,000 shRNAmir constructs. Cells were transduced with 100,000 transduction units per pool, which allowed for a 10-fold coverage of each clone, and at an MOI of 0.3, reduced the risk of any single cell being multiply transduced to less than 4% of the cell population according to Poisson Distribution. After 48 h to allow proper knockdown of transductants, the transduced cells were then infected with influenza virus strain A/New York/55/2004(H3N2) (NY55) at an MOI of 7 PFU/cell to ensure > 99% of cells were initially infected. The infection was carried out for 72 h to kill off irrelevant knockdown cells. Illumina®-mediated high-throughput sequencing of

all 7 pools was then individually performed on two independent replicate screens of surviving transduced, infected cells.

The shRNAmir constructs also expressed GFP, which allowed us to confirm that the A549 cells were successfully transduced with the lentiviruses of each pool (Figure 12A). At 72 hpi, cells transduced with a non-targeting shRNA control all showed virus-induced cytopathologic effect (CPE), which were observed as rounded and floating cells. These cells were assumed to be killed off by the viral infection. In each of the infected cell populations that were transduced with 1 of the 7 pools, I observed individual cells that remained attached to the plate with little phenotypic change associated with CPE (Figure 12A, black arrows). These surviving cells were harvested for genomic extraction to identify the specific shRNA that resulted in the KD which allowed for the cell to survive the viral infection. In confirmation that these cells were indeed alive and capable of replicating, I had manually sequenced 95 clones and identified 80 genes from a total of 421 isolated clones in 3 shRNA pools (Table 7). These manually isolated survivors were amplified in culture into thriving monolayers. Given the onerous tasks of isolating and cloning each individual clone, a high-throughput readout was developed that involved sequencing entire surviving populations as a pool with Illumina® high-throughput sequencing. The combined high-throughput genome-wide screens identified 1256 potential targets (annotated and unannotated, Figure 12B, Tables A1 and A2) from a total of ~21,415 genes screened in 2 independent replicates. Of these, ~ 138 (70 annotated and



**Figure 11. Outline of methodology.** Independent RNAi global screens were carried out by transducing A549 cells with shRNAmir-lentivirus pools, infected with NY55, genomic DNA of surviving infected cells were harvested and sequenced by Illumina® high-throughput sequencing. siRNA arrays of 147 genes as well as 5 individual genes were chosen for further functional validation. Network analyses and PANTHER gene ontology were conducted on the positive hits.



**Figure 12. Global RNAi screens identify genes significant for influenza virus-induced cell death and replication.** (A) Images of transduced and infected cells were taken for each shRNA pool at 72 hpi. Cytopathic effect of cells were examined with a Nikon Eclipse TE2000-S inverted microscope and images obtained with a Canon PowerShot A700 digital camera. Shown are images representative of 2 independent replicates. Scale bar represents 100  $\mu$ M. GFP fluorescence was detected with Axio Observer.Z1 fitted with Plan-Apochromat 10x/0.45 M27 objective (Carl Zeiss MicroImaging GmbH, Germany), AxioCamHR3 and AxioVision imaging software. Images were collected at 1388  $\times$  1040-pixel resolution. The black arrows indicate surviving cells. The images were minimally rendered on Adobe® Photoshop® for publication purpose. (B) Venn diagram indicates the number of genes identified in each replicate screen and the number of genes identified in both replicates.

**Table 7. List of 80 annotated and unannotated genes identified from manual RNAi screen of 3 shRNA-lentivirus pools.**

Clone #	Oligo ID	shRNA Sense Sequence	Gene Symbol*
P2A-4D5	V2LHS_94280	GACAAAGCAAACCTTCAACA	ACYPI
P2S-2C1	V2LHS_137822	GACATAAGAGTGATGGAAA	ADAMTS12
P2S-5B3	V2LHS_243025	CAGTGACCTTCGCTCCACA	BAD
P1-1C6	V2LHS_150110	CACCAGAAGTCCTGAAACT	BTC
P2S-2E2	V2LHS_87831	CTCTCCTTGGCAGATACTA	C17orf75
P2S-5E1	V2LHS_51307	CAGTTAACCTGATTTCAAT	CA10
P2S-2F4	V2LHS_62457	GACTGCACTTGCTCGAATA	CD180
P3S-1B5	V2LHS_117063	CTCAATCGCTACCTAGATA	CD1E
P2S-5F7	V2LHS_150519	CAAGTGATTATTAGAGAAA	CEBPG
P1-1D4	V2LHS_88640	CTTTAACACTCATACTGAA	CLCN5
P2S-2A8	V2LHS_95583	GAAACAAGGGTTATGCTTA	DDX46
P1-1A6	V2LHS_115547	GAGTTCTGCTGTTCTGTAA	DDX47
P2A-1D5	V2LHS_202767	CAGTGACCTTCATCACCTA	DYNC1H1
P1-3A2	V2LHS_50572	CTACTTATGGATTCATCAA	FEZ2
P1-1E6	V2LHS_38043	CGTCTACGTTTATATCCTA	FLJ23834
P1-1A2	V2LHS_29650	GTGCTTTATTCAAATCTAA	hCG_1986447
P2S-6A4	V2LHS_84337	GTCTTTAAATGAAATGGTT	HCN1
P2S-5B7	V2LHS_117482	CCTTTAGGAAGAACTATAA	IFT81
P2A-4C8	V2LHS_24258	CTATCTATGTCCTGGAAGT	JRK
P2A-3E1	V2LHS_63527	CAGATATCATGTA CTGTAA	KIAA1804
P2A-4C5	V2LHS_133869	CCTATACATACATATACAA	LAIR2
P2S-5A3	V2LHS_140578	CTGCCAGTGTTCTGGAGAA	LOC284297
P1-3C8	V2LHS_74321	CCGCACATCAGGAACCTCA	LOC728965
P1-2A8	V2LHS_84222	CCAATATTGTGTTTGGATA	MALT1
P2S-5A7	V2LHS_115863	GTCATGAAATCCAGATATA	MPP6
P2A-4F8	V2LHS_152031	GACAAGATGTTCTTTCTAA	MX2
P1-1D2	V2LHS_87406	GCCATAATCTTCAAGTTAA	MYSM1
P1-2A3	V2LHS_156036	GTGTTTCAGAAATATTTCA	NUDT11
P1-3B8	V2LHS_27973	GTATCAAGTTCGAATCTTT	OFD1

P1-3F7	V2LHS_139107	CAGAGGCTCTGCTTCCAGT	OR2B2
P2S-2D1	V2LHS_72606	GATGGAGATTCTTCTCAA	PGM5
P2S-2D5	V2LHS_267223	CCACGTCTGGAAAGCTAAT	PGPEP1
P2A-3E3	V2LHS_77345	GATGGTAGTTTATTTGAAT	PHLPPL
P2A-1D6	V2LHS_170021	CAAGTTATTTCACAACTTA	PIK3CG
P2A-4C3	V2LHS_222992	GCGCCTATAAGATCTATGT	PRPSAP1
P1-3F6	V2LHS_95200	GAATCGGCATTCCAAGTCA	PTDSS1
P1-3E4	V2LHS_173277	CCGGACAGGCTTCTTTCCA	PYY
P2S-5C2	V2LHS_196272	CAATGTATTTGACTACTAA	RBBP9
P2A-3E7	V2LHS_201681	CAAACGCATTGATGGATT	RORA
P1-1A4	V2LHS_31837	GACATGTACCTGCAGAATA	S100A8
P2S-2D6	V2LHS_263543	CCATTTAATGATTGATTAT	SERPINA5
P2A-4D8	V2LHS_260346	CTTTCTATTTGTTAAGTAT	SH3BP4
P2A-4C7	V2LHS_15769	CCCATATATTCTATCTAGA	SLC2A12
P3S-1B6	V2LHS_263089	CGAATGCAGGTTGCAAATA	SPG7
P2A-3A2	V2LHS_153678	CAGAAATACTTCTCAGCAA	SUPV3L1
P2-4C9	V2LHS_66700	CGCAATGGGCTGGATTATA	TBC1D25
P1-2F7	V2LHS_228809	CTCACTGAGATGTGTGAAA	TCN2
P1-4D2	V2LHS_198493	GGGACTCTATTTATTCTGA	TMC6
P1-2E3	V2LHS_17314	GATATTCTGAGTGTCATAA	TNFSF12- TNFSF13, TNFSF13
P1-3B6	V2LHS_18602	AGCATACAGTGTCTGGAA	TRDMT1
P2A-3C2	V2LHS_174640	CTTATAAGATGATGGATT	USP47, Usp47
P2S-5F8	V2LHS_179763	CCTGCTCCATCGGCACTAA	ZNF213
P2S-5E8	V2LHS_65911	AACGTCTCCTCCTGTACTA	ZP4
P1-4D6	V2LHS_257969	CCTTATTTGACTTCAAAGA	ZSCAN12
P2A-1F2	V2LHS_206752	CTGAGACAGGATTATCAAT	ZWILCH, LCTL
P1-3A7	V2LHS_169189	CAAACCTTTGCATTAATAAT	-
P1-3A1	V2LHS_187381	CTGCAGTCATCTTATGATA	-
P1-1B4	V2LHS_193088	CTAAATAAACGTTATCGAT	-
P1-1D6	V2LHS_193556	GAAAGGAGGATTAGGAATA	-
P1-1E3	V2LHS_19513	CTACTCAATGCTTGGCCCT	-

P1-1D3	V2LHS_23062	CTAATGAGCTGCCACATAA	-
P1-2E7	V2LHS_73553	GCCTTTGAGTCCCCTACA	-
P1-2E6	V2LHS_79588	CAGAGACAATCCAAAGCCT	-
P1-4C1	V2LHS_80262	AAGATGCACTCATTTGCGA	-
P2A-4A1	V2LHS_105347	CTCACCAGGAGCAAGTTTA	-
P2S-5E7	V2LHS_107967	CAGAGAAGGAGCTGAACCA	-
P2S-2A4	V2LHS_124124	GCCAAAGGTACAAGTGTA	-
P2A-1E7	V2LHS_169606	CTAAGTCATGTTTCTTGAT	-
P2S-2C6	V2LHS_21591	CTCCTAAAGCCAAGGCCAA	-
P2A-3D7	V2LHS_229575	CAATACCAGCTAAATTAAT	-
P2A-4D6	V2LHS_25880	CTCATAACACCCTGTTTCT	-
P2A-1F7	V2LHS_266396	CAAGTTTATCCATTCTCCA	-
P2A-4B1	V2LHS_26934	GAGCTATTCGTTTCATATA	-
P2S-5F4	V2LHS_60207	GTTGCAATGTCTCCAGTTT	-
P2A-3F6	V2LHS_66414	CTAGAAGTGTCAAGGGAAT	-
P2S-6B5	V2LHS_79527	GTTTAAGTGTCCAAGGGAA	-
P2A-4E7	V2LHS_88625	CATTTCTTCTGTGACAACT	-
P3S-1D2	V2LHS_165318	GCATTCTACTCATGGAACA	-
P2S-6B1	V2LHS_149376	CAAATAATAAACTCTTAGA	-

\* a hyphen (-) indicates unannotated genes; no gene symbol or gene ID is associated.

**Table 8. List of annotated and unannotated genes identified in both independent replicate RNAi screens.**

Oligo ID	Sense Sequence	Gene IDs*	Gene Symbols*	Identified in Manual Screen
V2HS_15289	CTCACTACCAAATGTTAAT	572	BAD	Yes
V2HS_254648	CACAAAGTGTGACCACATA	672	BRCA1	
V2HS_150519	CAAGTGATTATTAGAGAAA	1054	CEBPG	Yes
V2HS_64314	CCCTCTATGATGCAACCTA	1072	CFL1	
V2HS_11917	CATATGATATGTAAACCAA	1787	TRDMT1	Yes
V2HS_151315	CTGCGAACTTGTGTTTATA	1907	EDN2	
V2HS_42837	GAGAATAGCTTATGAGTTA	2620	GAS2	
V2HS_84939	ATGTGTTATTTGTCACAAA	3084	NRG1	
V2HS_218617	GGCACTACATAAATGTGAA	3174	HNF4G	
V2HS_128339	CTCCTATACCCAATGACCT	4128	MAOA	
V2HS_131202	CAGTCTCCAGTCCCAATAA	4306	NR3C2	
V2HS_152028	CTGCCAGGCTTTGTGAATT	4599	MX1	
V2HS_152031	GACAAGATGTTCTTTCTAA	4600	MX2	Yes
V2HS_203345	GCGGAGATCTCCAGTGATA	5468	PPARG	
V2HS_57273	CAGTGAAAGTCCATCTATT	5783	PTPN13	
V2HS_171000	GGAAGTCACGTATTTGAAT	5795	PTPRJ	
V2HS_94799	CAATCTTAGCTTTGAAGAA	6013	RLN1	
V2HS_33937	CCCAGAAACTGCAATGTAT	6809	STX3	
V2HS_171633	GTTCCCGACTTGAAATGAA	7273	TTN	
V2HS_172400	CTCCTATGTATGAAGAGAA	7857	SCG2	
V2HS_33954	CATCATAAGGCCAAGGGAA	8335	HIST1H2AB	
V2HS_100766	CACCTGAAGCTCTTCAAGA	8844	KSR1	
V2HS_52981	CAGGTACTCACTCTGGTTT	8858	PROZ	
V2HS_130541	CTGGCAAGCTTGAATCCAA	9320	TRIP12	
V2HS_35328	CTTCTAATCTTAAACTTCA	9372	ZFYVE9	
V2HS_18674	CATTCTATCTTTGCTCAAA	9643	MORF4L2	
V2HS_196634	ACCAGTTCGTTATATTAGA	10152	ABI2	
V2HS_65277	GCCATAAACACTGAGATGT	10455	PECI	
V2HS_1643	CTGCATACTTACTTACTTT	10461	MERTK	
V2HS_84222	CCAATATTGTGTTTGGATA	10892	MALT1	Yes
V2HS_198493	GGGACTCTATTTATTCTGA	11322	TMC6	Yes
V2HS_254695	GGCTTCAGTTATTTAAAGT	23219	FBXO28	
V2HS_100836	CTCACATAATCAAACCTTA	23235	SNF1LK2	
V2HS_244561	GCTAGGTTTAAAGCATTCA	23538	OR52A1	
V2HS_260346	CTTTCTATTTGTTAAGTAT	23677	SH3BP4	Yes
V2HS_96523	GTTCTTCATCCATTAGTTA	25897	RNF19A	
V2HS_130208	CATTATTTGGTGCTCCTAA	26043	UBXN7	

V2HS_114879	GGAAGATCTTCCATACCAT	51046	ST8SIA3	
V2HS_138350	CAGGTGAGAGCTGGAGAAT	54856	GON4L	
V2HS_173612	CCAGGAAGAAGGTTCTCAA	54884	RETSAT	
V2HS_173728	CTGAGATGAGCAGTGAAC	54900	LAX1	
V2HS_174637	GAATCTGTCTTCAAACCAA	55031	USP47	Yes
V2HS_176062	CCGATACTCGGGAGAAGAA	55421	C17orf85	
V2HS_35766	GTGTTCTTATAGTTATTTA	57150	C6orf162	
V2HS_46786	CTCTGGATTTAGAGATATA	57475	PLEKHH1	
V2HS_217697	GGCCTTGGCTACTACCAGT	57561	ARRDC3	
V2HS_57109	GTGTTGACAATATATTGAT	57709	SLC7A14	
V2HS_78473	GGGTCTACATGTTTCCAAT	64857	PLEKHG2	
V2HS_116332	CTGCCCTTAAGGAAATTGT	64946	CENPH	
V2HS_238923	GTCTGAGAGAGTGACCTAT	65268	WNK2	
V2HS_136349	CTCAGCACGTGTATTGAAA	79616	CCNJL	
V2HS_177667	CTTTATTCCTCAAGGCAAT	84899	TMTC4	
V2HS_159971	CTCTTGGTGTGATCACATA	85481	PSKH2	
V2HS_70495	GCCTATCCGTGTATATGGA	89781	HPS4	
V2HS_215547	AATATTACTGGCAAAGTAA	90987	ZNF251	
V2HS_247493	GGGATACAGAGATAAACAA	91464	ISX	
V2HS_161523	GCTGGTTATGGATATTCAT	120526	DNAJC24	
V2HS_18851	ACCTTGATCTGAGTATGAA	127495	LRRC39	
V2HS_29175	CCTTTGTAGTGTTGTTTAT	138065	RNF183	
V2HS_225634	CAGTGAGGGCCAATAATTA	142685	ASB15	
V2HS_44617	CGGAGGAACCTCTGTTAGAA	163882	C1orf71	
V2HS_77698	CTCTTTAACTCAGTAGTTA	388403	YPEL2	
V2HS_75780	ACCATTCTGAGTTTATTAA	431707	LHX8	
V2HS_165267	CTGAGATTGCTCACAATGT	728572	LOC728572	
V2HS_29650	GTGCTTTATTCAAATCTAA	729324	hCG_1986447	Yes
V2HS_25168	GTGTACACATGCTGACACA	730139	LOC730139	
V2HS_93855	CTTAGTAAAGGACTTATCA	6230, 100131196	LOC100131196, RPS25	
V2HS_202686	GGCGCCTAATGATGTCTAA	7335, 387522	TMEM189- UBE2V1, UBE2V1	
V2HS_17313	GCCGCCCTCTGCTAGGGAA	8741, 407977	TNFSF12- TNFSF13, TNFSF13	Yes
V2HS_23046	GGACCAGTAGCAAAGGAGT	9349, 646949	LOC646949, RPL23	
V2HS_101147	GAAATTCTGATGAAGTTAT	-	-	
V2HS_101982	CAGGGAACATCATTGGATA	-	-	
V2HS_104782	CAGAGAGAATCTCTGTTGA	-	-	
V2HS_106016	CACAGTCTAAGACTGCTCA	-	-	
V2HS_106193	CACAAACGCTGAGAGACAA	-	-	
V2HS_106421	CATCGAACAGGACCACAAA	-	-	
V2HS_106978	GGATTACAGAGAAGAAAGA	-	-	

V2HS_107757	CAATCACAACGAGACAACA	-	-
V2HS_108407	CCAAGTTCCATACAGGAGT	-	-
V2HS_108849	CCACTTTCATCTAAAGTTT	-	-
V2HS_108975	CAGCTGCTCACACTGACTA	-	-
V2HS_109586	CAGACACCACGATGCCAAA	-	-
V2HS_110305	GTCAGCACCCTTAAGAGA	-	-
V2HS_117073	GACTGTAAAGTTGTAGGTA	-	-
V2HS_120960	GCTTCTGTTCTCACAGAT	-	-
V2HS_121207	CACAGGGTCTTTGATGAGA	-	-
V2HS_122101	CGGACAGAGGTGACATTTA	-	-
V2HS_122678	CCAGTGGAGAACATGGAAA	-	-
V2HS_123869	CAAGGTCCTGACACAGAA	-	-
V2HS_124057	CCCAACATATGTTCTCTTT	-	-
V2HS_124124	GCCAAAGGTACAAGTGTA	-	-
V2HS_124718	CCCTCCTCGTTTCTCCATA	-	-
V2HS_125389	CAGGTCTGCAAATACCAGA	-	-
V2HS_125487	CAAAGAGAGCAGAGACTTA	-	-
V2HS_125812	GACAGGACATCTCTGGGAA	-	-
V2HS_127050	CCTTAATTCTCAGCTTAAT	-	-
V2HS_129204	CGAGCAGTCTAGAGCAGGA	-	-
V2HS_129365	CCCTTGCAGGGCTATGGGA	-	-
V2HS_129996	CTTCTGGAAAGTGGCACTA	-	-
V2HS_130639	GTTGGGTTACTAACTTTA	-	-
V2HS_141402	CTGGTGCTCACCTGAGTAT	-	-
V2HS_141961	GTTGAGGACAGTAGAAGAA	-	-
V2HS_142348	CATGTAGAGCTGTTGGTCA	-	-
V2HS_142455	CGAAGGAGAAGATCTATCT	-	-
V2HS_144903	GGATGACACTGAAAGGTGT	-	-
V2HS_145518	CAGCAGAAGTAATCAGAAA	-	-
V2HS_145592	CACTAAATCTGCTTGGAGA	-	-
V2HS_145643	CAAGCCAATTCAGTGGAA	-	-
V2HS_145685	GCCAGTTTAGCAGTTTCAA	-	-
V2HS_145727	GCCTCAGGACAAAGATGAA	-	-
V2HS_145885	GAATGAACAACCTGGTGTA	-	-
V2HS_146271	CAAGAACCTTTGCTGTAA	-	-
V2HS_146395	CGAGCTATAGGTCTAAGAA	-	-
V2HS_146489	CCGCCAAGACTCTTACTCA	-	-
V2HS_146766	ACTGGAAACTCTATCATGA	-	-
V2HS_147063	CAGACACATGTGGATCTGT	-	-
V2HS_14715	CAACATATCTCACTCTAGA	-	-
V2HS_147212	CTCATCTTGAAACACCAGA	-	-
V2HS_147399	CAAAGAGGGAGATAGAACA	-	-
V2HS_147422	GCTAAAGGAAGAAATGCAT	-	-
V2HS_147584	CTGCTATTAATGACAATCA	-	-

V2HS_147618	GCCTTTAGCTACACCTTTA	-	-
V2HS_147773	CCATCACTGTCATAGTTAT	-	-
V2HS_147823	CTAAGGTGTCTCTGAGGAA	-	-
V2HS_147868	CTGCCATAAGTGGAACATA	-	-
V2HS_148105	GATAAGAAGCTGTGAAGAA	-	-
V2HS_149821	GCTTAAACATTTATGGAAT	-	-
V2HS_15255	CCACTCCTCACCGAGATGA	-	-
V2HS_15504	GAGATTACAGGACCTAAGA	-	-
V2HS_15783	GACCTGATATATAGATTTA	-	-
V2HS_162781	GAATGCAGGTACTTACATA	-	-
V2HS_162949	GCTTATACGTGCGAAGAGA	-	-
V2HS_163380	CTTTATGTCTTACATGGAA	-	-
V2HS_163662	CATTTAGAATGCAGCAAAT	-	-
V2HS_165173	GCTACATATCGAAATCGAA	-	-
V2HS_165684	CAATCACGTGCCAATGATA	-	-
V2HS_167941	CATGCAAGGAGAGCCTCAT	-	-
V2HS_168208	GAGTAAGAGGATATTATAA	-	-
V2HS_177112	CAGTAACTTTCAGAATCAA	-	-
V2HS_178691	CAAATTAGTAAGAGACCAA	-	-
V2HS_179613	CTTAGTATGAATTACTGCT	-	-
V2HS_181197	CTGAAATTGAGCAAAGGCA	-	-
V2HS_181516	CAGTACCAACTGCTTTGAA	-	-
V2HS_181529	CCACCTCCACTCATAGCTT	-	-
V2HS_183124	CTCTTTCTACTACCACACA	-	-
V2HS_184474	CGACTTTGGGTCTCATATA	-	-
V2HS_184487	CCGCGTAGTAATTCCTAAT	-	-
V2HS_184488	CGCGTAGTAATTCCTAATT	-	-
V2HS_184760	CATAGGTAGCGATTTCTTA	-	-
V2HS_187876	CACAGGAATGTTTAGAAAT	-	-
V2HS_190852	CAGAGTTACGAATTACGTT	-	-
V2HS_203877	GGACAATTATGCTGGCTAT	-	-
V2HS_204070	GAGTACCTATCTGGTGGTA	-	-
V2HS_204240	CTATGGATGGCATATGGAA	-	-
V2HS_207290	CTCATCGCTGAATACCTTA	-	-
V2HS_209142	CCAAGTATACTTAAAGCAA	-	-
V2HS_209302	GCTACATTATATATAGGAT	-	-
V2HS_210659	GGCCTTAGGGCAACCTTTA	-	-
V2HS_212808	CCCTTTGTATCAAGGTAGT	-	-
V2HS_213004	GTTGTATTGCTTGTITCAA	-	-
V2HS_214255	CATTGTTGATAGTAGAATA	-	-
V2HS_215196	CTCTTTATAGCAGTTGCCA	-	-
V2HS_215437	AGTTGAATTTAGTAATGGA	-	-
V2HS_21547	GCTACATTATATATAGGAT	-	-
V2HS_215556	CTGTTGGCATGTATCTTAA	-	-

V2HS_21710	GATATTAGCTTAAACTATG	-	-
V2HS_217354	CTGGCACTTGAATTAAGTA	-	-
V2HS_217793	CAATGATTCTCAAGTTCCT	-	-
V2HS_217839	CAGATTCTTCCTATAAAGT	-	-
V2HS_219564	GAAGAAGTATGAGAATTAA	-	-
V2HS_219766	CACTTTATGAACACTAGGA	-	-
V2HS_220203	GAAATAACATGAGCTTGGA	-	-
V2HS_22091	CACGGAATGCTGGTCATTC	-	-
V2HS_221340	CCTTAGCAGAGAGAATATT	-	-
V2HS_223954	CTTCACTTCTTAAGATAAT	-	-
V2HS_224571	CTGGAATAGAGATTCCTAA	-	-
V2HS_224677	GTGCTAGCCTCATTGAGGA	-	-
V2HS_225141	CTTGCTCTATGCCAATCCA	-	-
V2HS_226760	CAGATTCCTCATTAATTAT	-	-
V2HS_228360	CTCTTATGCAATTGATGGA	-	-
V2HS_229435	CTGGTGATATCCCAATTAT	-	-
V2HS_231328	GAAGCACATTGTATTTGAT	-	-
V2HS_23175	CCCTGCCAAGGAATCATT	-	-
V2HS_233616	GTCTTATCTAGTTTAATTA	-	-
V2HS_236504	CAAATAAGATCCAAGATCA	-	-
V2HS_236523	CTCTATCCTACAGAAGAAT	-	-
V2HS_237211	GTGAGGGAGAGTAATTTGA	-	-
V2HS_241584	CATTAAAGCTTCGTAAATA	-	-
V2HS_243248	CTGTAATAACTTAGCTATT	-	-
V2HS_244118	CAAATGATGCTGCAATGTA	-	-
V2HS_247631	CCCTATTCCTCTGAAGTT	-	-
V2HS_250042	CAATCAGAGAGGAGACCCT	-	-
V2HS_250064	CAGTATCTATCAGTGGGAA	-	-
V2HS_250087	CATTGGTAGAATTTGAGAA	-	-
V2HS_25043	GCCATTGGATGCAGTCTTT	-	-
V2HS_251063	CCCTTTCCCTTGACATCCT	-	-
V2HS_251570	GGAATTAACCCGAAAGTTT	-	-
V2HS_252148	GTGGTGATGAATATTGGGA	-	-
V2HS_252474	CACAGTGATGATCTTTGTA	-	-
V2HS_256866	CAATGCAAATGTAGGGTTA	-	-
V2HS_256884	CACAAAGAATCAACCTGAT	-	-
V2HS_259103	CTCACCAGCTTATGCCTAT	-	-
V2HS_25956	CAGACCACAGGTCTCCTTA	-	-
V2HS_26033	CAGGGAAATTGTGCTGGAT	-	-
V2HS_26100	ATTAGAGTCCCTATTTCTA	-	-
V2HS_265230	GCAGAATGCTGTCACCTTA	-	-
V2HS_266928	GAGTGGATCTTGGAATAA	-	-
V2HS_268909	GCTGATACTTTCATCTCAA	-	-
V2HS_269067	TTGTGTCAGCATATGTGAT	-	-

V2HS_274387	GGAATCATTGGGAAATATT	-	-
V2HS_274959	CTCGTGCTGACTTGTAAGT	-	-
V2HS_275375	GAGGCTGAAACCTTTAAGA	-	-
V2HS_276130	GGGTTCACTTACTTGGA	-	-
V2HS_277428	GAGGTGGAAGCATGTAAGA	-	-
V2HS_29742	GGAGGTTTCATCACATAGAA	-	-
V2HS_29828	GCTTTGCTGGTTTCTATTA	-	-
V2HS_31146	AGTACAAATTCCTATGCCA	-	-
V2HS_31615	CACAGAACCTCAATTGGAT	-	-
V2HS_31714	CCATTGATCAAGTGTTTAA	-	-
V2HS_32064	GCAATATTGTGTTGAGAGA	-	-
V2HS_32355	CTGAAAGACTCCTTGCATA	-	-
V2HS_34104	CGCTGGAAATCAACTCAAT	-	-
V2HS_36976	GCTATGTTGTCAAGACTTT	-	-
V2HS_37229	GTCTGATAGTTTCCATTTA	-	-
V2HS_37358	CTGGTGAAGTGTACATCA	-	-
V2HS_40503	GAGAGACTAGCTTGTATTG	-	-
V2HS_44335	ATATTCAGCAGGTTACTAA	-	-
V2HS_44343	GCCAGAATTAGATCCTCAA	-	-
V2HS_44947	AGCTCTGCCTCCTCTCTAA	-	-
V2HS_45001	CCTTCAGCATTATACAGCA	-	-
V2HS_45437	ACCATTACCTCATTAGATT	-	-
V2HS_46260	CTCTTACAATCAATGGGAT	-	-
V2HS_52653	CATCTCAGTAATGCTAAGT	-	-
V2HS_52852	CTCGTTGCCAGCAATTATT	-	-
V2HS_53386	CCCGCTGGGACCTTGCTAA	-	-
V2HS_53517	CATCTATGATGAAAGCTCT	-	-
V2HS_54194	GAAATGAACCTGAAACTTA	-	-
V2HS_54310	CACACACGATTGCAACTTA	-	-
V2HS_55108	CAGTTCAAATATCTTGATA	-	-
V2HS_55354	CTCATCTTCTGGTGAGTTC	-	-
V2HS_56093	GTATCACCTCCAAGACTGA	-	-
V2HS_58165	AAGGTAGACCCAAATATGT	-	-
V2HS_59571	CCCATTAGAGGAGATCCAT	-	-
V2HS_62166	CAAACCTATGGCCTGTCCAT	-	-
V2HS_65234	CTACGAACAGTGTAGGCAA	-	-
V2HS_65880	GTCCTATTCTGCATGCATT	-	-
V2HS_66555	GACTAAACCTTGGAGAAAT	-	-
V2HS_67012	GCCACAATCCAACCTATCAA	-	-
V2HS_67065	ATTACCAGATCAATGAGTA	-	-
V2HS_67275	GGAGTCATTTGGATGGATA	-	-
V2HS_70405	AGCATCTGCCCAAACCTAA	-	-
V2HS_71627	CTTGCCAATGCTAAGGCAA	-	-
V2HS_72211	CTCAATCTGTATATACAAT	-	-

V2HS_73163	GAGGGATGTAGTTACATAT	-	-
V2HS_74133	CAGTTCAACGGGAGATGAA	-	-
V2HS_74979	GAACTCCCTGTATACCACA	-	-
V2HS_75519	CCTTTAAGACGTTCTGGAA	-	-
V2HS_75791	GTCTATACTTGTTTCCTCAA	-	-
V2HS_75869	GACTTCAGTTGGTTGTCAT	-	-
V2HS_77299	CAAACAAATTGAATTAGAG	-	-
V2HS_79147	CTGCTGGATTGTTATGAGG	-	-
V2HS_79504	GGGACTCAGGGTACAATAA	-	-
V2HS_80097	GCCAGTCACTTCTCACAAT	-	-
V2HS_80340	CTATTACCGTTACAGTGTT	-	-
V2HS_82244	CACACATTGACACATATGT	-	-
V2HS_83964	GAAGTGCATTTATCAAGAT	-	-
V2HS_84055	CTGAGGACTTTGATGATGA	-	-
V2HS_85063	CAGAAGTCTTGGA ACTATA	-	-
V2HS_85210	CAAGGCAGATTAAAGAGAT	-	-
V2HS_85992	AGCTTGAAGATCGAGGTAA	-	-
V2HS_90801	GGATAAGATTCACTAGATA	-	-
V2HS_90995	AGAATATACTCTGTTCATA	-	-
V2HS_91534	AGAACCATGTAAGGGTAAA	-	-

\* a hyphen (-) indicates unannotated genes; no gene symbol or gene ID is associated.

~ 68 unannotated) genes were identified in both trials (Table 8). The identification of the 68 unannotated genes was based solely on the unique sequences of the shRNA species identified due to the lack of annotated information that would allow for better classification. Thus, the number of unannotated genes, genes for which there are no currently reported or associated biological functions, was calculated based on the general assumption that an average of ~ 3 shRNA species are representative per predicted gene.

In comparison with the 11 previously published RNAi screens (which included influenza, human immunodeficiency virus (HIV), West Nile Virus (WNV), Vaccinia virus (VV), and Hepatitis C virus (HCV) (19, 20, 94, 119, 137, 141, 185, 205, 282, 307, 343)), only 3 of the 70 annotated genes overlapped: TRDMT1, GON4L, and HPS4 were also identified in König et. al. (influenza screen, (137)), Sui et. al. (influenza screen, (307)), and Tai et. al. (HCV screen, (311)), respectively. I further determined that 146 annotated genes identified in my screen (from the total 1256 potential targets) were also reported in the other 11 published siRNA screens (Table 9). My data suggest that over 100 genes are required for influenza virus regulation of host cell death.

As a second validation, I carried out an siRNA array on a total of 147 genes. The 147 genes included: the 70 annotated genes (from the 138 genes identified in both my replicate screens), 57 genes (randomly selected from the 146 identified in both my screen and published screens), and 20 genes randomly selected from the lists of genes not identified in my screens but amongst those identified in other published siRNA global screens. In other words, the siRNA array consisted of 127 genes identified in my screen and 20 genes identified in the published screens but not found in my screen. I determined NY55 viral production in each of these knockdown cells by a standard plaque assay on

MDCK cells and the viability of infected knockdown cells with WST-1. The knockdown of these genes did not affect the viability of most of the A549 KD cells; only EIF4A3 was an exception (Figure 13A).

Of the 127 genes that were identified in my screen and included in the siRNA array, only 9 genes (7%, indicated as \*\* in Figure 13B) showed similar levels of virus-induced cell death relative to the non-targeting siRNA control (Figure 13B, statistically significant cell viability is above the red line). The remaining genes showed significantly greater cell viability after influenza virus infection relative to the non-targeting control. This confirms that the genes I identified in my screen were enriched in genes required for the induction of host cell death by influenza viruses. In comparison, 6 (30%, indicated as \*\* in Figure 13B, magenta bars) of the 20 chosen genes uniquely identified in other screens, but not in ours, did not protect infected cells from virus-induced cell death (Figure 13B, magenta bars), despite having significant reduction in viral titre (Figure 13C, statistically significant reduction in viral titre is below the red line). Interestingly, the knockdown of PTPN13 showed significant enhancement in viral production but very little cell death (Figure 13, \*).

Of the 127 genes included in the siRNA array that were identified in my screen (exclusion of the 20 genes uniquely identified in other published screens), knockdown of 24 (19%) of the genes did not significantly reduce viral titre (Figure 13C, green bars) but these genes did show significant resistance to virus-induced cell death (Figure 13B). This suggests that specific host factors are involved in killing the host cell during viral infection, independent of viral production.

**Table 9. Pairwise comparison of hit-list of genes identified in 11 published screens to both of my replicate screens.\***

	Tran	Shapira	Konig	Karlas	Brass (Flu)	Sui	Hao	Mao	Brass (HIV)	Krishnan (WNV)	Tai (HCV)	Mercer (VV)
Tran		38	20	18	9	6	6	1	20	11	7	10
Shapira			16	8	16	5	5	0	12	12	5	8
Konig				14	10	2	7	0	10	20	6	14
Karlas					7	2	6	0	4	1	1	16
Brass (Flu)						2	11	0	4	8	7	6
Sui							0	0	2	2	0	1
Hao								0	2	2	3	6
Mao									0	0	0	0
Brass (HIV)										9	2	4
Krishnan (WNV)											1	8
Tai (HCV)												1
Mercer (VV)												

Genes Identified in Both Our shRNA Screens and Published siRNA Screens											
ABCC10	ACTC1	ABCB10	ATF1	BPTF	DPF2	TNRC18	CRIPAK	ABCB10	CHMP2A	EGFR	
AMIB2	AKAP13	CD58	CRNKL1	C21orf33	HSPAR		DOX53	ABLIM1	FANCE	GUCY1B2	
AP0B53G	BMPRI3	CNGB1	DCI1E1A	FBX17	NUP43		DPM1	ANKRD31	HAMP	MAST4	
ARMC45	C5orf38	DERL3	GSK3A	GORN4L	NNT2		EGFR	AP52	HPS1	MYLIP	
C22orf33	CCL13	FROD1	NUP1	PXD2L2	PSMB4		ET1	GAB1	NUF2	POLE	
CA1M1 (includes others)	CDK4/BPA	IGSF1	PAGE5	SLC30A9	RPS3A		FBXW11	IFP1	RAN	PRKAR1A	
CDKN2AIP	CLOCK	IL1A	PPARA				IQJB	PCDHGA10	SSPO	PSMB4	
CLEC2B	DCLK1	LOC100510692/NAIP	STARD5				KLHDC2	PLK4		RPK3	
DHCR24	EPHA7	MDM2	ZNF354				LPL	RNF44		WTAP	
FBXO34	FGFR2	ME16					ME16	SP100		ZDHHC3	
FGFR2	IGSF1	MER8A					NGL1	USP16			
HIST1H2AC	KHSRP	NOF3					NUP85				
HMGGA2	MDM2	P2RY12					PHF3				
HIT2	NUP214	RBM3					PRDM7				
ILIRAP	POK3	SERPINA1					SPTBN1				
IRF6	PLK1	SIGMAR1					THAP3				
ITPK3	SCNSA	TK2					TMED2				
LYN	SORL9	TRIM21					TMEM132C				
MAPK13	SIGMAR1						XBR4				
NOD1	TROMT1						ZNF354A				
NRAA2											
PDGFRA											
PHF3											
PKM1											
PCOLCE											
PPP2R2B											
POLC1											
PTPN6											
RIOK3											
RNF44											
RPF2											
SAMHD1											
SRSF6											
SRSF7											
STARD5											
TOPORS											
TRIM21											
WTAP											

\* reference: Shapira et. al. (282), Konig et. al. (137), Karlas et. al. (119), Brass et. al. (flu) (20), Sui et. al. (307), Hao et. al. (94), Mao et. al. (185), Brass et. al. (HIV) (19), Krishnan et. al. (141), Tai et. al. (311), Mercer et. al. (205)



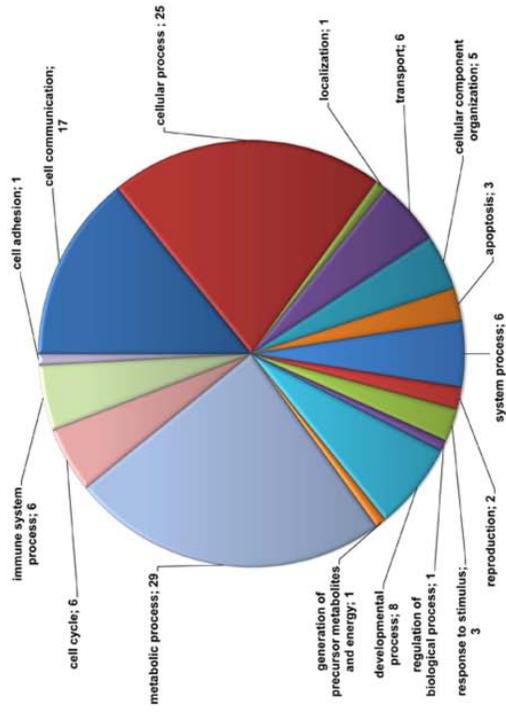
**Figure 13. Secondary validation of candidate genes with siRNA array.** Viability of siRNA transfected cells was determined with WST-1 assay for (A) uninfected cells 48 h after transfection or (B) 72 h after infection, \*\* indicates comparable cell death to control. (C) Virus replication was determined at 72 hpi by titring infected supernatants on MDCK cells by plaque assay. Magenta colored bars indicate genes uniquely identified in the 11 published screens (19, 20, 94, 119, 137, 141, 185, 205, 282, 307, 343), green colored bars indicate genes whose knockdown does not significantly affect virus replication. \* indicates significant increase in virus titre. Horizontal red line indicates statistical value of  $P < 0.05$ . Significant cell death or significant reduction in virus titres are considered for all samples below the red line. Error bars represent the mean  $\pm$  standard error of the mean from 3 independent replicates.

## 4.1.2 Network Analysis of 138 Genes Identified in Both Screen Replicates

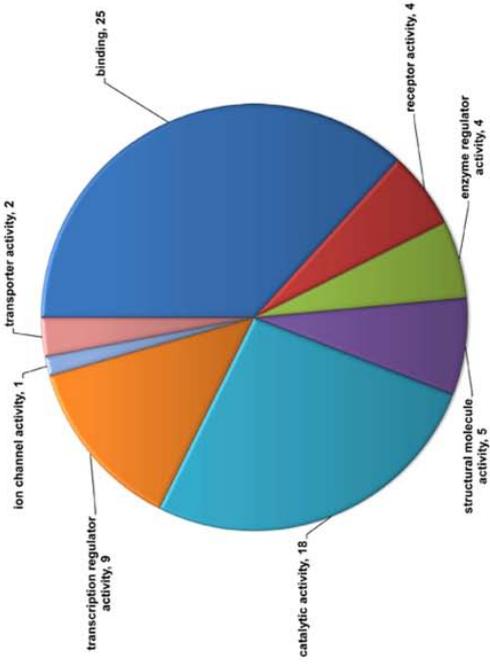
Influenza virus infection results in the induction of a number of host cell signaling pathways. I wanted to identify the protein networks to which the identified 138 genes belonged in order to assess how influenza viruses may directly or indirectly instigate the induction of host cell death. PANTHER GO analysis showed diverse molecular, biological and protein class categories to which these 138 genes were mapped (Figure 14). The greatest number of genes were mapped to 3 broad molecular categories: metabolic processing, cellular process, and cell communication (Figure 14A). Similarly, I determined that the genes also fall into 3 broad biological categories: transcription regulator activity, binding, and catalytic activity (Figure 14B). The majority of the protein products encoded by these genes were placed into 5 broad protein classes: transferase, transcription factor, hydrolase, enzyme modulator, and nucleic acid binding (Figure 14C).

I mapped the genes to 2 connecting networks with Ingenuity Pathway Analysis® (Figure 15). More specifically, these genes are involved in 2 major protein complexes: PI3K and NF- $\kappa$ B complexes (Figure 15A). Both complexes are reported to be induced during influenza virus replication (62, 226). More importantly, both PI3K and NF- $\kappa$ B signaling cascades are known to be involved in cell death and survival (59, 62). Other key host factors also known to be involved in the regulation of cellular death and survival are Akt and ERK1/2 (Figure 15B). BAD is an important pro-apoptotic protein identified as part of the 138 genes in the screen, appears to be involved in all 4 protein networks: PI3K, NF- $\kappa$ B, Akt, and ERK1/2 (Figure 15B). I have recently shown that BAD is

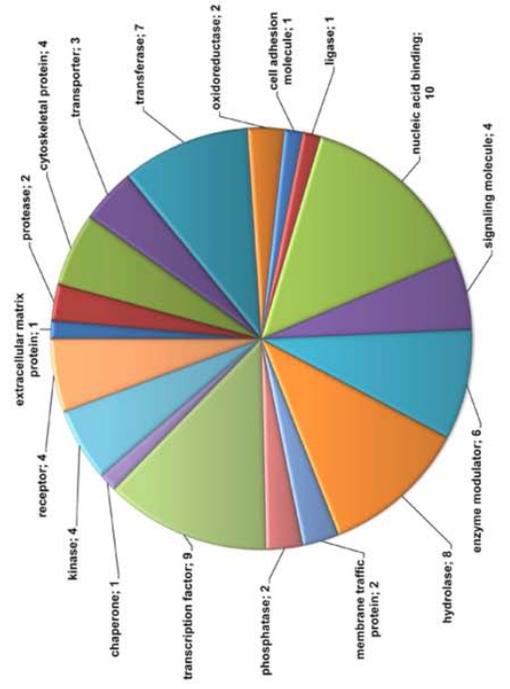
**A**



**B**

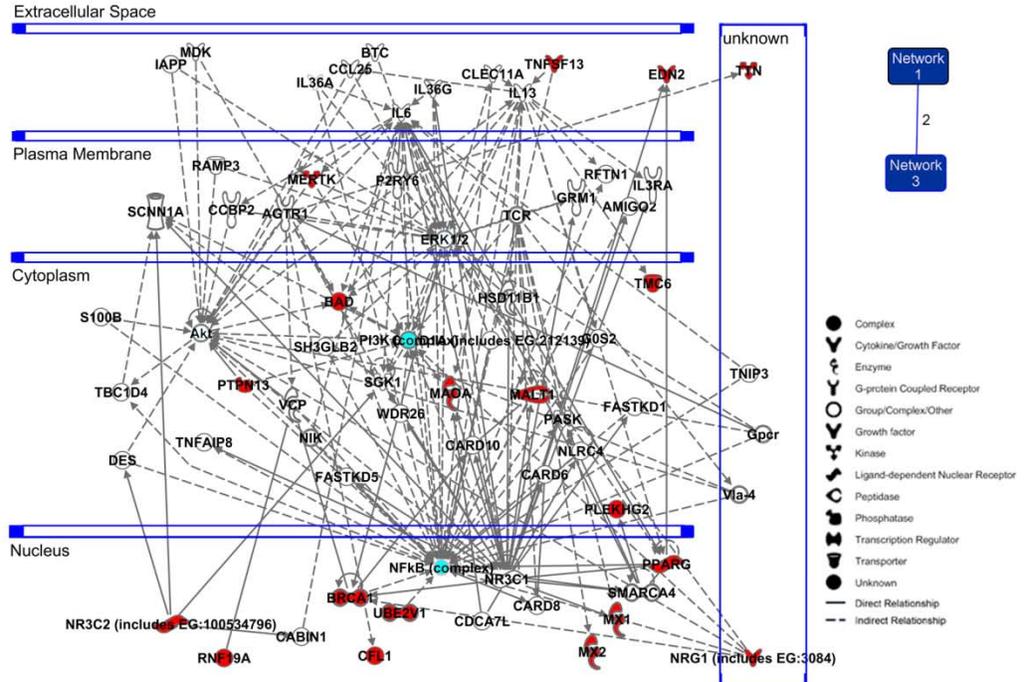


**C**

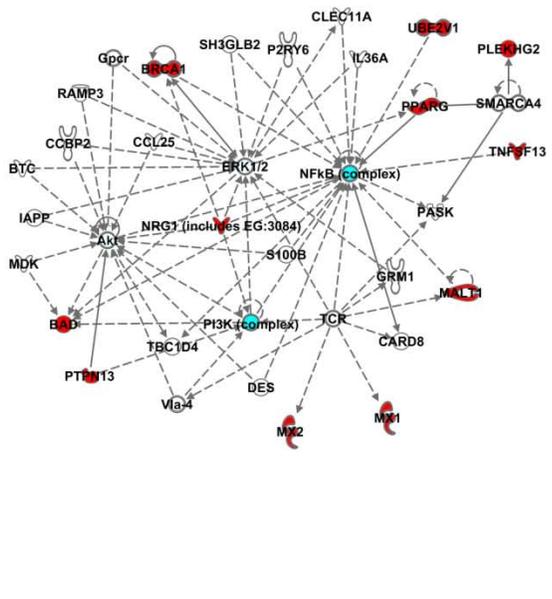


**Figure 14. Molecular and biological functions and protein class of 138 genes enriched in 2 genome-wide screen replicates.** The 70 annotated of the 138 genes identified were mapped with PANTHER ontology classification system to (A) Molecular functions, (B) Biological Functions, and (C) Protein classes.

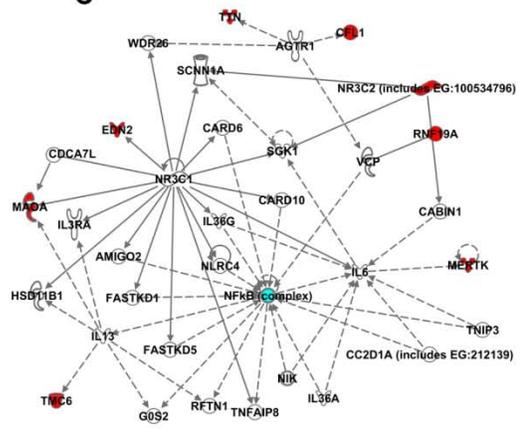
A



B



C



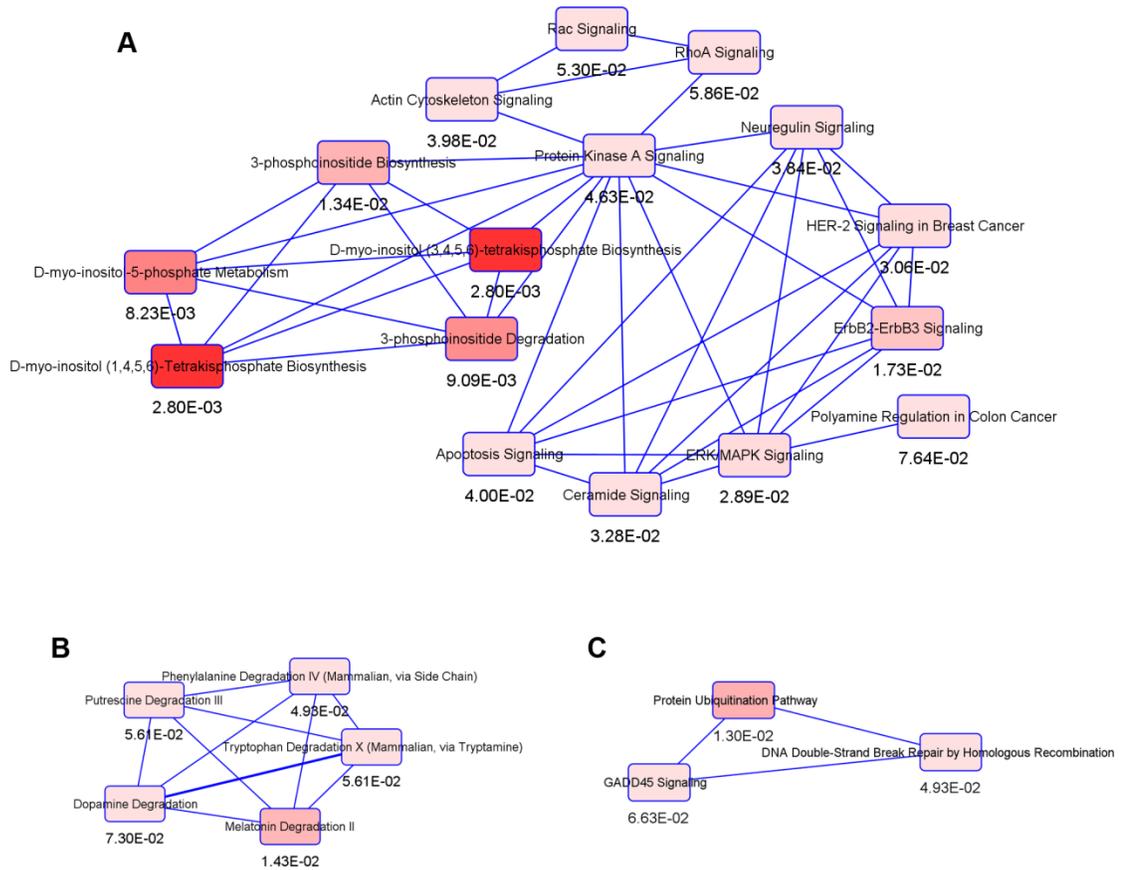
**Figure 15. 138 enriched genes from replicate screens were mapped to 2 major protein complexes.** Interacting networks were determined with Ingenuity Pathway Analysis for the 70 annotated of the 138 genes identified in the RNAi screen. Red are genes identified in my screens, white are unidentified genes, and cyan highlights PI3K and NF- $\kappa$ B complexes. (A) Shows both Networks 1 and 3 (right inset) as a single merged network. The number between the inset Networks 1 and 3 schematic indicates overlapping genes. (B) and (C) shows Network 1 and Network 3, respectively.

required for efficient influenza virus replication and induction of cell death (322). My analysis here, thus, suggested that BAD may be a central regulator of influenza-virus induction of host cell death, which involves the virus' control of the communication between these 4 protein networks.

Analysis of canonical signaling pathways showed my 138 genes are mapped to 3 separate pathway complexes. In the largest overlapping signaling pathway complex, Protein Kinase A signaling appears to act as a central hub that interacts with all the other interconnected pathways (Figure 16A). As suggested from the network analysis, the involvement of the ERK/MAPK signaling pathway, into which ERK1/2 are categorized, and apoptosis signaling were also identified. The second overlapping pathway complex shows 5 inter-connected biochemical pathways (Figure 16B). Interestingly, Protein Ubiquitination was identified in the last overlapping pathway complex (Figure 16C). Protein ubiquitination is an important process known to control the signal activation or inhibition of different pathways, including interferon (362), NF- $\kappa$ B (283), and apoptosis (262) signaling cascades.

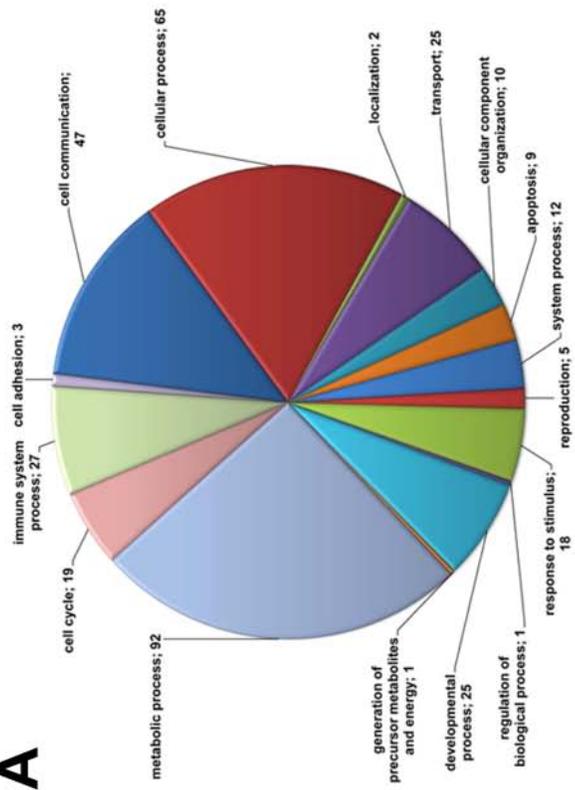
### **4.1.3 Network Analysis of 146 Genes Identified with Other RNAi Screens**

As mentioned above, 146 annotated genes identified from the total 1256 potential targets identified in my screen were also reported in the other 11 published siRNA screens (19, 20, 94, 119, 137, 141, 185, 205, 282, 307, 343). PANTHER analysis of these 146 genes showed an enrichment of the same 3 biological categories listed in section 4.2 (Figure 17A). However, in addition to the 3 molecular categories listed in section 4.2, these genes were also enriched in receptor and structural molecular activities

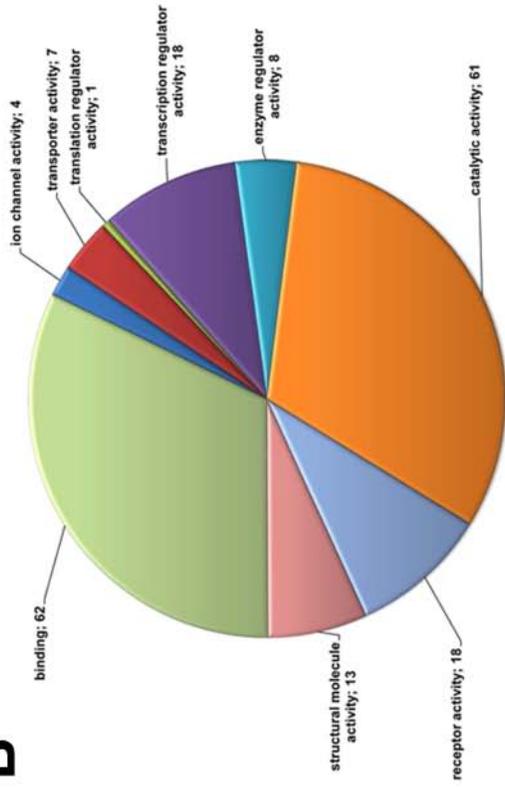


**Figure 16. Overlapping canonical signaling pathways of the 138 genes enriched in the RNAi screens.** Interconnecting signaling pathways were determined with Ingenuity Pathway Analysis for the 138 genes identified in the RNAi screen. Intensity of red highlight is representative of the degree of P-value, darker red indicates greater statistical significance. P-values are indicated below each node. (A-C) shows the 3 major overlapping signaling pathways.

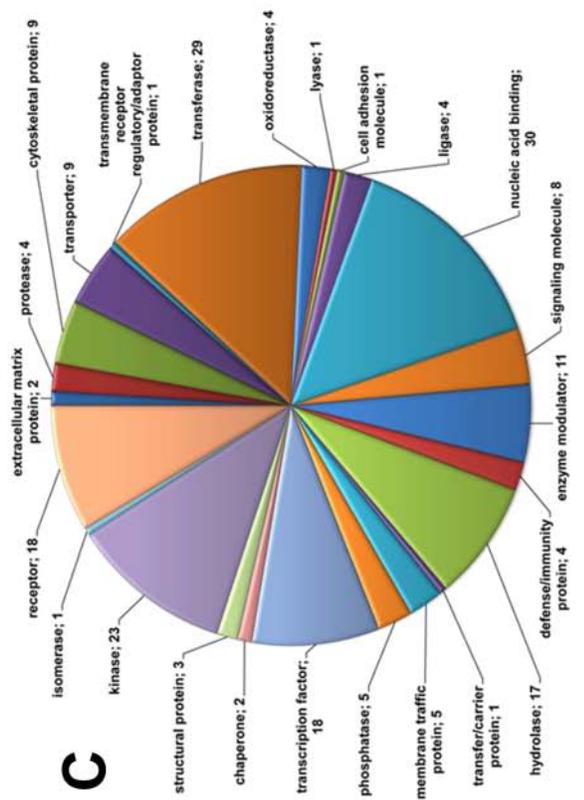
**A**



**B**



**C**



**Figure 17. Molecular and biological functions and protein class of 146 genes overlapping with other published screens.** The 146 annotated genes identified in all 11 RNAi screens and my screen were mapped with PANTHER ontology classification system to (A) Molecular functions, (B) Biological Functions, and (C) Protein classes.

(Figure 17B). In regards to protein classes, the 146 genes were also categorized into the same 5 broad protein classes listed in section 4.2 with an additional category, kinases (Figure 17C).

Interestingly, network analysis of these 146 genes with Ingenuity Pathway Analysis® resulted in 3 connecting networks (Figure 18A, B). Unlike the genes discussed in section 4.2, these 146 genes are connected to a single major hub called the ubiquitin c (UBC) protein complex (Figure 18). Network 7 showed 2 additional minor protein hubs identified as NEDD4 and SMAD1 (Figure 18E). NEDD4 is an E3 ubiquitylase, and SMAD1 protein belongs to a group of proteins involved in signal transduction and transcription regulation of multiple signaling pathways. Network analysis of the 97 genes identified in our screen and in only the 6 influenza RNAi screens also highlighted the same single major hub: the UBC protein complex (Figure A1).

A single large interconnecting network of canonical signaling pathways was identified for these 146 genes (Figure 19). Specifically, the network showed 4 central pathways: acute phase response signaling, IL-1 signaling, cell junction signaling and glioma signaling (Figure 19). Notably, NF- $\kappa$ B signaling was also identified. The NF- $\kappa$ B signaling pathway also was one of the major pathways identified in section 4.2 for the 138 genes enriched in my RNAi screen. This suggests that NF- $\kappa$ B plays a significant role in influenza virus replication, possibly at different stages during the virus lifecycle. A manuscript describing the RNAi screens has recently been submitted to *Cell Death and Disease*.

As mentioned earlier, the design of my RNAi screen was to identify genes that are required for virus-induced cell death. Of the 138 genes identified in both high-

throughput replicate screens, I chose 5 genes – BAD, MxB, TNFSF12-TNFSF13, TNFSF13, and USP47 – to further investigate their individual roles in influenza virus induction of host cell death because cells in which these genes were knocked down appeared to completely resist influenza virus-mediated cytopathology (Figure 13).

BAD was chosen for further study because of its well-known role in apoptosis regulation. TNFSF12-13, TNFSF13, and USP47 are suggested to be involved in NF- $\kappa$ B and deubiquitylation signaling, 2 major pathways highlighted in my network analysis (Section 4.2). Moreover, all 5 genes also were identified in the preliminary manual screen of 3 library pools discussed in Section 4.1.

## **4.2. Pro-apoptotic BAD Involved in Virus Regulation of Apoptosis**

### **4.2.1 BAD Regulation of Virus-induced Cytopathology and Cell Death**

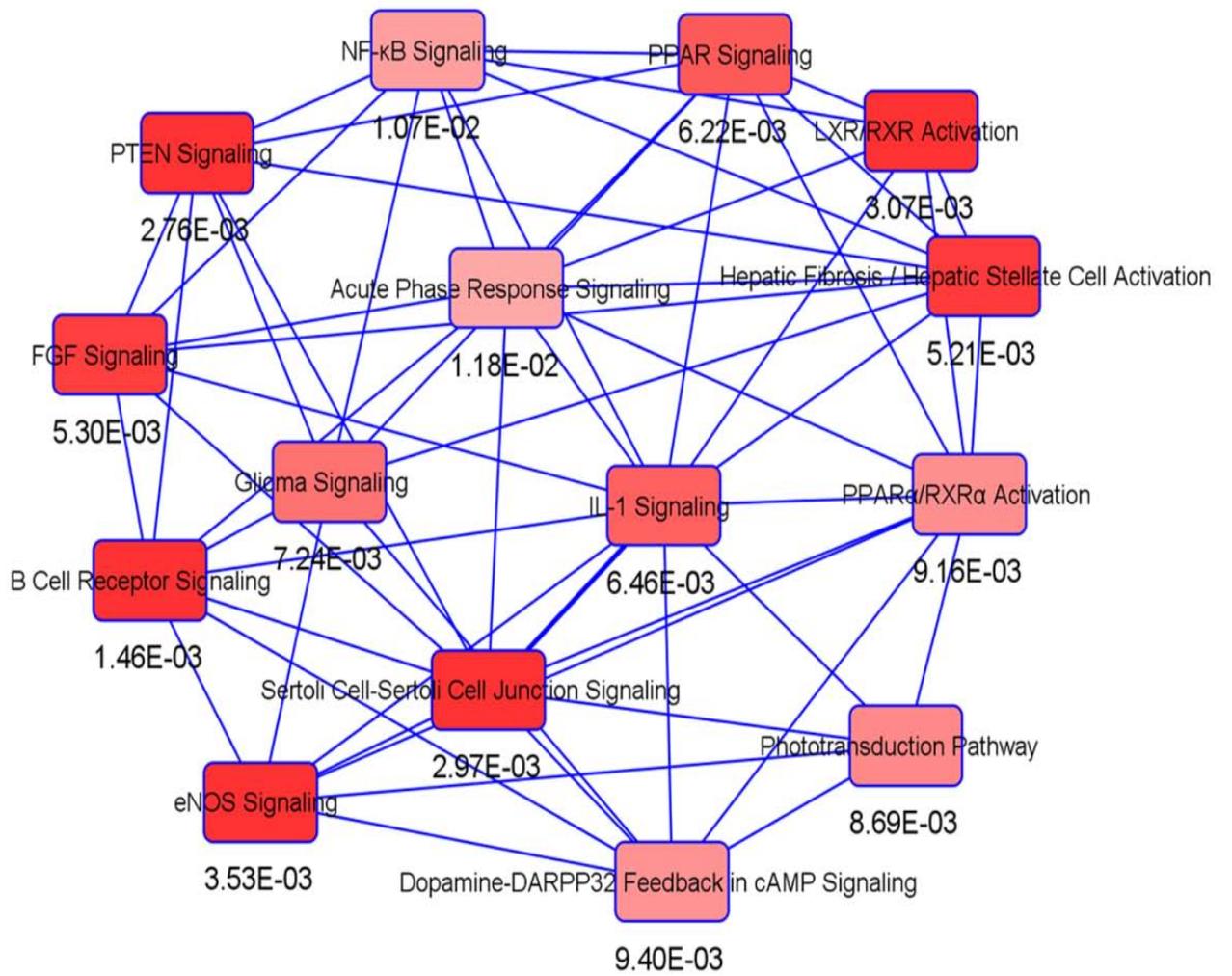
Influenza virus killing of host cells is known to occur through the activation of the apoptotic signaling pathway (101, 199). BAD is an important regulator of anti-apoptotic Bcl-2 and Bcl-xL proteins. Its blockage of the latter two factors defines the fate of the host cell towards apoptosis. In order to determine the effect of BAD on influenza virus replication, I generated BAD knockdown (KD) cells. A549 cells were treated with shRNA, or siRNA oligomers, that target BAD transcripts to create stable or transient knockdown cells, respectively. BAD-shRNA KD cells were infected with A/New York/55/2004 (H3N2) virus (NY55), A/Puerto Rico/8/1934 (H1N1) virus (PR8) or the 2009 pandemic SOIV (A/California/07/2009). Infected cells were examined visually for demonstration of CPE, which phenotypically manifests as rounding up and detachment of infected cells as well as abnormal cellular structural morphology. Non-transduced cells



**Figure 18. 146 genes overlapping with other published screens were mapped to a single major protein complex.** Interacting networks were determined with Ingenuity Pathway Analysis for the 146 genes identified in all 11 RNAi screens and my screen. Red are genes identified in my screen, white are unidentified genes, and cyan highlights major protein complex. (A) Shows connecting Networks 4,6, and 7 with number of common genes indicated along connector line. (B) Merge of all 3 networks from (A). (C-E) shows Network 4, 6 and 7, respectively.

and non-targeting shRNA transduced controls infected with any tested influenza virus subtype (NY55, SOIV, or PR8) showed extensive CPE indicative of virus-induced cytopathology (Figure 20A). In contrast, there was no observable CPE in influenza virus-infected cells that had been transduced with BAD-specific shRNAs. To ensure that my observation was not biased by artefacts derived from the use of shRNA constructs and lentivirus transductions, and to determine if the same observations would manifest in transient KD, I used siRNA duplexes to knockdown BAD in another set of A549 cells and infected these with the same virus subtypes. Comparable results were observed with siRNA-treated cells (Figure 20B). This lack of CPE development in virus-infected BAD KD cells suggests inhibition of the virus' capacity to induce cytopathology in BAD-deficient cells.

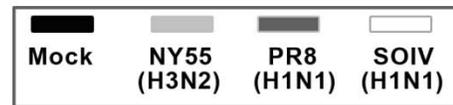
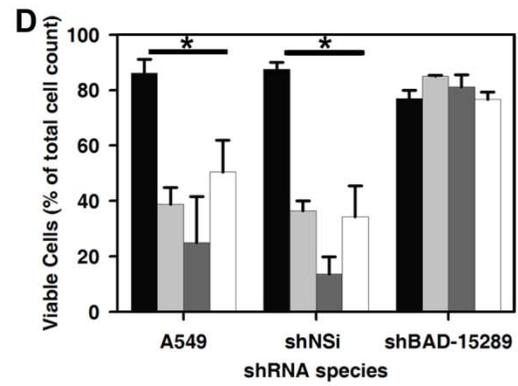
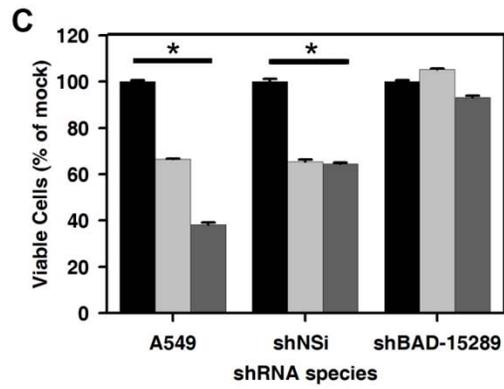
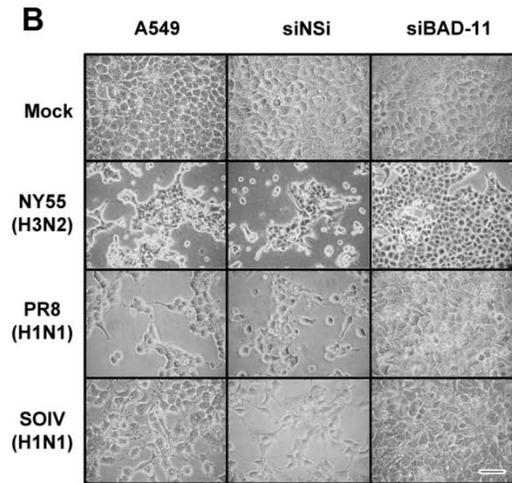
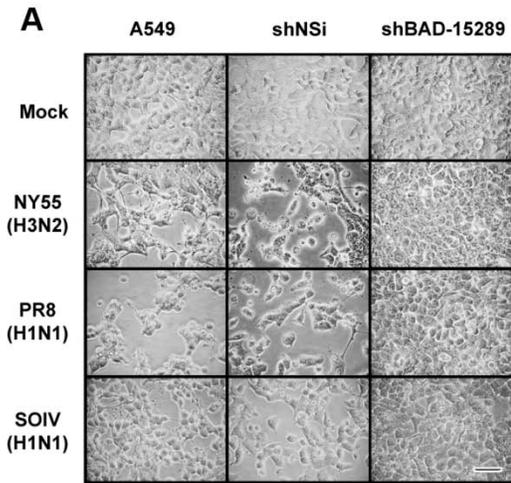
CPE development does not necessarily correlate with cell viability; therefore, a more quantitative means of determining cell viability was carried out by measurement of mitochondrial activity (WST-1) and by trypan blue exclusion. Both assays showed greater viability of virus-infected BAD KD cells compared to non-targeting shRNA and untransduced cell controls. In NY55 infected cells, 100% of the BAD KD cells survived viral infection, as measured by mitochondrial activity, and over 93% of cells survived PR8 infection; this is compared to controls with less than 65% survival in non-targeting and untransduced cells ( $P < 0.001$ ) (Figure 20C). Similarly, greater than 76% of the BAD KD cells survived influenza virus infection at 72 hpi for all three virus subtypes (Figure 20D), as measured by trypan blue exclusion, compared to less than 40% of the cells surviving infection in the non-targeting and untransduced controls.



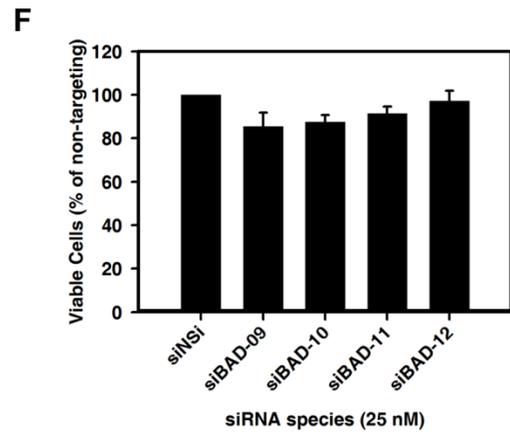
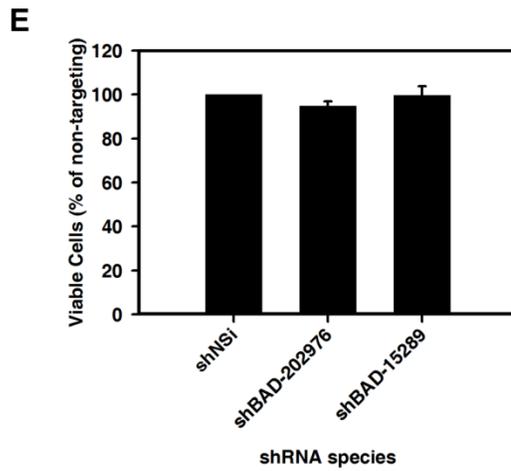
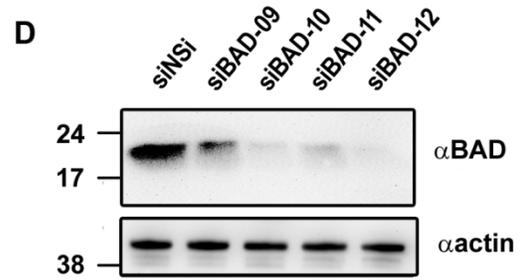
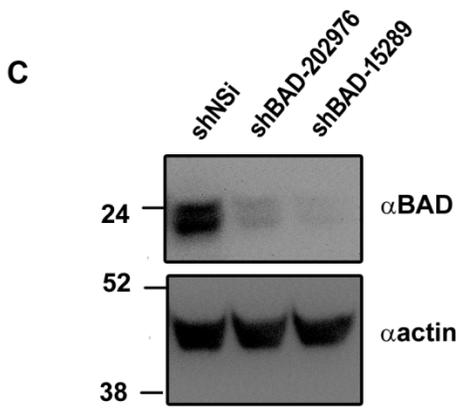
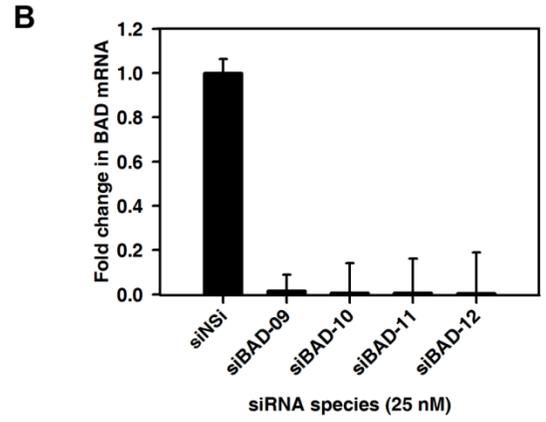
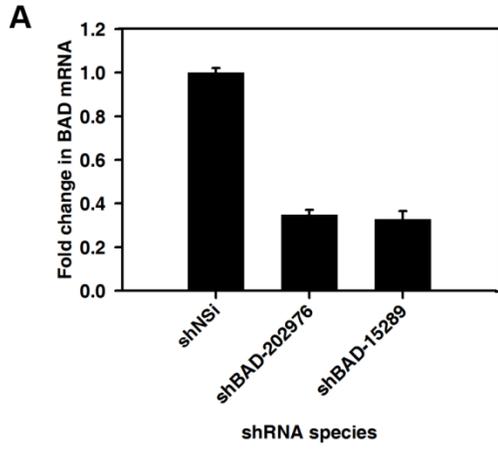
**Figure 19. Overlapping canonical signaling pathways of the 146 genes that overlap with other published screens.** Interconnecting signaling pathways were determined with Ingenuity Pathway Analysis the 146 genes identified in all 11 RNAi screens and my screen. Intensity of red highlight is representative of the degree of P-value, darker red indicates greater statistical significance. P-values are indicated below each node.

Efficient KD of endogenous BAD was validated by quantitative real-time PCR and Western blot to ensure the effectiveness of the shRNA or siRNA treatment. BAD mRNA KD was confirmed by qRT-PCR for both shRNA and siRNA treatments (Figure 21A, B). The different fold-change in mRNA levels between shRNA treated cells and siRNA treated cells might be due to the difference in knockdown efficiencies as a result of different shRNA and siRNA sequences. Additionally, shRNA expression is dependent on the region of integration in the genome. Expression will be greater in more active promoter regions, and the opposite is true in less active regions.

Western blots for total endogenous BAD protein supports the real-time PCR results (Figure 21C, D). Total BAD protein was significantly reduced for both shRNA species (Figure 21C) as well as for all 4 siRNA oligomers tested (Figure 21D). BAD protein appeared as a doublet in Figure 21C and only as a singlet in Figure 21D. Possible explanations for this discrepancy may include: a difference in how the samples ran through the gel during electrophoresis since the separation of the ladders appeared different between the 2 gels (refer to Figure A2 in the Appendix) and more samples were loaded in Figure 21C compared to Figure 21D based on the actin loading control. Phosphorylated BAD may exist as a smaller population, which may not be detected when less sample was loaded in Figure 21D. Treatment of A549 cells with shRNA or siRNA alone did not affect cell viability as determined by the WST-1 cell proliferation assay (Figure 21E, F). For proper comparison of cell viability and BAD knockdown, both cell viability and BAD mRNA isolation was carried out at the same time—2 weeks after shRNA transduction, which allows for the establishment of a stable knockdown cell line, and 48 h after the initial siRNA transfection.



**Figure 20. BAD is required for influenza virus-induced cytopathology and cell death.** Cytopathic effect in infected (A) BAD-shRNA or (B) BAD-siRNA knockdown A549 cells that were infected with NY55, PR8, or SOIV. At 72 hpi, cytopathic effect of cells were examined with a Nikon Eclipse TE2000-S inverted microscope and images obtained with a Canon PowerShot A700 digital camera. Shown are images representative of 3 independent replicates. Scale bar represents 100  $\mu$ M. Cell viability at 72 h after influenza virus infection was determined for shRNA-treated cells by (C) WST-1 assay and by (D) trypan blue exclusion assay. A total of 200 cells were counted and the percentage of trypan blue-excluding (viable) cells was determined. NSi is nontargeting shRNA or siRNA control. Shown is the mean from 3 independent replicates with error bars representing standard deviation (\*  $P < 0.001$ ). (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12)



**Figure 21. Efficiency of BAD knockdown in A549 cells.** BAD transcripts were determined by real time PCR for (A) stably transduced shRNA cells ( $P < 0.001$ ) or (B) siRNA-transfected cells ( $P = 0.002$ ) and non-targeting (NSi) shRNA or siRNA control cells, respectively.  $C_t$  values were normalized to 18S rRNA control and compared to non-targeting (NSi) control. BAD protein knockdown was confirmed with western blot in (C) shRNA- and (D) siRNA-treated cells. Effect of BAD knockdown on cell viability was assessed by WST-1 for (E) stably transduced shRNA cells and (F) siRNA-treated cells. Both cell viability and BAD mRNA quantification was determined 2 weeks after stably transduced cells were produced and 48 h after transfection with siRNA. NSi is nontargeting shRNA or siRNA control. Shown is the mean from duplicate runs with error bars representing standard deviation. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12). (Refer to Appendix Figure A1 for full Western blot.)

The greater percentage of cell survival, and lack of CPE development despite viral infection, suggests BAD is involved in promoting virus-induced cytopathology and cell death. Therefore, my next objective was to assess the capacity for influenza viruses to induce apoptosis in BAD KD cells.

#### **4.2.2 Inhibition of Virus Replication in BAD Knockdown Cells**

A number of studies have reported the importance apoptosis plays in promoting efficient influenza virus replication (356, 357). Given the critical pro-apoptotic nature of BAD and the lack of CPE and cell death development I observed in BAD KD cells, I hypothesized that influenza virus replication was suppressed in BAD-deficient cells. To explore this possibility, stable BAD KD and non-targeting shRNA A549 cells were infected with NY55, and virus replication was followed over a 72 h period. Virus progeny yield was also determined for PR8 and SOIV. Virus progeny production was titered via plaque assay on MDCK cells.

Initial round of virus replication from 0 hpi to 12 hpi was comparable in shRNA BAD KD cells and shRNA non-targeting control (shNSi); however, subsequent viral replication after 12 hpi showed less efficient viral replication in BAD KD cells compared to the control (Figure 22A). NY55 production was significantly reduced to less than 37% and 15% of the non-targeting shRNA control with BAD-specific 202976-shRNA and 15289-shRNA, respectively (Figure 22B,  $P = 0.011$ ). Reduction of viral titre was much more dramatic with SOIV and PR8, which replicated to only about 1% of levels seen in the shRNA non-targeting controls (Figure 22D,  $P < 0.001$ ).

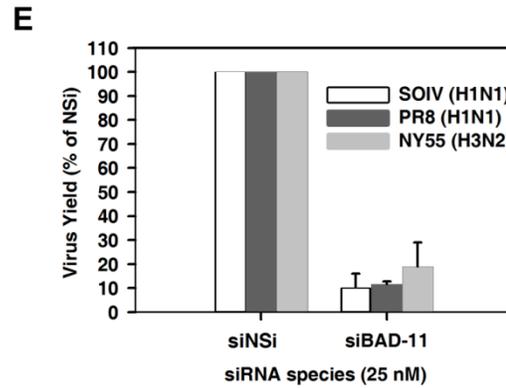
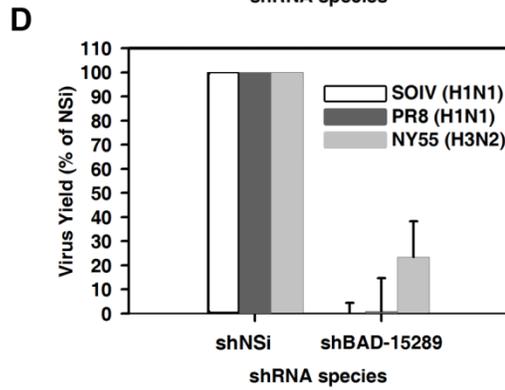
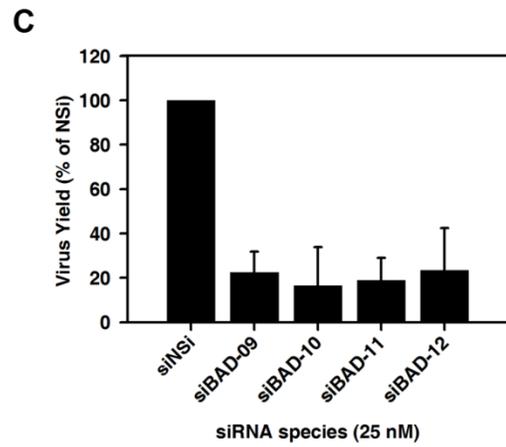
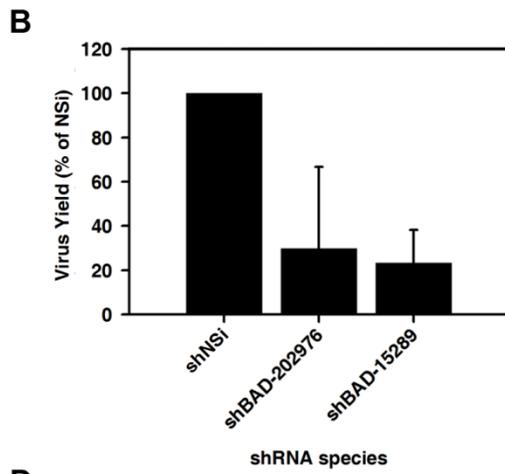
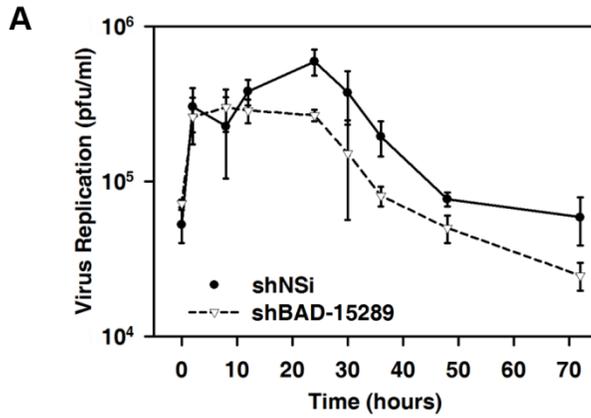
To ensure that a reduction in viral replication was not due to the effect of shRNA and/or lentivirus treatment, I repeated the infections in siRNA transiently transfected

A549 cells. Cells were sequentially treated twice with each of four distinct siRNAs that target BAD (plus a non-targeting siRNA control) 24 h apart, and after a further 24 h, were infected with NY55, PR8, or SOIV influenza viruses. Virus titre was determined at 72 hpi.

Replication of all three virus strains was dramatically reduced in siRNA BAD KD cells (Figure 22C, E). NY55 production was reduced to less than 24% of the non-targeting control by all four siRNA species (Figure 22C,  $P < 0.001$ ). SOIV and PR8 titers were less than 10% and 11% of the control, respectively; this was slightly lower than that detected for NY55 titre, which was  $\sim 19\%$  of the control (Figure 22E,  $P < 0.001$ ). My observation with siRNA confirms the results determined with the infection of lentivirus-mediated BAD shRNA knockdown. These data strongly indicate that BAD is a significant host factor required for efficient influenza virus replication. Importantly, my results show that BAD contributes a vital role in the lifecycles of different influenza virus subtypes.

### **4.2.3 Reduction of Virus Protein in Absence of BAD**

Influenza virus-induced cell death occurs in late viral lifecycle; as late as 15 hpi has been reported (369). The lack of cell death coupled to significant reduction in viral replication in BAD KD cells raises the question of whether influenza virus replication in BAD-deficient cells may be inhibited early in the viral lifecycle. In order to explore this, I carried out western blot assays on whole cell lysates of stable shRNA BAD KD and non-targeting shRNA control cells that were infected with NY55. Viral protein production was determined at specific time points over a 72 h period. The membrane was probed for influenza virus proteins NS1, NP, and HA. The NS1 mouse monoclonal

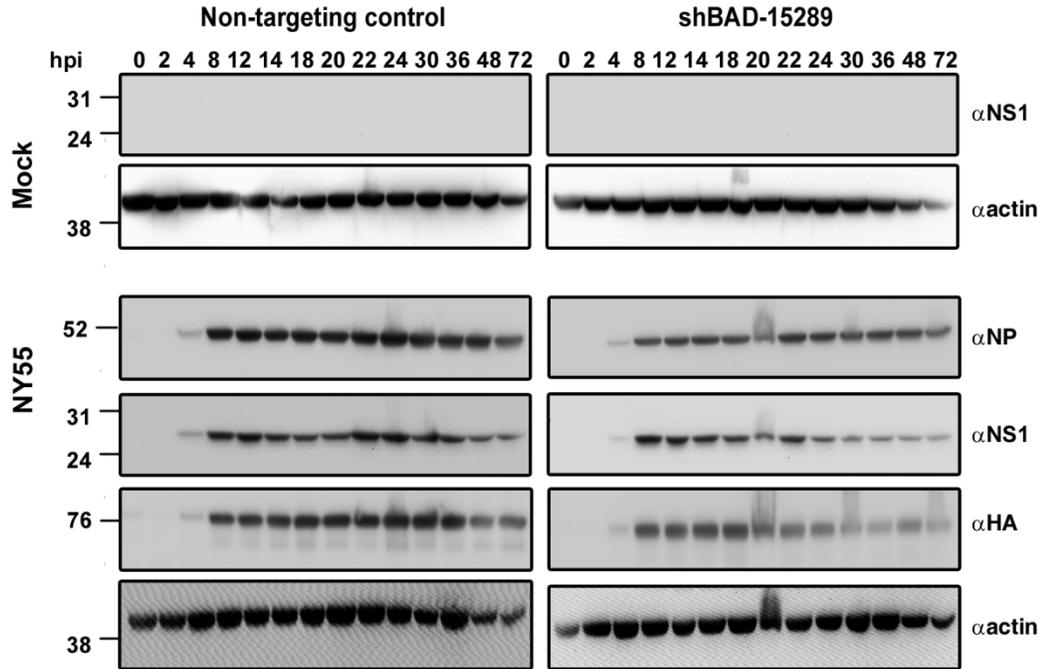
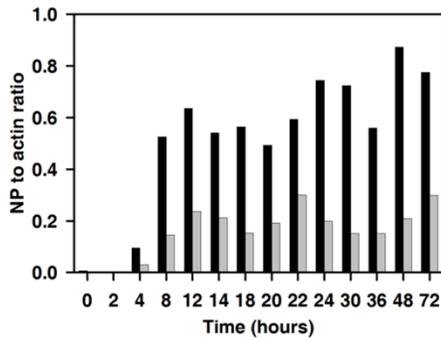
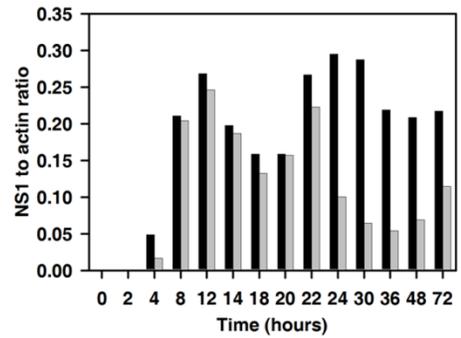
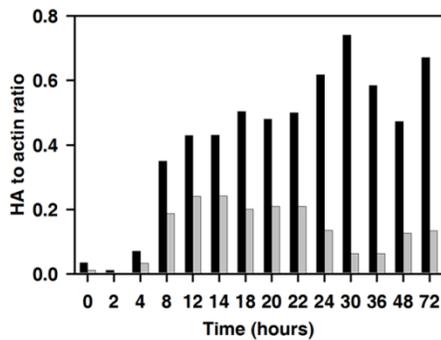


**Figure 22. Inhibition of influenza virus replication in BAD knockdown cells.** (A) NY55 virus growth curve in shRNA knockdown cells. NY55 virus yield was determined at 72 hpi in indicated (B) shRNA or in indicated (C) siRNA knockdown cells ( $P < 0.011$ ). Virus yields of NY55, PR8, and SOIV replication at 72 hpi in (D) shRNA or in (E) siRNA knockdown cells ( $P < 0.001$ ). NSi is the nontargeting shRNA or siRNA control. Values are means plus standard deviations (error bars) from 3 independent experiments. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12).

antibody was generated and characterized in my lab (266), and the characterization of the mouse monoclonal NP antibody was previously described (359). In the stably expressing non-targeting shRNA control cells, NS1, NP, and HA viral proteins were detected as early as 4 hpi, and strongly detected at 8 hpi onwards (Figure 23A, left column). Protein bands were faintly detected as early as 0 hpi for the HA viral protein. Since HA is incorporated into virion particles, the early detection could be due to the infecting viral population initially introduced. In contrast, the production of all three viral proteins was clearly reduced in infected BAD KD cells (Figure 23A, right column). Densitometric analysis of the viral protein bands showed an average of 5-fold and 4-fold reduction of NP and HA protein, respectively, in BAD KD cells compared to the non-targeting (shNSi) shRNA control (Figure 23B, D). NS1 protein production between BAD KD cells and non-targeting shRNA control is comparable from 8 hpi to 22 hpi but NS1 protein level dramatically dipped lower in BAD KD cells at 24 hpi up to 72 hpi, with an average of 3-fold reduction in BAD KD cells (Figure 23C). The results suggest that BAD KD significantly reduces the efficiency of viral replication.

#### **4.2.4 Influenza Induction of BAD Phosphorylation and Cleavage**

BAD's capacity to bind and neutralize anti-apoptotic proteins is inhibited upon phosphorylation (46). Given that my results showed BAD as a valuable cellular factor required for influenza virus replication, I suspected that viral replication might affect BAD activity and how BAD is regulated. I infected A549 cells with NY55 and harvested samples at specific time points post-infection. The samples were subjected to Western blot analysis and probed for BAD phosphorylation at sites S112 and S136. Total BAD was also determined.

**A****B****C****D**

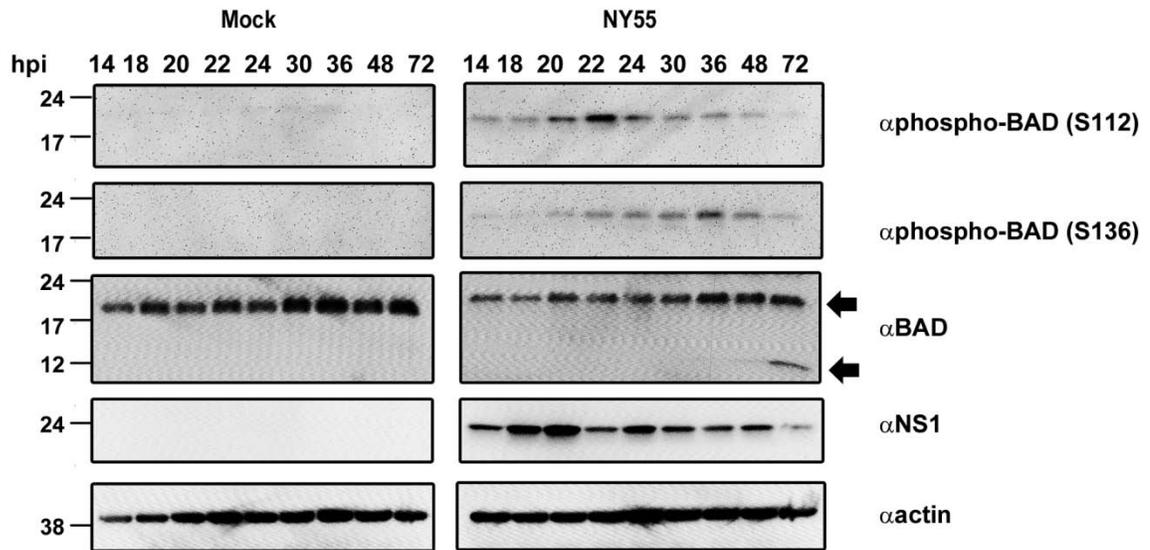
shNSi  
 shBAD-15289

**Figure 23. Reduction of influenza virus protein in BAD knockdown cells.** (A) Cells were infected with NY55 at MOI 1 and whole cell lysate was obtained at the indicated times. Western blot was probed with  $\alpha$ -NS1,  $\alpha$ -NP, and  $\alpha$ -HA antibodies. (B) to (D) Densitometric quantitation of bands in infected lanes were done with Alpha Innotech FluorChem® Q Imaging System and normalized to  $\beta$ -actin. NSi is non-targeting shRNA control. Mock is uninfected control. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12). (Refer to Appendix Figure A2 for full Western blot.)

My results showed that influenza virus infection induces BAD phosphorylation at both S112 and S136 but in a sequential manner (Figure 24). Phosphorylation at S112 occurred as early as 14 hpi, with most intense bands observed at 20-22 hpi, and was gradually reduced after 24 hpi. Phosphorylation at S136 was not detected until 20 hpi; the signal was maintained until 48 hpi but then decreased by 72 hpi. Total BAD, including unphosphorylated BAD, showed a gradual increase towards the late time points. Interestingly, I detected the smaller cleaved form of BAD at 48 hpi and 72 hpi, with the latest time point showing the greatest cleavage of BAD (Figure 24). All these modifications observed during influenza virus infection were not detected in uninfected (mock) controls. My results suggest that influenza viruses tightly control BAD activity via phosphorylation and cleavage to regulate the intrinsic apoptotic signaling cascade.

#### **4.2.5 Suppression of Cytochrome c Release in BAD Knockdown Cells**

Given that BAD is a well-known regulator of the mitochondria-dependent apoptosis pathway (46, 142), I wanted to determine whether a deficiency in BAD would suppress influenza virus' capacity to induce this signaling pathway during infection. Cytochrome c release from the mitochondria as a result of the organelle's dysregulation is a hallmark of the intrinsic apoptosis signaling pathway (142). To address this issue, I carried out a cytochrome c release assay, which briefly involved the following steps: infection of BAD KD and non-targeting control cells with NY55, cells were harvested at specific time points post-infection, and gently lysed to obtain the cytosolic and organelle fractions (labeled as mitochondrial pellet in Figure 25).



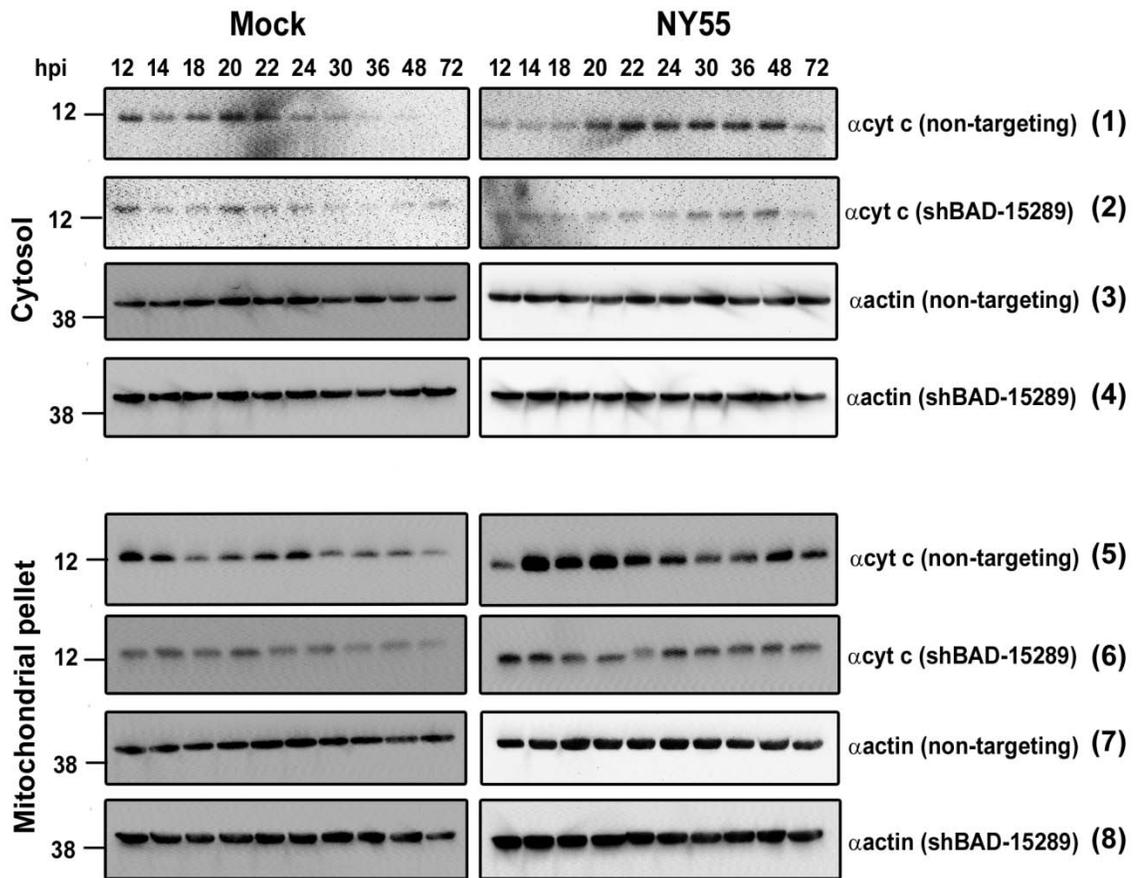
**Figure 24. Influenza virus induces BAD phosphorylation and cleavage.** A549 cells were infected with NY55 at MOI 3, harvested at indicated time points post-infection, and protein samples probed for phosphorylated BAD at residues S112 and S136, total BAD, and viral NS1 protein. Mock is uninfected. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12). (Refer to Appendix Figure A3 for full Western blot.)

I observed an increase in the release of cytochrome c into the cytosol of infected non-targeting control at 20 hpi to 48 hpi relative to the uninfected (mock) samples (Figure 25, row 1). As expected, the corresponding mitochondrial pellet of the infected non-targeting control showed a decrease in cytochrome c at 20 hpi to 48 hpi (Figure 25, row 5). I did not observe a similar increase in cytochrome c release into the cytosol of infected BAD KD cells (Figure 25, row 2), and the amount of cytochrome c in the mitochondrial pellets was similar for the infected and uninfected BAD KD cells (Figure 25, row 6). These data suggest that influenza viruses induce mitochondria dysregulation late in the virus replication cycle, as observed by cytochrome c released into the cytosol of infected cells, and that BAD is required for virus-induced dysregulation of the mitochondria.

#### **4.2.6 Virus-induced Caspase Activity Requires BAD Protein**

Influenza virus infection results in the activation of apoptosis both *in vivo* and *in vitro* (213, 313). I determined caspase activity in BAD KD A549 cells infected with NY55 at an MOI of 1. Caspase activity was measured at 72 hpi using the Promega Caspase-Glo 3/7 kit. Non-transduced and stably transduced non-targeting shRNA cells were used as controls. Staurosporine-treated cells were included as positive controls for caspase-3/7 activity.

Infection of non-transduced and non-targeting shRNA cells resulted in an increase in caspase 3/7 activity by approximately 1.5-fold ( $P = 0.001$ ; Figure 26A). I did not detect any significant difference in caspase activity induced by NY55 infection in BAD-deficient cells compared to uninfected mock (Figure 26A). Although there appeared to be a difference in caspase activity between the uninfected (mock) non-targeting shRNA



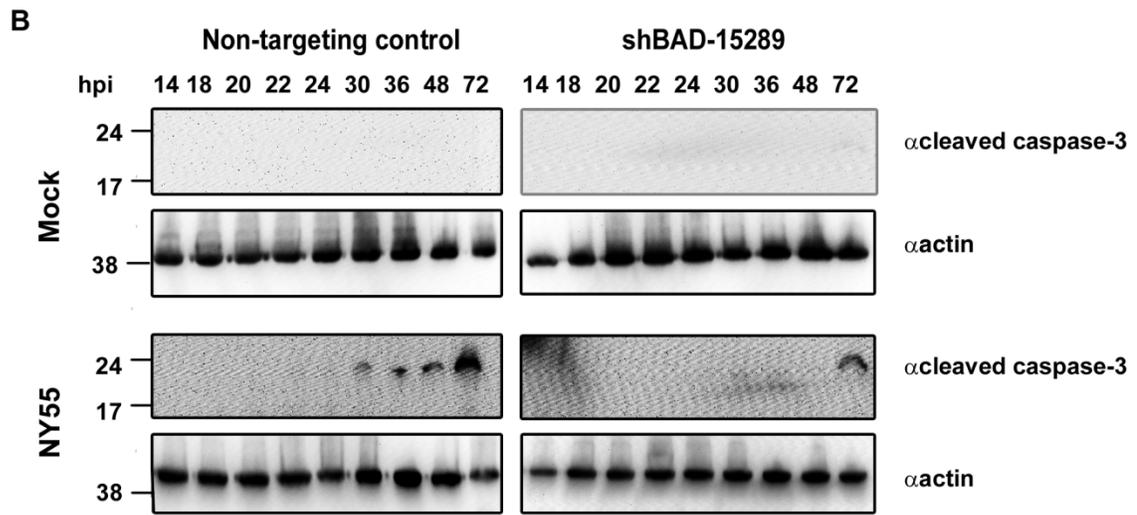
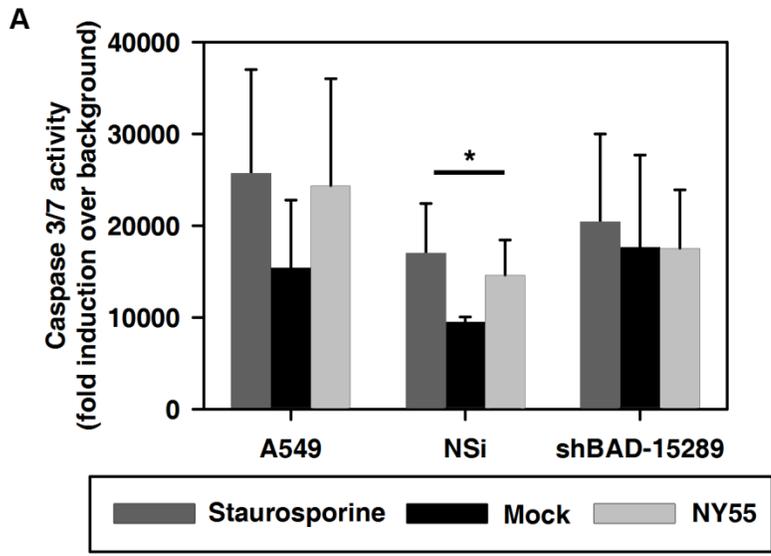
**Figure 25. Deficiency in BAD inhibits virus-induced Cytochrome c release.** Cytochrome c release was determined in NY55 infected cells at MOI 3. Cytosolic and mitochondrial fractions were obtained and blotted for cytochrome c at specific time points post-infection. Non-targeting represents non-targeting shRNA control. Mock is uninfected control. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12). (Refer to Appendix Figure A4 for full Western blot.)

control and BAD KD cells, this difference was not statistically significant. Overall, this indicates that BAD is necessary for influenza virus activation of caspase 3/7 activity.

#### **4.2.7 Virus Induction of Caspase-3 and Caspase-7 Activity**

The Caspase-Glo assay does not differentiate between the activities of caspase-3 and caspase-7. Given that caspase-3 activation is necessary for efficient influenza virus infection (357), I carried out immunoblotting for cleaved caspase-3 and caspase-7 protein products. Caspase activation requires proteolytic cleavage of zymogens into smaller, enzymatically active fragments (257). Cytoplasmic lysates were obtained from shRNA non-targeting control and BAD KD cells infected with NY55. Cleaved caspase-3 products were readily detected in the infected shRNA non-targeting control at 30 hpi, and most strongly visible at 72 hpi (Figure 26B, left column). However, caspase-3 cleavage was undetected at times points before 72 hpi and significantly reduced cleaved caspase-3 was detected in the infected BAD KD lane at 72 hpi (Figure 26B, right column).

I also observed caspase-7 cleavage in the infected non-targeting control, with significant cleavage occurring between 30 hpi to 72 hpi (Figure 27A, left column). Similar to my observation with caspase-3, cleavage of caspase-7 was suppressed in BAD KD cells compared to the non-targeting control (Figure 27A, right column). Densitometric analysis of cleaved caspase-7 in the infected lanes showed on average a 5-fold reduction in caspase-7 cleavage compared to the shRNA non-targeting control cells (Figure 27B). These results suggest that influenza viruses need BAD in order to induce caspase activation.

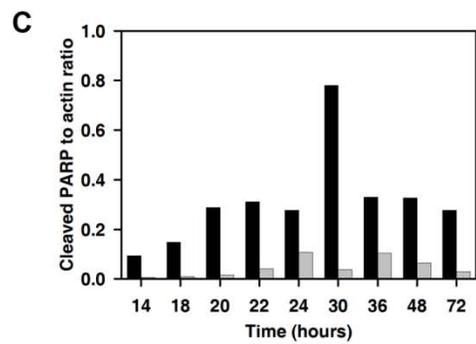
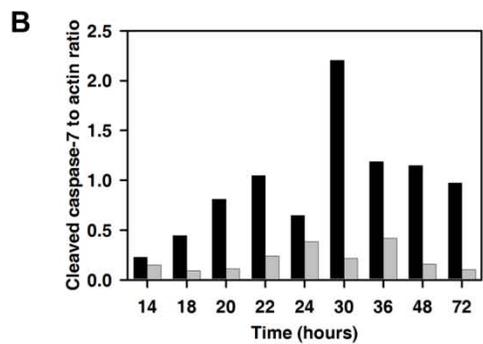
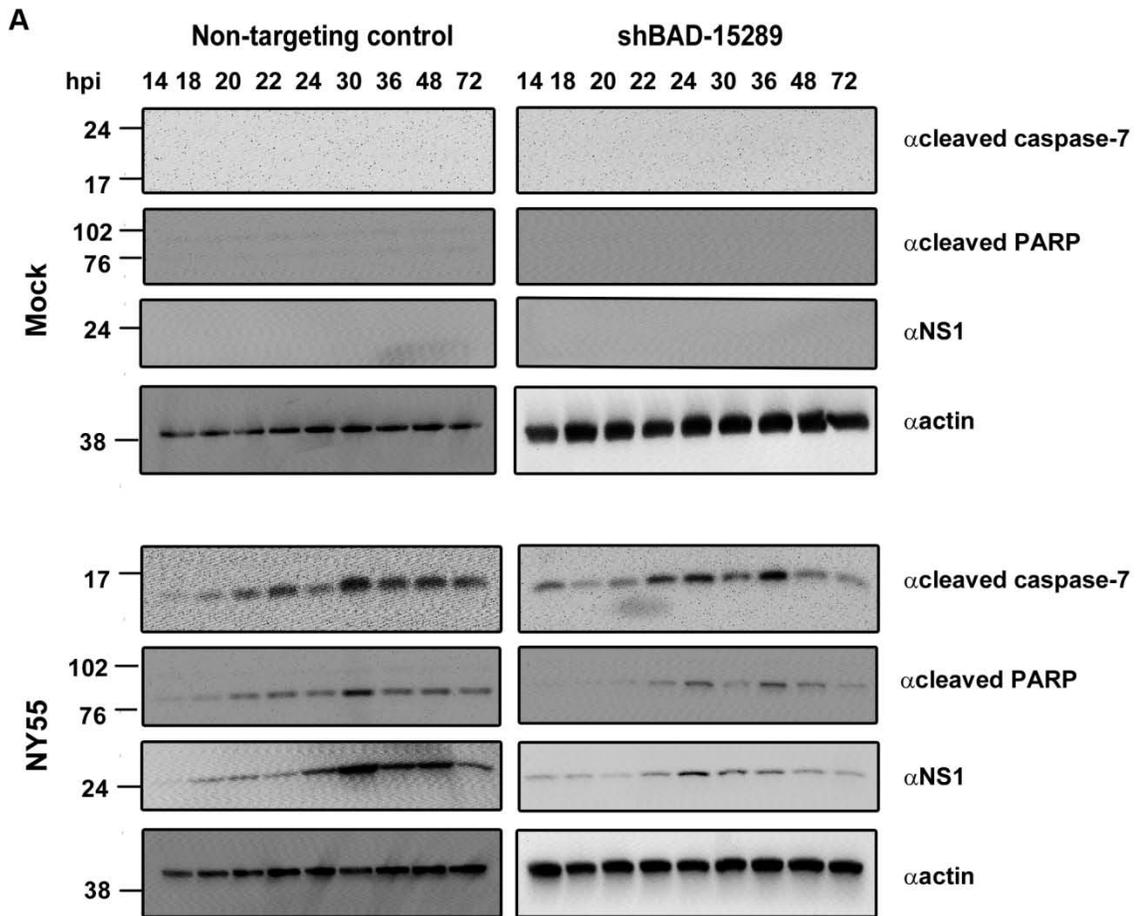


**Figure 26. BAD is required for influenza virus activation of caspase-3.** (A) Measurement of caspase-3/7 activity using Caspase-Glo assay at 72 hpi in A549 cells infected with NY55 at MOI 1, or at 24 h post-treatment of cells with 1  $\mu$ M staurosporine. Shown is the mean from 3 independent replicates with error bars representing standard deviation (\*  $P < 0.001$ ). (B) Caspase-3 cleavage was assessed via Western blot for the small, cleaved fragment of caspase-3. Non-targeting control (NSi) and BAD-shRNA KD A549 cells were infected with NY55 at MOI of 3 and cytoplasmic lysate was obtained at 72 hpi. NSi is non-targeting shRNA control. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12). (Refer to Appendix Figure A5 for full Western blot.)

Caspase-3 and -7 are effector caspases that cleave other proteins downstream of the apoptotic signaling pathway. One of these downstream substrates is PARP. I looked at PARP cleavage during influenza virus infection at specific time points late in the replication cycle in both the non-targeting control and BAD KD cells. My results showed that influenza virus replication induces increasingly greater PARP cleavage from 14 hpi to 72 hpi (Figure 27A). However, cleaved PARP was only faintly detected in western blots of infected BAD KD cell lysates. Densitometric analysis showed more than a 10-fold reduction of PARP cleaved in the knockdown cells (Figure 27C). Interestingly, greater caspase-7 and PARP cleavage in the non-targeting control corresponded with an elevated expression of NS1 (Figure 23 and Figure 27). The cleavage of both caspase-7 and PARP appeared most elevated at 30 hpi, suggesting this to be a critical period for apoptosis induction and viral replication. Further studies will provide a greater understanding of the critical molecular changes that may occur around this time period. Similar to my observation earlier, NS1 expression is reduced in BAD KD cells. In all, my data suggest that influenza viruses require the presence of BAD to efficiently induce the intrinsic apoptotic signaling pathway.

#### **4.2.8 Change in BAD Co-localization Between Bcl-2 and Bcl-xL**

Studies have shown that overexpression of Bcl-2 inhibits influenza virus replication and causes an overall reduction in viral protein production (233). This raises the question whether influenza viruses may redirect BAD to inhibit Bcl-2 or Bcl-xL to promote apoptosis for viral propagation. I carried out immunofluorescence assays to look at the co-localization of BAD with Bcl-2 or Bcl-xL in the presence of influenza virus infection in A549 cells. Cells were infected with NY55 at an MOI of 1 and processed at 24 hpi for



■ shNSi    ■ shBAD-15289

**Figure 27. Cleavage of caspase-7 and PARP is inhibited in influenza virus-infected BAD knockdown cells.** (A) Non-targeting shRNA control (NSi) and BAD knockdown cells were infected with NY55 at MOI 3 and cells were harvested at specific times points. Whole cell lysates were subjected to Western blotting with antibodies to cleaved caspase-7, cleaved PARP, and viral NS1 proteins. (B) and (C) Densitometric quantitation of bands in infected lanes were done with Alpha Innotech FluorChem® Q Imaging System and normalized to  $\beta$ -actin. NSi is non-targeting shRNA control. Mock is uninfected control. (Used with permission from: Copyright © American Society for Microbiology, (322), doi:10.1128/JVI.02017-12). (Refer to Appendix Figure A6 for full Western blot.)

imaging. Using the Zen (Zeiss) software, co-localization images were generated. Co-localization is determined by the software when colors emitted by 2 or more fluorescent molecules are detected in the same pixel. Zen designates BAD and Bcl-2 (or Bcl-xL) channels as blue and red in the co-localization image, respectively. Regions of co-localization are shown in yellow, and irrelevant background is white.

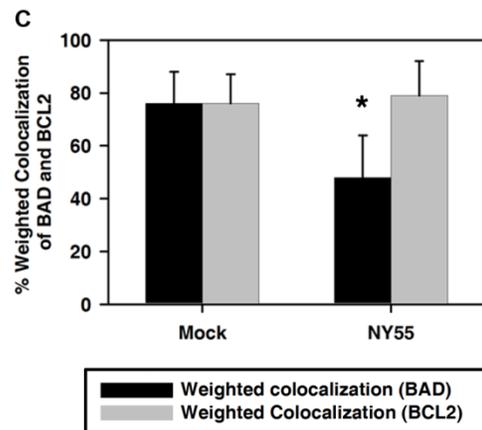
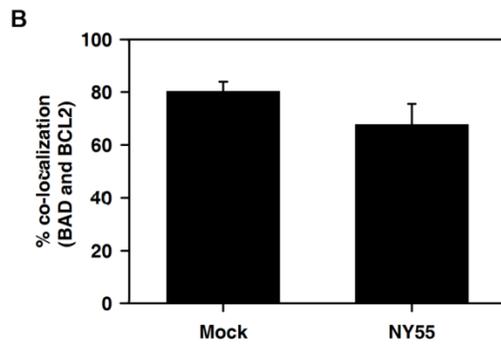
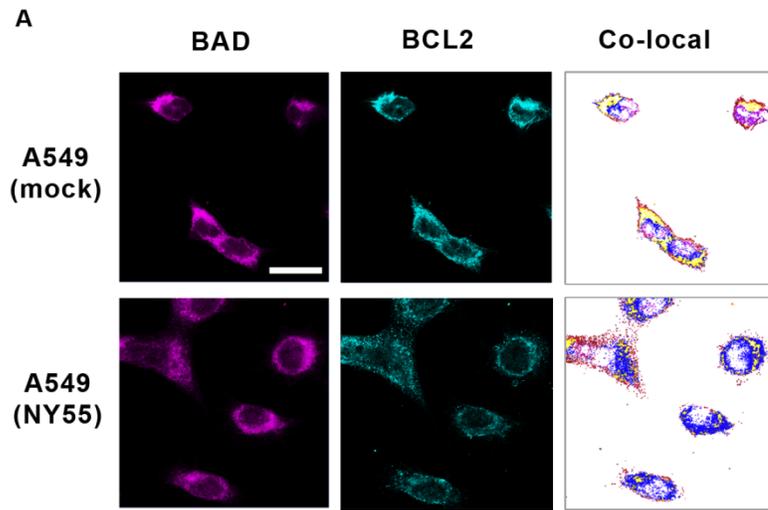
Co-localization of BAD and Bcl-2 is observed in uninfected A549 cells, but the regions of co-localization are significantly reduced in influenza virus infected cells as observed by a reduction in proportion of yellow pixels (Figure 28A). I determined the Manders Overlap Coefficient (183) to measure the degree of co-localization of BAD and Bcl-2 in a given area. The degree of co-localization is denoted as a value ranging from 0 (no co-localization) to 1 (complete co-localization). This value can be interpreted as the percentage of co-localization observed in the sampled region. Manders Overlay Coefficient showed the co-localization of BAD and Bcl-2 was reduced by more than 10% in virus-infected cells compared to uninfected cells ( $P = 0.01$ , Figure 28B).

I also calculated the Weighted Colocalization Coefficient (183, 374) for the same set of images to quantitatively determine the proportion of either BAD or Bcl-2 that contribute to the overall co-localization. The coefficient value can be interpreted as a percentage of the sampled population. My results indicated the proportion of BAD that actually co-localizes with Bcl-2 was significantly lower in virus-infected cells compared to uninfected cells ( $P=0.014$ , Figure 28C). However, the Bcl-2 Weighted Colocalization Coefficient did not significantly differ between uninfected and infected samples (Figure 28C). Yang et al (2005) determined that BAD associates with Bcl-2 at ~ 1:3 ratio, with excess BAD remaining unbound in the cytosol (358). This may explain why a decrease

in BAD participation did not affect the percentage of Bcl-2 that participated in the overall colocalization. Moreover, I may interpret the observed colocalization of BAD with Bcl-2 to suggest that BAD has an antagonistic role with Bcl-2 activity during influenza virus replication. Several studies have shown that expression of Bcl-2 results in suppression of vRNP translocation in the cytoplasm (223). A deficiency in BAD could allow Bcl-2 to mount a stronger anti-viral activity against the virus. This would certainly explain my observation in BAD KD cells where viral replication was dramatically inhibited, accompanied by a significant diminishment of influenza virus protein levels.

In contrast to what was observed with Bcl-2, the area of colocalization (yellow) between BAD and Bcl-xL remain unchanged from uninfected to influenza virus infected cells (Figure 29A). The Manders Overlap Coefficient for BAD co-localization with Bcl-xL did not significantly differ between uninfected and infected samples (Figure 29B). There were also no observed differences in the Weighted Colocalization Coefficient of uninfected (mock) and infected samples. This indicates that the proportion of Bcl-xL that contribute to the co-localization with BAD is unaffected by influenza virus infection. This suggests that the association between BAD and Bcl-2 may play a more significant role in influenza virus replication than BAD association with Bcl-xL.

Most the data obtained for the BAD protein described above was recently accepted for publication and are expected to appear in print in the 2<sup>nd</sup> January 2013 issue of the Journal of Virology (322).

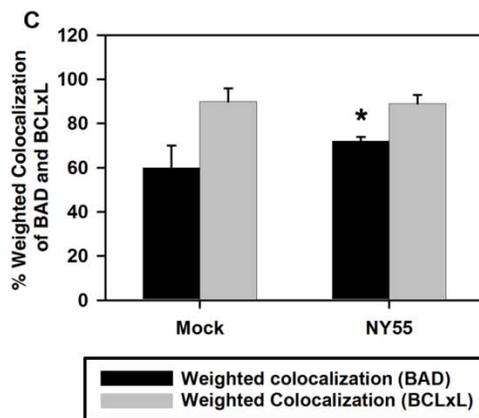
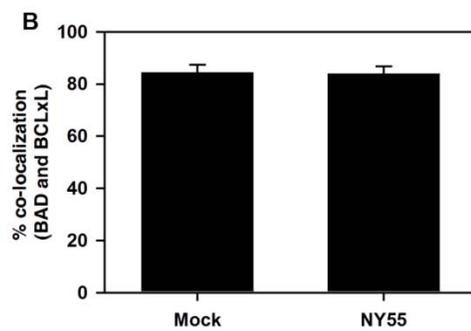
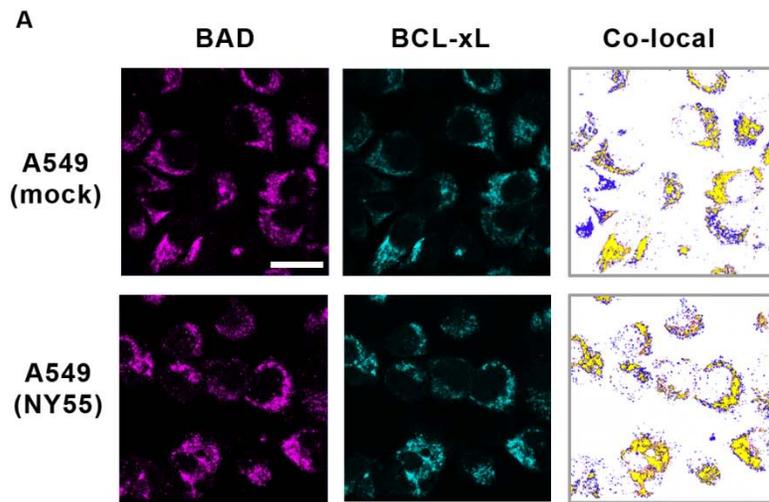


**Figure 28. Reduction in Bcl-2 and BAD co-localization near early stage of virus infection.** A549 cells were infected with NY55 at MOI 1 and fixed at 24 hpi. (A) Cells were stained with  $\alpha$ -BAD (magenta) or  $\alpha$ -Bcl-2 (cyan) antibodies. Zen software (Zeiss) was used to produce the co-localization (co-local) image. BAD and Bcl-2 channels are blue and red in the co-localization image, respectively. Regions of co-localization are shown in yellow and irrelevant background is white. (B) Manders Overlay Coefficient (P=0.01) and (C) Weighted Colocalization Coefficient (\* P=0.014) was determined from images taken at five different areas of each sample. Mock represents uninfected cells. White bar represents 100  $\mu$ M scale bar.

## 4.3. Role of MxB in Promoting Influenza A Virus Infection

### 4.3.1 MxB Affects Virus-induced Cytopathology and Cell Death

The activity of Mx proteins on influenza virus replication was initially established by Pavlovic and colleagues (247) using mouse 3T3 cells overexpressing MxA or MxB, from which it was determined that MxB had no anti-viral activity. However, to my knowledge, there have not been any studies that examined effects on influenza virus replication when MxB protein is depleted. If MxB activity supports influenza virus replication, overexpression assays may mask this possible observation. Moreover, overexpression of a protagonistic factor may not always result in enhanced viral replication. I therefore re-examined the involvement of human MxB protein in influenza virus replication. A549 cells treated with MxB-specific shRNA or siRNA oligomers were infected with PR8, NY55, or with the recent SOIV (A/California/07/2009). Virus progeny production was determined by plaque assay on MDCK cells. Given that *mxh* is an IFN-regulated gene (295), I infected KD cells with influenza virus (a potent IFN activator (354)) to induce MxB expression in order to determine efficiency of knockdown. Real-time PCR confirmed that efficient knockdown of targeted mRNA transcripts was achieved with all the shRNA and siRNA species (Figure 30A, B). Knockdown of target mRNA transcripts was achieved at more than 3-fold reduction with the shRNA oligomer and on average at more than 2-fold reduction in 3 of the 4 siRNA species tested. Efficiency of MxB protein knockdown was determined by Western blot (Figure 30C, D). As expected of an inducible gene, MxB was detected during viral infection but not detected in the uninfected control lanes. I did not detect any MxB in the MxB knockdown lanes infected with virus. Surprisingly, MxB transcript level was not

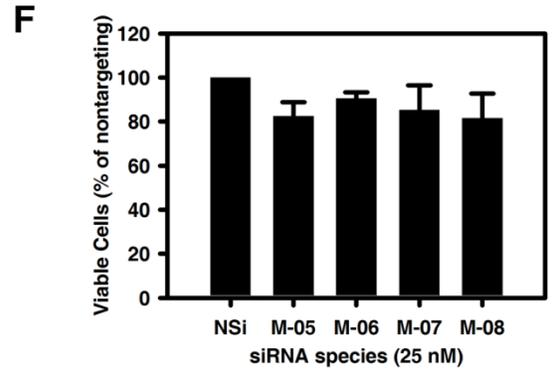
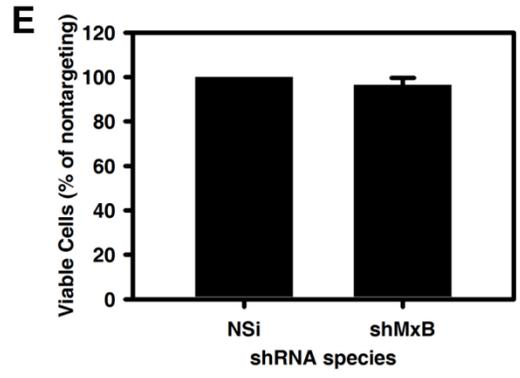
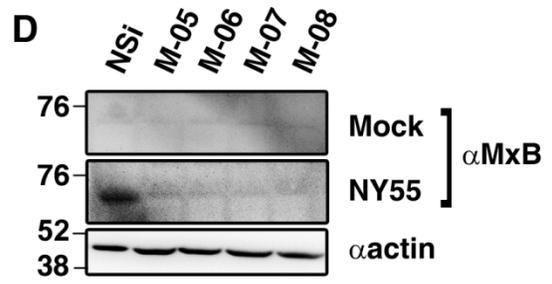
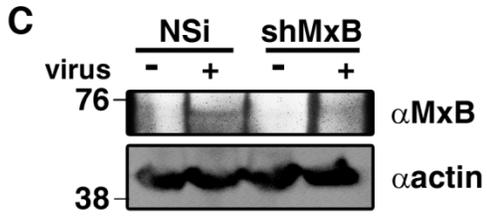
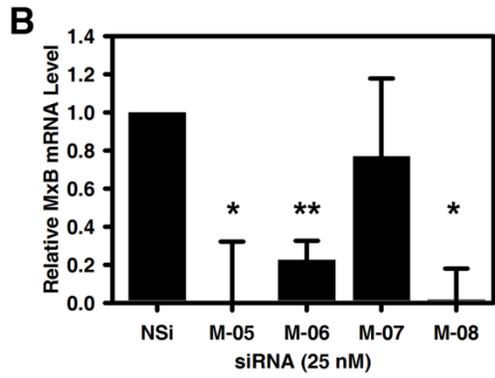
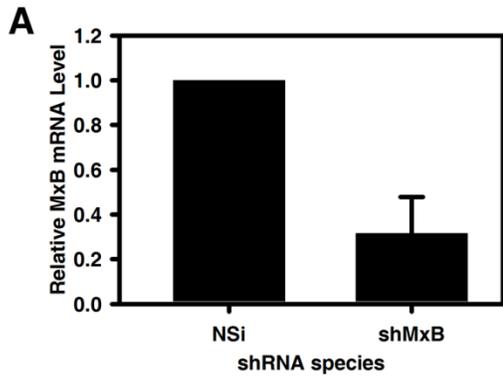


**Figure 29. Influenza virus infection does not affect BAD colocalization with Bcl-xL.**

A549 cells were infected with NY55 at MOI 1 and fixed at 24 hpi. (A) Cells were stained with  $\alpha$ -BAD (magenta) or  $\alpha$ -Bcl-xL (cyan) antibodies. Zen software (Zeiss) was used to produce the co-localization (co-local) image. BAD and Bcl-2 channels are blue and red in the co-localization image, respectively. Regions of co-localization are shown in yellow and irrelevant background is white. (B) Manders Overlay Coefficient ( $P=0.01$ ) and (C) Weighted Colocalization Coefficient (\*  $P=0.014$ ) was determined from images taken at five different areas of each sample (\*  $P=0.03$ ). Mock represents uninfected cells. White bar represents 100  $\mu$ M scale bar.

significantly reduced in M-07 (Figure 30B) but MxB protein was clearly knocked down in the siRNA treated cells. A possible explanation may be attributed to the sensitivity of real-time PCR compared to Western blot. Perhaps the reduction of a small amount of MxB mRNA may be enough to reduce MxB protein below the detection limit of a Western blot assay. Additionally, the sequence specificity of M-07 siRNA duplex may result in translation inhibition of the target transcript but does not necessarily lead to degradation of the host mRNA, which is a characteristic that has been observed in miRNA systems (107). Moreover, studies now show that there is poor correlation between mRNA levels and protein levels (71, 305). Nevertheless, my data showed that efficient knockdown of MxB was achieved in both shRNA- and siRNA-treated cells. WST-1 cell viability assay (Roche) showed no significant reduction in knockdown cell viability compared to controls (Figure 30E, F).

I consistently identified MxB shRNAmir in cells that survived the transduction and high MOI influenza virus infection in both independent RNAi replicate screens as well as in a preliminary RNAi manual screen of 3 shRNA-lentiviral pools, which suggested that MxB knockdown protected the cells from influenza virus infection. To more directly confirm these results, I then treated A549 cells with MxB-specific shRNA, or siRNA oligomers, that target MxB transcripts to create stable or transient knockdown cells, respectively. MxB KD cells were infected with NY55 and with A/Puerto Rico/8/1934 (H1N1) virus (PR8) at an MOI of 1 and 0.1, respectively. CPE was documented every 24 hpi up to 72 hpi. Non-transduced cells, as well as cells transduced with an irrelevant non-targeting shRNA (NSi), exhibited CPE by 48 hpi (Figure 31A). In contrast, there was no



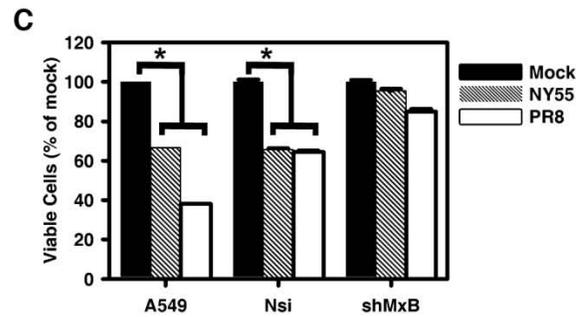
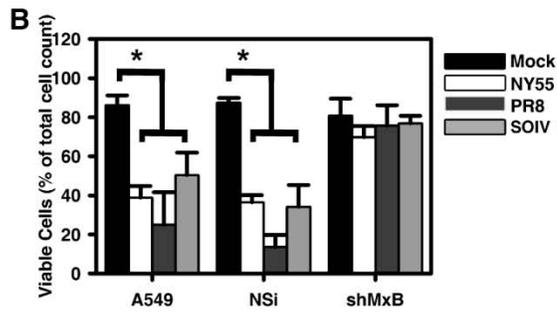
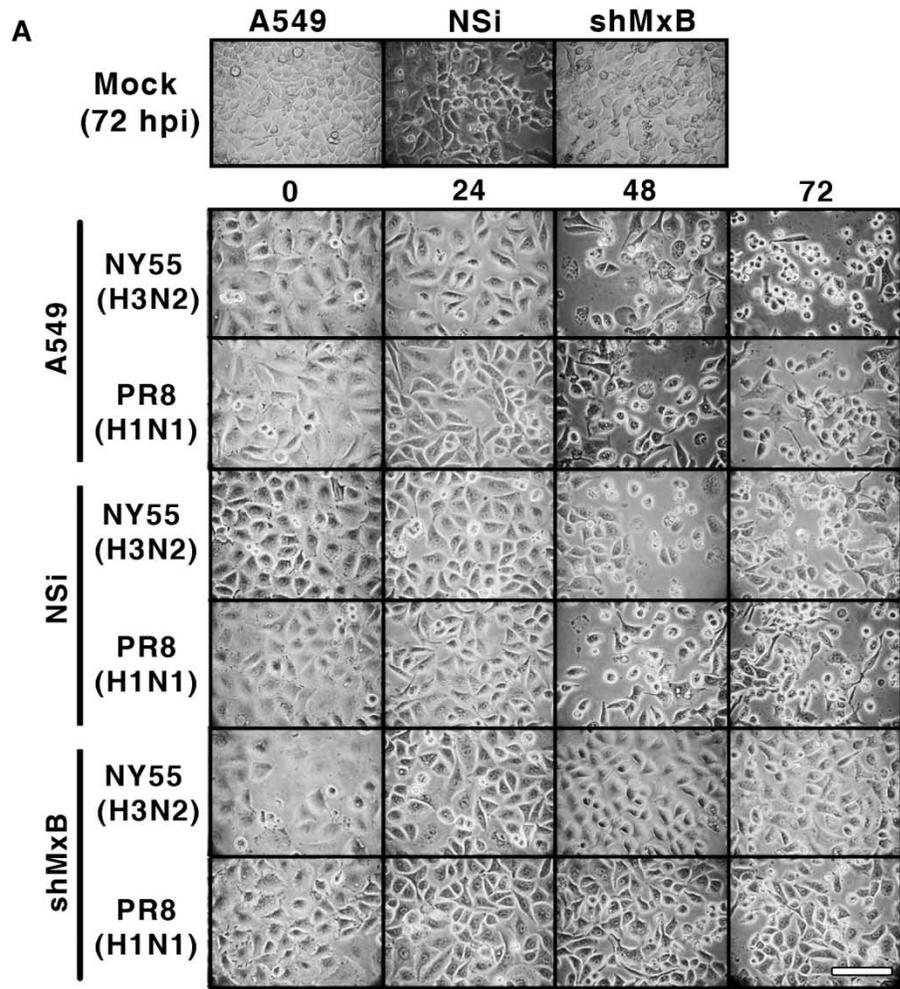
**Figure 30. Efficiency of MxB knockdown in A549 cells.** MxB transcripts were determined by real time PCR for (A) shRNA-treated ( $P < 0.001$ ) or (B) siRNA-treated (\*  $P = 0.006$ , \*\*  $P = 0.026$ ) A549 cells and non-targeting (NSi) control cells.  $C_t$  values were normalized to 18S rRNA control and compared to non-targeting control. Endogenous MxB protein level was assessed by Western blot and densitometry was determined for protein bands in (C) shRNA or (D) siRNA treated samples. Effect of BAD knockdown on cell viability was assessed by WST-1 for (E) shRNA-treated and (F) siRNA-treated cells. Shown is the mean from triplicate runs with error bars representing standard deviation. (Refer to Appendix Figure A7 for full Western blot.)

observable CPE development in influenza virus-infected cells that had been transduced with MxB-specific shRNAs.

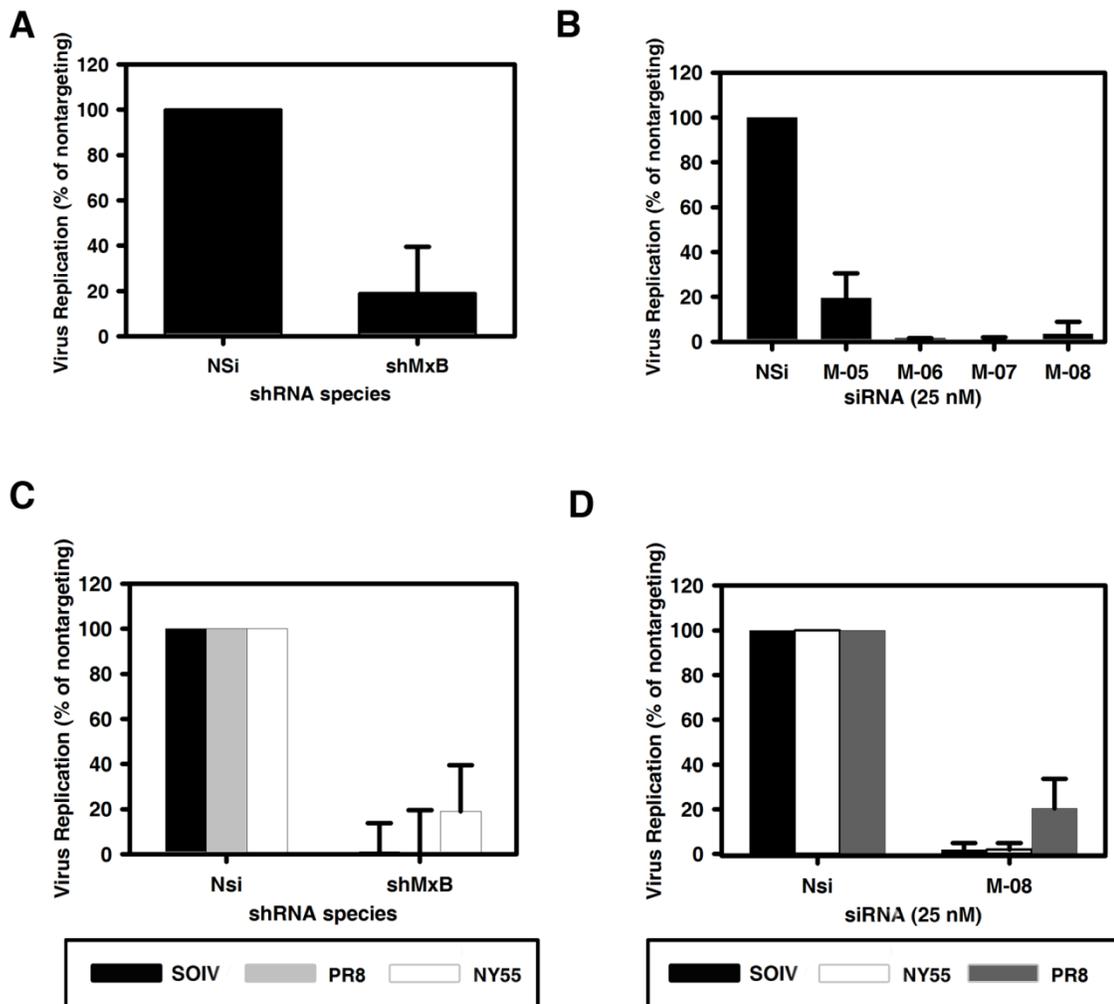
To provide a more quantitative analysis of cell survival after influenza virus exposure, I determined cell viability after virus infection with WST-1, which measures mitochondrial activity, and by trypan blue exclusion. Viability of untransduced and non-targeting control cells infected with influenza virus, as determined by trypan blue exclusion assay, showed more than 2-fold reduction (Figure 31B,  $P \leq 0.029$ ). However, virus infection of MxB knockdown cells showed nearly complete viability of the infected cell population (Figure 31B). This observation is corroborated by results with the WST-1 assay (Figure 31C,  $P < 0.001$ ). Regardless of viral subtype, influenza virus infection of MxB knockdown cells consistently showed greater viability compared to non-knockdown cells. These results indicate that MxB knockdown cells are more resistant to virus-induced cytopathic effect and virus killing.

#### **4.3.2 Reduction of Virus Replication in MxB Knockdown Cells**

Knockdown of MxB resulted in significant reduction in virus titre compared to non-transduced and non-targeting shRNA-treated controls (Figure 32). Given that only one shRNA species was available for MxB, I decided to confirm my observation using 4 different siRNA duplexes to MxB. I replicated the shRNA assays using these siRNA duplexes and the results supported my observation with the shRNA oligomer. NY55 virus yield was dramatically inhibited by more than 5-fold in both shRNA- and siRNA-treated cells (Figure 32A, B,  $P < 0.001$ ). Expanding my observation to other virus subtypes, I looked at both PR8 and SOIV infection of MxB knockdown cells; both virus types showed an even greater reduction in virus production. PR8 and SOIV virus yields



**Figure 31. Inhibition of virus-induced cytopathology and cell death in MxB knockdown cells.** (A) Cytopathic progression in virus infected cells over a 72 h period was imaged with a Nikon Eclipse TE2000-S inverted microscope and a Canon PowerShot A700 digital camera. Shown are images representative of 3 independent replicates. White bar represents 100  $\mu\text{m}$  scale bar. Cell viability at 72 hpi was determined for shRNA-treated cells by (B) trypan blue exclusion assay ( $P \leq 0.029$ ) and (C) WST-1 assay (\*  $P < 0.001$ ). NSi is nontargeting shRNA. Shown is the mean from triplicate runs with error bars representing standard deviation.



**Figure 32. Inhibition of influenza virus replication in MxB knockdown cells.** (A) NY55 virus yield was determined at 72 hpi in shRNA-knockdown ( $P < 0.001$ ) and (B) siRNA-knockdown ( $P < 0.001$ ). (C) Virus yield of NY55, PR8, and SOIV replication at 72 hpi in shRNA-knockdown ( $P < 0.001$ ) and (D) siRNA-knockdown A549 cells ( $P < 0.001$ ). NSi is nontargeting or scrambled shRNA. Error bars represent standard deviation from 3 independent experiments.

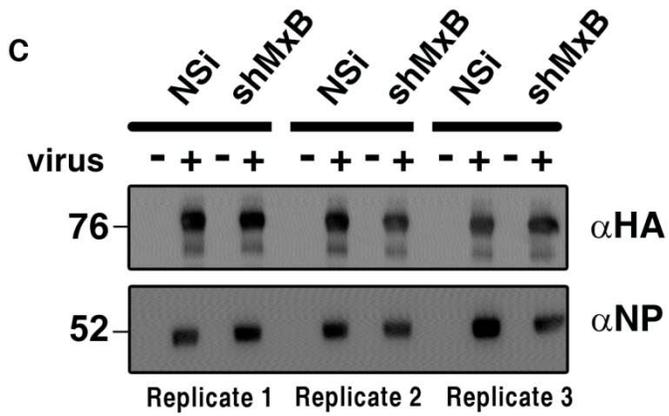
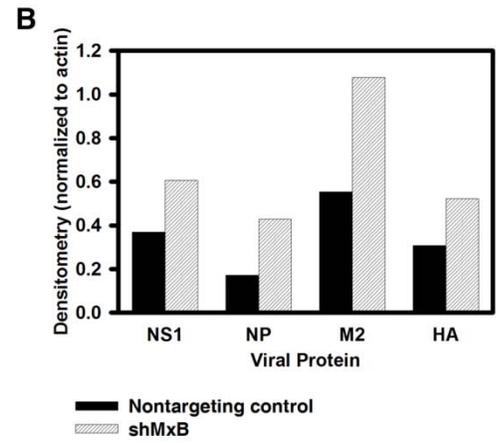
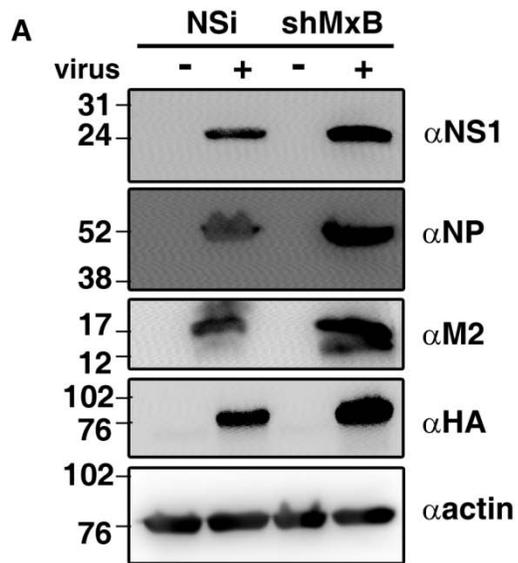
were reduced by 100-fold in shRNA-treated cells (Figure 32C,  $P < 0.001$ ). In siRNA-treated cells (M-08), PR8 virus productivity was inhibited by 5-fold more than the non-targeting control and SOIV yield was reduced more than 50-fold compared to the non-targeting control (Figure 32D,  $P < 0.001$ ). There appeared to be a slight difference in the degree to which PR8 and NY55 replication is repressed in shRNA knockdown cells relative to siRNA knockdown cells. PR8 replication appeared to be reduced more in the shRNA knockdown cells than in the siRNA knockdown cells. A possible explanation could be that initial lentivirus transduction and continual production of shRNA in the stable knockdown cell line may induce host anti-viral responses that could affect PR8 replication. Although siRNA treatments are reported to have greater impact on the induction of cellular anti-viral signaling, this impact for both shRNA and siRNA is sequence-dependent (264). Therefore, the difference in siRNA and shRNA sequences used may result in differentiated induction of anti-viral signals that affect PR8 replication. However, the reverse appears to be true for NY55 replication. A possible explanation in this case could be that NY55, being a patient isolate and more pathogenic than mouse-adapted PR8, may have greater resistance to some anti-viral activities. Another possible explanation could be that NY55 may depend on MxB activity more than PR8, since MxB knockdown in siRNA-treated cells were achieved to a greater degree than shRNA treated cells. Despite the slight differences, both shRNA and siRNA knockdown cells showed significant reduction of all three influenza virus strains tested. Moreover, by creating two different knockdown conditions via siRNA and shRNA, I ruled out the possibility that the lentivirus or the shRNA constructs created artefacts that

might have radically skewed my data. Thus, these results strongly indicate that MxB is a significant host factor that supports, rather than antagonizes, influenza virus replication.

### **4.3.3 Virus Protein Production was not Inhibited in MxB Deficient Cells**

MxB has been reported to have a biological function in nucleocytoplasmic trafficking (129). As such I wanted to determine whether early import of incoming viral ribonucleoprotein (vRNP) was inhibited in MxB KD cells by analyzing viral protein production. If vRNP import into the nucleus is blocked, transcription should not occur, which would result in significant decline of viral protein production. Stable MxB KD cells were infected with NY55 at an MOI of 1, and whole cell lysate was obtained at 24 hpi. Western blot was probed for early influenza virus proteins (NS1 and NP) and late viral proteins (HA and M2). All four viral proteins were clearly detected in infected lanes (Figure 33). This indicates that a deficiency in MxB did not abrogate the translocation of incoming vRNP into the nucleus; this further suggests that export of viral mRNA transcripts from the nucleus to the cytoplasm was also unhindered. However, there was an increase in viral protein levels in MxB knockdown cells compared to the non-targeting shRNA control. A deficiency in MxB may affect protein trafficking and result in the accumulation of viral protein in infected host cells.

Given that infectious virus yield was reduced despite an increase in virus protein production in infected MxB KD cells, I suspected that MxB depletion might affect the particle-to-PFU (plaque forming unit) ratio of released virions. Virions were purified from the supernatants of MxB KD or non-targeting shRNA-treated cells by ultracentrifugation. I did not observe any difference in amount of HA and NP virion



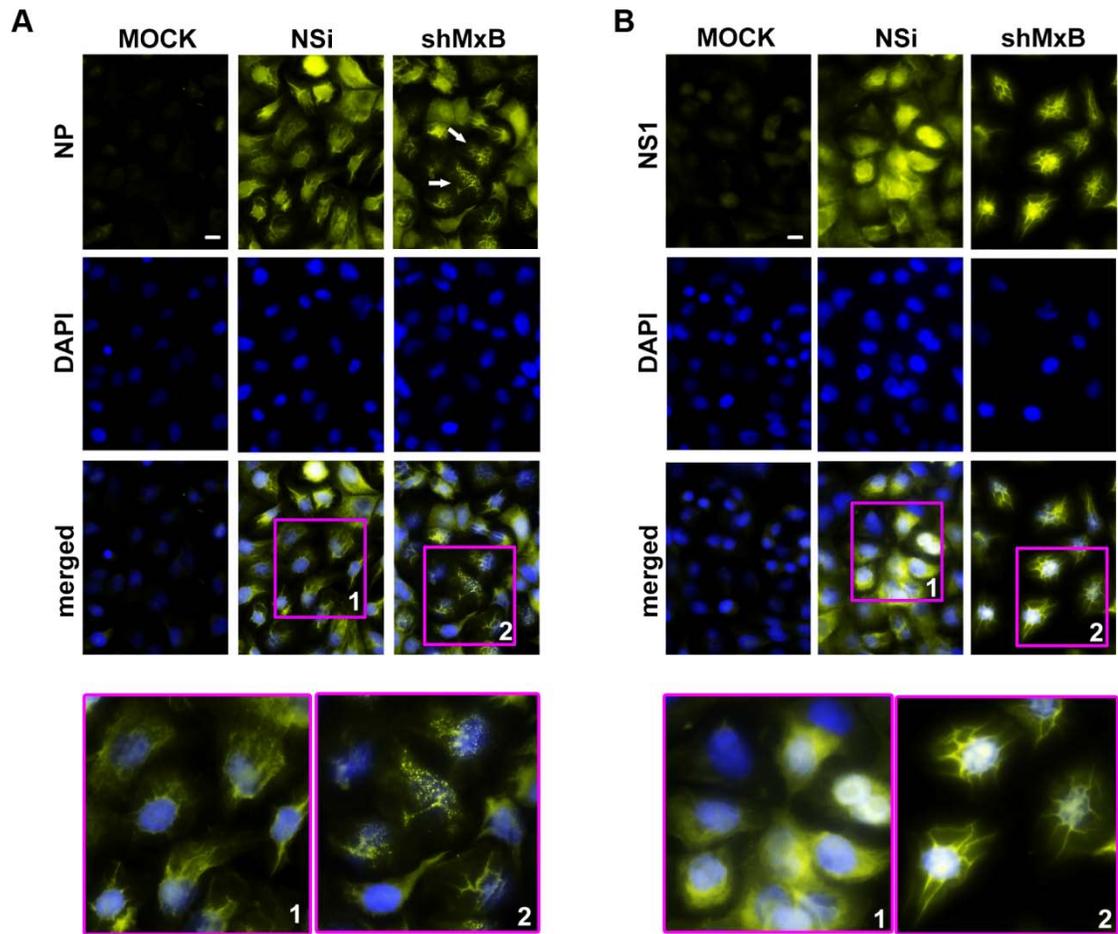
**Figure 33. Influenza virus protein production is unperturbed in MxB knockdown cells.** (A) Western blot was carried out to determine protein production in NY55 infected MxB knockdown cells and control cells. Whole cell lysate of cells infected for 24 h was electrophoresed on SDS-PAGE and blot was probed with antibodies against influenza virus proteins. (B) Densitometry was determined for infected bands from Western blot in (A). Protein levels were normalized to actin. (C) Western blot was carried out to determine amount of virion protein obtained from infected supernatants, which would correspond to relative amounts of particles produced during infection. Virus samples were isolated from infected supernatants and equal volumes were loaded for Western blot. Blot was probed with mouse monoclonal antibody against NP and rabbit polyclonal antibody against HA. NSi is nontargeting shRNA. (Refer to Appendix Figures A7 and A8 for full Western blot.)

proteins in particles released by MxB KD cells and control samples (Figure 33C). This suggests that virion particle production and release into the supernatant were not hindered in MxB KD cells, but that the specific infectivity of the released virions was dramatically lower.

#### **4.3.4 MxB Affects Localization of Viral NP and NS1 Proteins**

MxB proteins have been shown to have significant homology to dynamins, which are enzymes involved in intracellular protein trafficking (297); therefore, I wanted to determine whether viral inhibition may be due to possible relocation of viral NP in infected host cells due to the absence of MxB. To investigate this, I carried out immunofluorescence (IF) microscopy with stably transduced A549 knockdown cells infected with NY55 at MOI 1. Images were taken 24 hpi. IF images showed accumulation of distinct punctate granular structures that corresponded to influenza virus NP protein in the cytosol of MxB KD cells at 24 hpi (Figure 34A). Similar punctate formations were not observed in non-targeting control cells, where an even pattern of NP protein distribution was visible. My assay cannot distinguish between soluble NP and vRNP complex and it does not provide direct evidence to either support or refute the possibility of impaired viral protein trafficking to or from the nucleus. However, my results do indicate that NP protein trafficking is altered in infected MxB KD cells.

I extended my analysis of viral protein localization to the viral non-structural protein NS1. NS1 localizes to both the cytoplasm and the nucleus (89). I carried out immunofluorescence analysis on stably transduced knockdown cells infected with NY55 at MOI 1 for 24 h. NS1 did not develop the same punctate granular pattern observed



**Figure 34. Aberrant localization of NP and NS1 in MxB knockdown cells.** Shown is immunofluorescence localization of (A) viral NP (yellow) and (B) NS1 (yellow) in uninfected (mock), infected non-targeting shRNA (NSi), and infected MxB-knockdown A549 cells. Magenta enclosed boxes indicate region in image enlarged in the numbered insets. Proteins were detected with Alexa Fluor 594 anti-NP or anti-NS1 and merged with DAPI stained DNA. White bar represents 100 μm.

with NP, but it did demonstrate a peculiar “threading” like pattern in the cytosol of infected MxB-knockdown cells; such patterns were not observed in any of the controls (Figure 34B). This result suggests that MxB may be involved in NS1 protein trafficking.

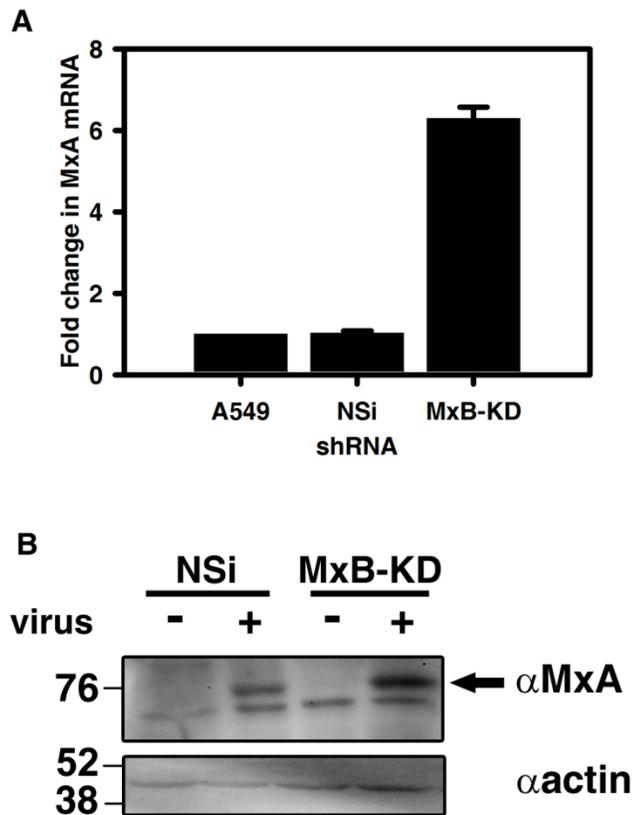
#### **4.3.5 Expression of MxA was not Affected by MxB Knockdown**

Although their functions are different (91), MxB and MxA have ~ 63% sequence homology (1). I wanted to confirm that my RNAi treatment specifically targets MxB and had no detrimental effect on MxA expression. I carried out Western blot analysis on MxA protein production and real-time PCR on MxA transcript levels in infected MxB stable KD cells. A deficiency in MxB did not affect the ability of MxA to be expressed upon viral infection (Figure 35); rather, MxA protein expression appeared elevated in MxB KD cells compared to the control (Figure 35A). Western blot also showed significant increase in MxA protein in infected samples (Figure 35B,  $P < 0.001$ ). These results confirm that the RNAi treatment was specific to MxB, and surprisingly showed a greater induction of MxA in MxB knockdown cells upon viral infection.

### **4.4. Role of TNFSF12-13 and TNFSF13 in Influenza Virus Infection**

#### **4.4.1 Protective Effect of TNFSF12-13 and TNFSF13 Knockdown**

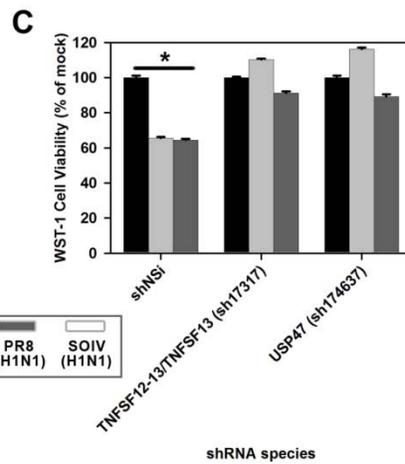
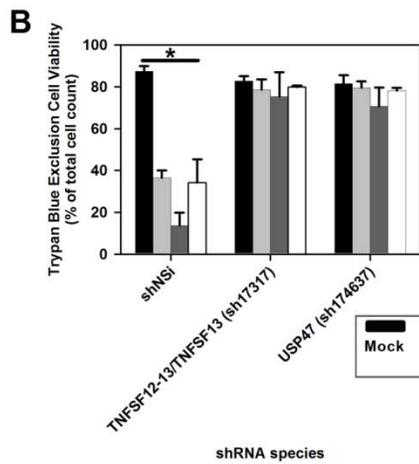
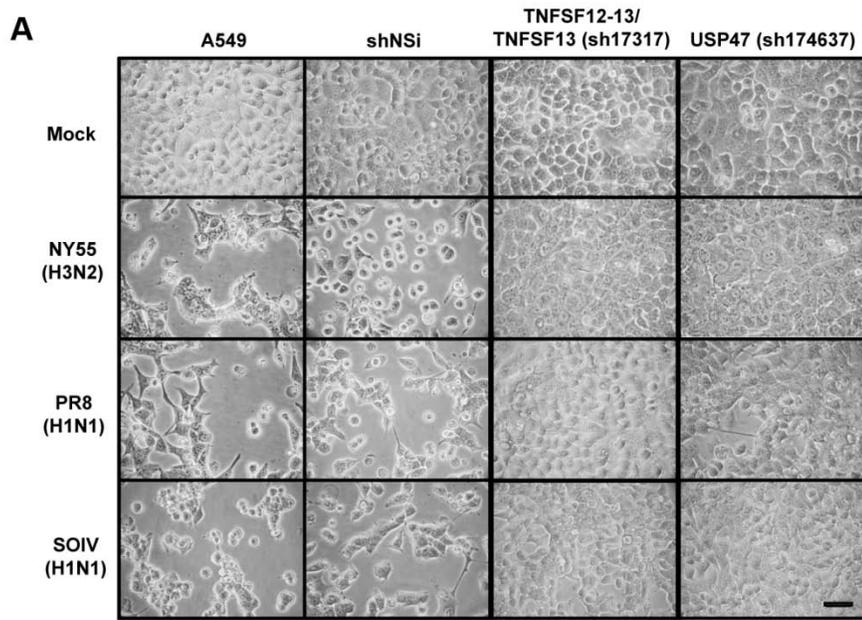
Of note, TNFSF12-13 is a fusion protein produced from the overlap of the TNFSF12 and TNFSF13 transcripts (49, 258). These 2 genes were also identified in a preliminary manual screen of 3 library pools in which transduced cell clones that survived NY55 infection were individually isolated, amplified, and sequenced to identify



**Figure 35. MxB knockdown did not affect induction of MxA expression.** (A) MxA mRNA levels were quantified by real-time PCR ( $P < 0.001$ ). Shown is the mean from triplicate runs with error bars representing standard deviation. (B) MxA protein production was detected by Western blot of whole cell lysate of infected or uninfected MxB knockdown cells. NSi is nontargeting shRNA. (Refer to Appendix Figure A9 for full Western blot.)

the shRNA insert (Table 7). The ability of each isolated clone to proliferate during the amplification step of the manual screen strongly demonstrated the survivability of the infected, knockdown cells.

I individually transduced sets of A549 cells with shRNA-lentiviruses specifically targeting each transcript. Of note, the shRNA designed to target TNFSF13, also targeted TNFSF12-13 fusion protein according to the manufacturer. The shRNA constructs contained puromycin markers for positive selection of transduced cells. Stably transduced cells were passaged in puromycin at least twice to remove non-transduced cells, followed by infection with NY55, PR8 or pandemic H1N1 (SOIV) influenza A viruses. Untransduced and non-targeting shRNA transduced controls (shNSi) showed extensive CPE indicative of virus-induced cytotoxicity (Figure 36A). In contrast, there was no observable CPE development in virus-infected KD cells with TNFSF12-TNFSF13/TNFSF13-specific shRNAs (Figure 36A). Quantitative assessment of cell viability 72 hpi by WST-1 or trypan blue further supported my observation that KD of these 3 genes transcripts resulted in the survival of these infected host cells. My results for the trypan blue exclusion assay showed less than 40% of the total cell count transduced with non-targeting shRNA control were viable compared to more than 80% viable cells in the mock (uninfected) for all the virus types tested (Figure 36B,  $P < 0.001$ ). In contrast, virus infection of the KD cells showed equivalent numbers of viable cells compared to mock. Similar observations were made with the WST-1 assay, which measured mitochondria activity as a representation of cell viability (Figure 36C). Due to biosafety regulations within my facility, I could not include the SOIV strain in this assay. However, my results with the trypan exclusion assay, which included SOIV, was



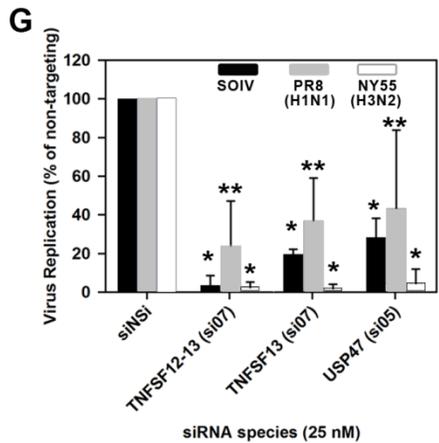
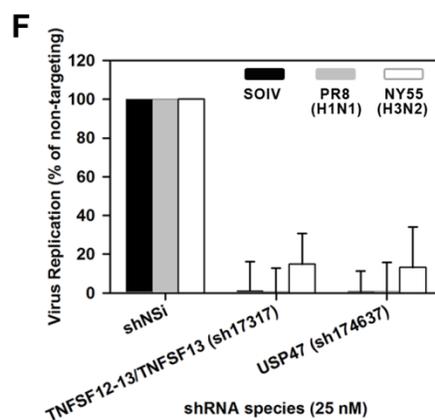
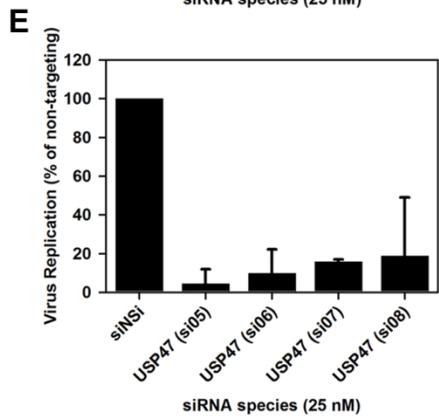
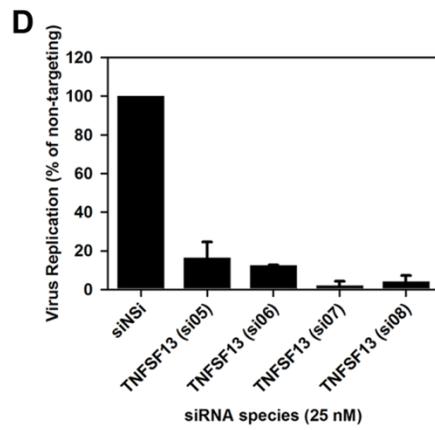
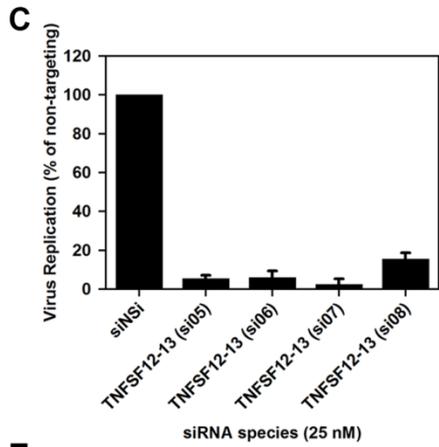
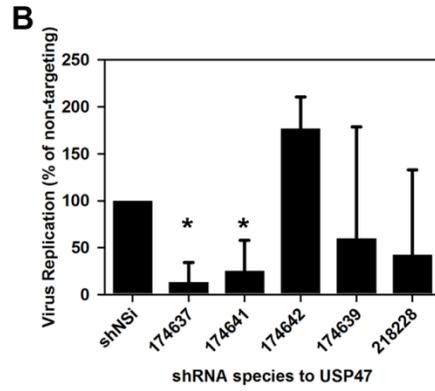
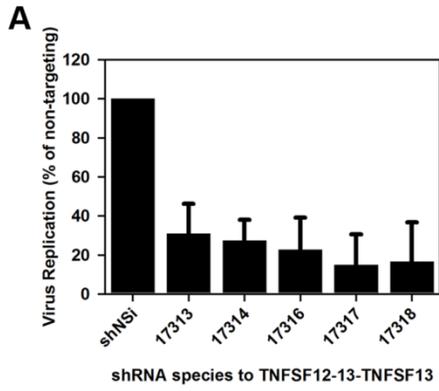
**Figure 36. Reduced cytotoxicity and cell death by influenza virus in TNFSF12-13/13 and USP47 knockdown cells.** Cytopathic effect in infected (A) TNFSF12-13/TNFSF13-shRNA or USP47-shRNA knockdown A549 cells that were infected with NY55, PR8, or SOIV. At 72 hpi, cytopathic effect of cells were examined with a Nikon Eclipse TE2000-S inverted microscope and images obtained with a Canon PowerShot A700 digital camera. Shown are images representative of 3 independent replicates. Scale bar represents 100  $\mu$ M. Cell viability at 72 h after influenza virus infection was determined for shRNA-treated cells by (B) trypan blue exclusion assay and by (C) WST-1 assay. A total of 200 cells were counted and the percentage of trypan blue-excluding (viable) cells was determined. shNSi is nontargeting shRNA control. Shown is the mean from 3 independent replicates with error bars representing standard deviation (\*  $P < 0.001$ ).

comparable to the WST-1 assay; that KD cells resisted virus killing regardless of virus subtype (Figure 36B).

#### **4.4.2 Virus Yield in TNFSF12-13 and TNFSF13 Knockdown Cells**

I showed in Figure 13 that genes identified in my screen support virus-induced cell death but not all of the identified genes necessarily influence the virus' ability to produce progeny virions. It is the ultimate goal, however, to identify genes involved in both cell killing and in virion production. Therefore, I set out to determine whether TNFSF12-13 and TNFSF13 would affect influenza virus replication. Stably transduced TNFSF12-13/TNFSF13 KD A549 cells and non-targeting shRNA control were infected with NY55, PR8 or SOIV for 72 h. Virus production was titered by plaque assay on MDCK cells.

My results showed that NY55 virus production was reduced to less than 30% of the non-targeting control for all 5 different shRNA species which target TNFSF12-13/TNSF13 (Figure 37A,  $P < 0.001$ ). Each shRNA species was designed to simultaneously target both TNFSF12-13 and TNFSF13 transcripts (i.e. double knockdown), which barred my ability to differentiate the effect of the individual proteins. Thus, I also repeated the experiments using transient transfection with siRNA duplexes that individually target TNFSF12-13 or TNFSF13. Viral production in siRNA transfected cells showed reduced virus titre, which corroborates that observed with shRNA stable knockdown cells. siRNA KD of TNFSF12-13 resulted reduced viral replication to less than 15% of the non-targeting siRNA control for all 4 different siRNA species tested (Figure 37C,  $P < 0.001$ ). Similarly, virus production was inhibited in TNFSF13 siRNA KD cells to less than 16% of the non-targeting control (Figure 37D,  $P < 0.001$ ).



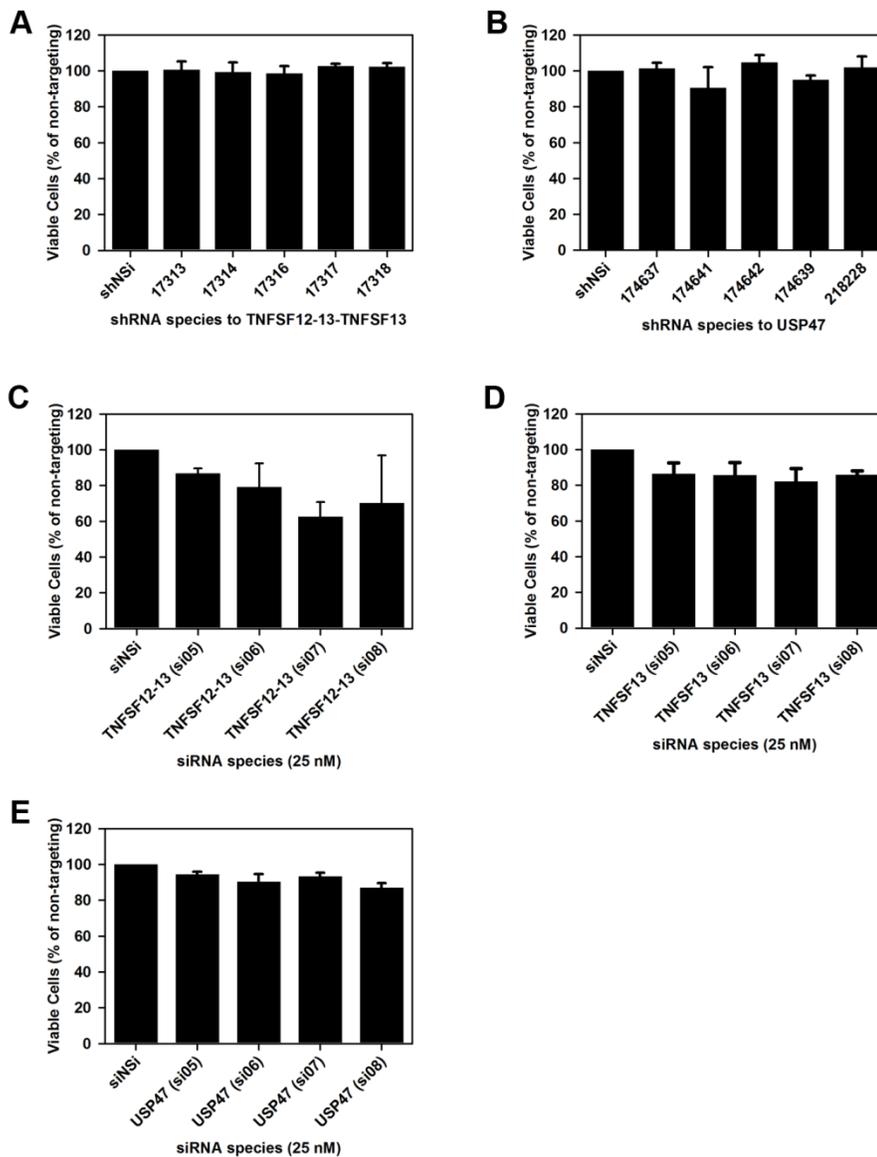
**Figure 37. Influenza virus propagation is inhibited in TNFSF12-13/-13 and USP47 knockdown cells.** Virus replication was determined at 72 hpi for NY55 in (A) TNFSF12-13/TNFSF13 ( $P < 0.001$ ) or (B) USP47 (\*  $P < 0.05$ ) shRNA KD. NY55 replication was determined in siRNA KD cells for (C) TNFSF12-13, (D) TNFSF13, or (E) USP47 ( $P < 0.001$ ). Virus replication for PR8 and SOIV of TNFSF12-13, TNFSF13, or USP47 (G) shRNA KD ( $P < 0.001$ ) and (F) siRNA KD (\*  $P < 0.001$ , \*\*  $P < 0.05$ ). NSi is nontargeting shRNA or siRNA control. Error bars represent standard deviation from 3 independent experiments.

shRNA KD of TNFSF12-13 and TNFSF13 also inhibited viral replication of other viral subtypes, namely PR8 and SOIV, to less than 10% of the non-targeting control (Figure 37F,  $P < 0.001$ ). Similarly, I observed significant reduction of both SOIV and PR8 viral production in all 3 siRNA KD cell lines: less than 30% of control for SOIV ( $P < 0.001$ ) and under 45% of the control for PR8 ( $P < 0.05$ ) (Figure 37G). I confirmed that the reduction of virus titre was not due to the effect of the RNAi treatments on cell viability (Figure 38). I also confirmed efficient knockdown of the targeted transcript and protein was achieved with real-time PCR and western blot (Figure 39). Presently, I am unable to find a commercial primary antibody that can effectively detect endogenous TNFSF13, and, to my knowledge, a primary antibody to TNFSF12-13 is currently unavailable. Overall, my results indicate that these host factors play significant roles in promoting influenza virus replication for different virus subtypes.

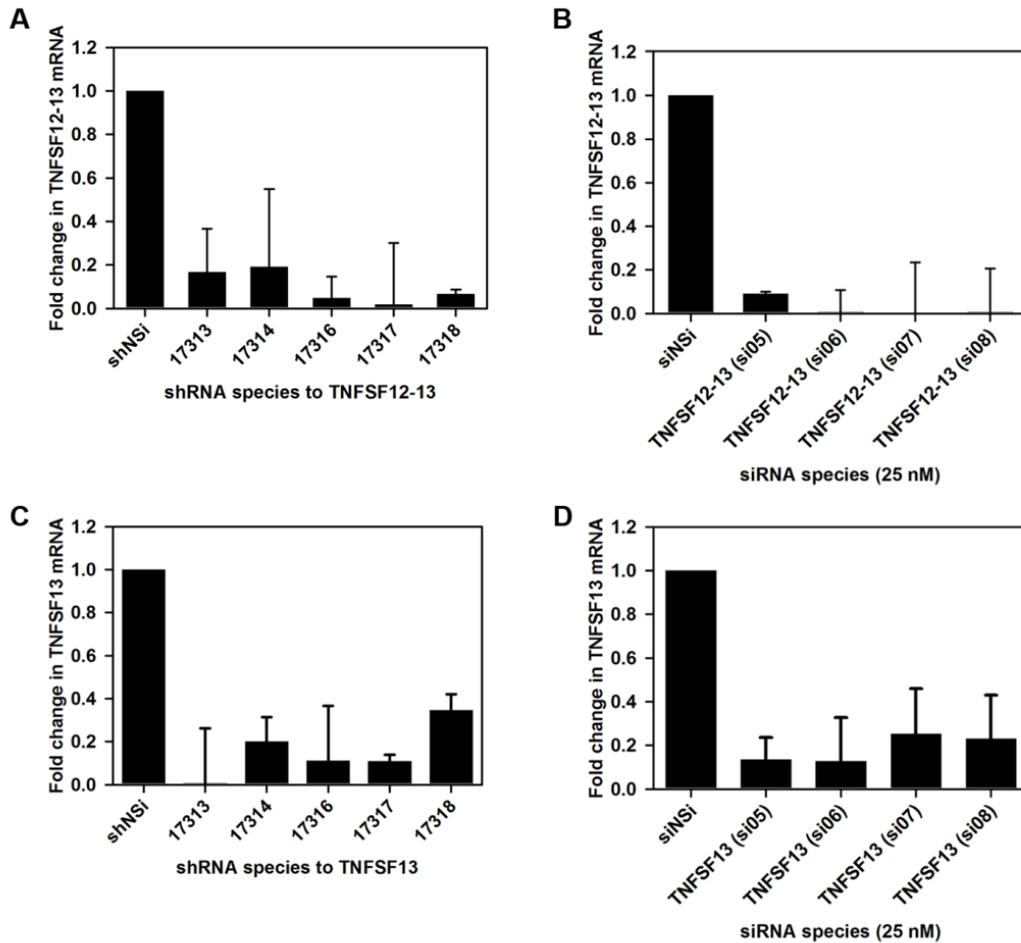
## **4.5. USP47 Contribution in Influenza Virus Infection**

### **4.5.1 Protective Effect of USP47 Knockdown**

The dynamic process of protein ubiquitylation and deubiquitylation through post-translational protein modification has emerged as a major regulatory mechanism in a number of cellular signaling pathways. Many viruses manipulate protein Ub and/or DUB in order to overcome host cell defense mechanisms that include apoptosis and type I interferon response. Although DUB is an essential process that regulates pathways important in viral infection, only a few examples are known of DUB proteins being



**Figure 38. Viability of TNFSF12-13/13 and USP47 knockdown cells.** Viability of knockdown cells were assessed by WST-1 for (A) stably transduced TNFSF12-13/TNFSF13 and (B) USP47 shRNA cells, (C) TNFSF12-13, (D) TNFSF13, and (E) USP47 siRNA-treated cells. NSi is nontargeting shRNA or siRNA control. Shown is the mean from duplicate runs with error bars representing standard deviation.



**Figure 39. TNFSF12-13 and TNFSF13 knockdown confirmation by real-time PCR.**

TNFSF12-13 and TNFSF13 transcripts were determined by real time PCR for TNFSF12-13 (A) stably transduced shRNA cells ( $P < 0.001$ ) or (B) siRNA-transfected cells ( $P < 0.001$ ); for TNFSF13 (C) stably transduced shRNA cells ( $P < 0.001$ ) or (D) siRNA-transfected cells ( $P < 0.001$ ) and non-targeting (NSi) shRNA or siRNA control cells, respectively.  $C_t$  values were normalized to 18S rRNA control and compared to non-targeting control. Shown is the mean from duplicate runs with error bars representing standard deviation. USP47 protein knockdown was confirmed with western blot in (G) shRNA- and (H) siRNA-treated cells.

involved in virus infection. My study further expands this list with the addition of the deubiquitinase USP47. Similar to the aforementioned TNFSF proteins, BAD, and MxB, USP47 also was among the genes identified in a preliminary manual screen of 3 library pools (Table 7).

A549 cells were individually transduced with shRNA-lentiviruses specifically targeting USP47 transcripts. The shRNA constructs contained puromycin markers for positive selection of transduced cells. Stably transduced cells were passaged in puromycin at least twice to remove non-transduced cells, followed by infection with NY55, PR8 or pandemic H1N1 (SOIV) influenza A viruses. Virus infections of non-transduced and non-targeting shRNA transduced controls (shNSi), showed extensive CPE indicative of virus-induced cytotoxicity (Figure 36A). In contrast, there were no observable CPE development in virus-infected KD cells with USP47-specific shRNAs (Figure 36A). Quantitative assessment of cell viability 72 hpi by WST-1 or trypan blue showed USP47 KD resulted in the survival of these host cells after influenza infection. Trypan blue exclusion assay showed fewer than 40% of total non-targeting shRNA control cells were viable compared to more than 80% viable cells in the mock (uninfected) for all the virus types tested (Figure 36B,  $P < 0.001$ ). In contrast, infected USP47 KD cells showed equivalent numbers of viable cells to that of mock. The protective effect of USP47 KD cells during influenza virus infection was corroborated by WST-1 assay, which measured mitochondria activity as a representation of cell viability (Figure 36C). Due to biosafety regulations within my facility, I could not include the SOIV strain in the WST-1 assay. However, my results with SOIV and NY55 infections

showed similar results observed with the trypan exclusion assay; that KD cells resisted virus killing regardless of virus subtype (Figure 36B).

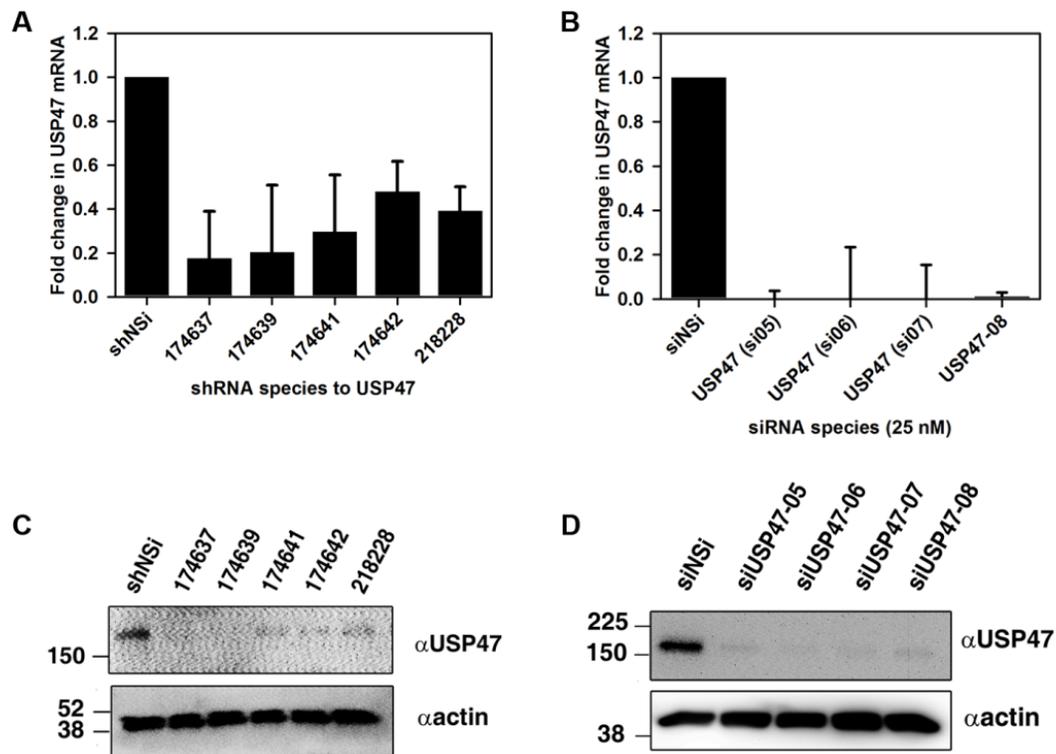
#### 4.5.2 Virus Yield in USP47 Knockdown Cells

My RNAi screens identified genes that support virus-induced cell death but not all of the identified genes necessarily affect the production of viral progeny virions. Hence, I followed up with further verifications to specifically assess the effect of USP47 KD on influenza virus replication. Stably transduced USP47 knockdown A549 cells and non-targeting shRNA control were infected with NY55, PR8 or SOIV for 72 h. Virus progeny production was titered via plaque assay on MDCK cells.

My results showed that NY55 virus production was reduced to less than 26% of the non-targeting control for 2 of the 5 different shRNA species targeting the USP47 transcript (Figure 38B,  $P=0.006$ ). I am uncertain why shRNA-174642 appeared to result in increased viral production but possible explanations include the consequence of the site of shRNA integration into the host genome or the shRNA sequence had a high off-target effect. For these reasons, I also repeated the experiments using transient transfection with 4 individual siRNA duplexes that target USP47. Viral production in siRNA transfected cells showed virus titre in USP47 KD was reduced to less than 19% of the non-targeting siRNA control (Figure 38E,  $P<0.001$ ), which corroborates the observation made with the 2 shRNA stable knockdown cells.

shRNA KD of USP47 also inhibited viral replication of other viral subtypes tested, namely PR8 and SOIV, to less than 14% of the non-targeting control (Figure 38F,  $P<0.001$ ). Similarly, I observed significant reduction of both SOIV and PR8 viral production in the siRNA KD cell lines: less than 29% of control for SOIV ( $P<0.001$ ) and

under 44% of the control for PR8 ( $P < 0.05$ ) (Figure 38G). I confirmed that the reduction of virus titre was not due to the effect of the RNAi treatments on cell viability (Figure 38). I also confirmed efficient knockdown of USP47 mRNA and protein levels by real-time PCR and Western blot, respectively (Figure 40). The shRNA I used in my studies is designed based on host miRNA oligomers; thus it is designated as shRNAmir. Cellular miRNA is well known to inhibit the translation of target transcripts without leading to the degradation of the mRNA, and this is sequence specificity dependent (107). Thus, the shRNAmir used in knocking down USP47 in Figure 40 led to effective reduction of protein levels (Figure 40C) but not equally effective reduction of transcript population. Additionally, real-time PCR is a more sensitive assay than Western blot. Thus, the difference in detection limit between the two assays may also explain the different observed levels of transcripts detected and that of its encoded protein. Additionally, there is poor correlation between transcript and protein levels (71, 305). My results indicate that USP47 has an important role in influenza virus replication for different virus subtypes. This further suggests that other as yet unidentified DUB activities also may significantly influence influenza virus infection.



**Figure 40. Efficiency of USP47 knockdown determined by real-time PCR and Western blot.** USP47 transcripts were determined by real time PCR for (A) stably transduced shRNA cells ( $P < 0.05$ ) or (B) siRNA-transfected cells ( $P < 0.001$ ) and non-targeting (NSi) shRNA or siRNA control cells, respectively.  $C_t$  values were normalized to 18S rRNA control and compared to non-targeting control. Shown is the mean from duplicate runs with error bars representing standard deviation. USP47 protein knockdown was confirmed with western blot in (D) shRNA- and (E) siRNA-treated cells. (Refer to Appendix Figure A10 for full Western blot.)

## Part V | Discussion

### 5.1. RNAi Screen

Influenza virus, a significant etiological agent with the potential to develop into pandemic outbreaks, has been a major focus of recent global screens. Presently, six different global screens have been published specifically for influenza virus (307) (Table 10). The criteria for a positive hit with these published screens was based on the knockdown of genes that resulted in distinct reduction of influenza virus titre. While the majority of these screens have proposed well over 100 genes as candidates that are necessary for influenza virus replication, the quandary is no single gene or groups of genes were identified across all six screens, and less than 33 genes were identified in two or more screens (343). A possible explanation for this lack of overlap may be due to differences in screen parameters and viruses used. However, as more global screens are carried out, potential overlaps with specific sets of genes may arise.

In the case of many viruses, successful replication results in the death of the infected host cell. Initially, it was thought that virus-induced cytopathology was a consequence of active immune response to viral infection; however, accumulating evidence, including my study on the effect of the pro-apoptotic protein BAD during influenza virus infection (322), strongly suggest influenza viruses manipulate the cell death signaling pathway(s) for their own benefit (166, 178, 199, 222, 233, 268, 356, 357). Viruses induce cell death either indirectly, by inducing severe cell stress due to intense viral replication (166), and/or directly, with specific pro-apoptotic viral proteins such as PB1-F2 (364) or induction of specific host pro-death proteins (322, 356). Therefore, I

**Table 10. Comparison of different influenza RNAi screen parameters.**

	Tran	Brass (20)	Hao (97)	Karlas (120)	Köing (138)	Shapira (275)	Sui (299)
<b>Approach</b>	<ul style="list-style-type: none"> <li>• RNAi screen</li> </ul>	<ul style="list-style-type: none"> <li>• RNAi screen</li> </ul>	<ul style="list-style-type: none"> <li>• RNAi screen</li> </ul>	<ul style="list-style-type: none"> <li>• RNAi screen</li> </ul>	<ul style="list-style-type: none"> <li>• RNAi screen</li> </ul>	<ul style="list-style-type: none"> <li>• Yeast-2-hybrid</li> <li>• RNAi screen</li> </ul>	<ul style="list-style-type: none"> <li>• random homozygous gene perturbation</li> </ul>
<b>Screen parameter</b>	<ul style="list-style-type: none"> <li>• Selection of surviving cells after influenza infection</li> </ul>	<ul style="list-style-type: none"> <li>• Stained for surface expression of HA</li> </ul>	<ul style="list-style-type: none"> <li>• Level of reporter gene expression</li> </ul>	<ul style="list-style-type: none"> <li>• level of <i>Renilla</i> luciferase expression in 293T reporter cells</li> </ul>	<ul style="list-style-type: none"> <li>• level of <i>Renilla</i> luciferase expression in 293T reporter cells</li> </ul>	<ul style="list-style-type: none"> <li>• level of <i>Renilla</i> luciferase expression in 293T reporter cells</li> </ul>	<ul style="list-style-type: none"> <li>• Host gene perturbation</li> </ul>
<b>Endpoint (hpi)</b>	<ul style="list-style-type: none"> <li>• 72 hpi</li> </ul>	<ul style="list-style-type: none"> <li>• 12 hpi</li> </ul>	<ul style="list-style-type: none"> <li>• 24-48 hpi</li> </ul>	<ul style="list-style-type: none"> <li>• 24 hpi</li> </ul>	<ul style="list-style-type: none"> <li>• 36 hpi</li> </ul>	<ul style="list-style-type: none"> <li>• 48 hpi</li> </ul>	<ul style="list-style-type: none"> <li>• 48 hpi</li> </ul>
<b>No. of genes screened</b>	<ul style="list-style-type: none"> <li>• 21,415</li> </ul>	<ul style="list-style-type: none"> <li>• 17,877</li> </ul>	<ul style="list-style-type: none"> <li>• 13,071</li> </ul>	<ul style="list-style-type: none"> <li>• 22, 843</li> </ul>	<ul style="list-style-type: none"> <li>• 19,628</li> </ul>	<ul style="list-style-type: none"> <li>• 1745</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>
<b>Delivery method</b>	<ul style="list-style-type: none"> <li>• lentiviral</li> </ul>	<ul style="list-style-type: none"> <li>• transfection</li> </ul>	<ul style="list-style-type: none"> <li>• infection with recombinant virus</li> </ul>	<ul style="list-style-type: none"> <li>• transfection</li> </ul>	<ul style="list-style-type: none"> <li>• transfection</li> </ul>	<ul style="list-style-type: none"> <li>• transfection</li> </ul>	<ul style="list-style-type: none"> <li>• lentiviral</li> </ul>
<b>Total candidates</b>	<ul style="list-style-type: none"> <li>• 1256 genes</li> </ul>	<ul style="list-style-type: none"> <li>• 312 genes</li> </ul>	<ul style="list-style-type: none"> <li>• 110</li> </ul>	<ul style="list-style-type: none"> <li>• 287 genes</li> </ul>	<ul style="list-style-type: none"> <li>• 295 genes</li> </ul>	<ul style="list-style-type: none"> <li>• 616 genes</li> </ul>	<ul style="list-style-type: none"> <li>• 110 clones</li> </ul>
<b>Genes in biological replicates</b>	<ul style="list-style-type: none"> <li>• 138</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>	<ul style="list-style-type: none"> <li>• N/A</li> </ul>
<b>Virus used</b>	<ul style="list-style-type: none"> <li>• NY55 (H3N2), PR8 (H3N2), pandemic California (H1N1)</li> </ul>	<ul style="list-style-type: none"> <li>• PR8 (H1N1)</li> </ul>	<ul style="list-style-type: none"> <li>• Recombinant WSN (H1N1) with VSV G protein and NA-luciferase</li> </ul>	<ul style="list-style-type: none"> <li>• WSN (H1N1)</li> </ul>	<ul style="list-style-type: none"> <li>• Recombinant WSN (H1N1), HA-luciferase</li> </ul>	<ul style="list-style-type: none"> <li>• PR8 (H1N1), Uorn(H3N2)</li> </ul>	<ul style="list-style-type: none"> <li>• Uorn (H3N2)</li> </ul>
<b>Cell line used in screen</b>	<ul style="list-style-type: none"> <li>• A549 cells</li> </ul>	<ul style="list-style-type: none"> <li>• U2OS cells</li> </ul>	<ul style="list-style-type: none"> <li>• Drosophila cells</li> </ul>	<ul style="list-style-type: none"> <li>• A549 and 293T cells</li> </ul>	<ul style="list-style-type: none"> <li>• A549 cells</li> </ul>	<ul style="list-style-type: none"> <li>• 293T and HBEC cells</li> </ul>	<ul style="list-style-type: none"> <li>• MDCK cells</li> </ul>

have taken a different approach with my global RNAi screen to encompass this aspect of influenza virus replication.

I report here an influenza virus RNAi screen using shRNAmir-harboured lentivirus pools that cover the entire human genome, as opposed to siRNA used in the previous six publications. Although I also use A549 cells, similar to König et. al (137) and Karlas et al. (119), my approach differs from the other six screens in that my criteria for a positive hit is the survivability of the knockdown cells against a lethal influenza virus infection. Regardless of viral titre, only the knockdown of genes that promote cell survival in the face of an influenza virus infection over a 72 h period were considered as possible candidates. With this approach, I expected to identify both populations of genes that promote and antagonize influenza virus infection, while permitting the survival of the infected host. The identification of both populations of genes (antagonistic and protagonistic) involved in influenza infection may allow for the development of drugs against specific targets that not only will inhibit viral replication but also reduce virus-induced tissue damage during disease progression.

My RNAi screen, based on cell survival of an influenza virus infection in the presence of an shRNA knockdown, resulted in the identification of 138 annotated and unannotated genes from a total of 1256 potential targets in 2 independent replicate screens. Further validation of these genes, in addition to 20 genes uniquely identified in the 11 published RNAi screens of other viruses (19, 20, 94, 119, 137, 141, 185, 205, 282, 307, 343), via an siRNA array, showed that my screen enriched for genes that are required for influenza virus' ability to kill its infected host cell; knockdown of the majority of the 138 genes identified conferred protective effects to host cells during

influenza virus infection, with only 7% of the genes identified in my screen resulting in significant cell death compared to 35% of the genes uniquely identified in other RNAi screens. Of interest also are the genes I found that protected host cells from virus-induced cell death but did not antagonize the virus' ability to efficiently replicate; this suggests that a small percentage of host genes are involved solely in the induction of cell death during a viral infection.

Bioinformatics analyses have revealed 2 major protein complexes, NF- $\kappa$ B and PI3K, suggested to be involved in the regulation of virus-induced host cell death. Both complexes are reported to be induced during influenza virus replication (62, 226); indeed, the activation of NF- $\kappa$ B is a requisite for efficient influenza virus replication (226). Both PI3K and NF- $\kappa$ B signaling cascades are known to be involved in cell death and survival (59, 62). Activation of NF- $\kappa$ B was commonly associated with anti-apoptotic and anti-viral properties (168); however, many studies have now strongly shown NF- $\kappa$ B activation can lead to the activation of apoptosis (59). Accumulating evidence suggest that influenza viruses (226, 356) and Dengue viruses (187) manipulate this signaling cascade to induce host cell death during viral infection.

Inhibition of PI3K with inhibitor LY294002 blocked viral RNA and protein synthesis as well as nuclear export of vRNPs (286). PI3K has been shown to regulate a very early step during viral entry (62) and also in the suppression of premature apoptosis at late stages of infection. This suppression of apoptosis is shown to occur through a direct interaction of viral NS1 protein and p85 to activate PI3K (62), which leads to cell survival through the activation of Akt; however, there is now evidence showing that PI3K, under hypoxia conditions, can lead to cell death (2, 3, 92). Thus, influenza viruses

may regulate the activation of NF- $\kappa$ B and PI3K signaling cascades to carefully control the degree and timing of cellular death responses to benefit its proliferation.

Interestingly, other RNAi screens enriched genes involved in other aspects of host process such as transcription regulation and signal transduction but not so much in apoptosis. As my work with the BAD protein, discussed in the next section, revealed that host signaling involving cell death occurs late in viral infection, most distinctly detected as late as 72 hpi. Previously published RNAi screens looked only at earlier time points from as early as 12 hpi to 48 hpi. This suggests that critical induction of cell death by influenza viruses may occur within the last 72 hpi.

## **5.2. BAD**

Virus-induced host cell death in WNV (245, 273), HIV (210, 265), and Hepatitis B and C viruses (99), are significant contributing factors to disease pathology. Similarly, influenza virus-induced cell death is a contributing factor to tissue damage and has been suggested to be a catalyst for aberrant host immune response during influenza disease progression (149). A study with the 1918 pandemic virus in macaques indicated up-regulation of cell death and inflammatory related genes (39).

Influenza virus induces cell death through activation of the apoptotic pathway, a process regarded as a major contributor to influenza virus pathogenesis that results in extensive lung tissue damage (24, 178). Blockage of the cell death pathway also leads to a significant decline in virus production (199). I report here that influenza virus-induced apoptosis requires BAD.

My results showed that influenza viruses failed to induced cytopathology and cell death in BAD-deficient cells. Similar observations were reported for WNV infection,

where inhibition of apoptosis prevents WNV induction of cell death (130). Infection of BAD KD cells resulted in significant reduction in virus yield for all three virus strains I looked at (H3N2, H1N1, pandemic H1N1), and both early and late viral protein production were dramatically reduced. There is the possibility that the observed low viral protein production was a result of low progeny yield from initial infections, and therefore reduced viral spread and infection of other cells in the vicinity. However, a general reduction in overall viral protein produced during infection was also observed in Bcl-2 overexpressed cells (233). Thus, it is highly possible that BAD-deficient cells may fail to properly inhibit Bcl-2 activity.

BAD and Bcl-2 co-localization in cells infected with influenza virus at 24 hpi and I showed a statistically significant decline in the co-localization of the two proteins relative to uninfected cells. This reduced co-localization corresponds to a decrease in the percentage of BAD participating in the co-localization with Bcl-2, which dropped considerably from 76% in uninfected mock to 48% in infected samples. Virus-induced phosphorylation of BAD at both S112 and S136 occurred at 24 hpi, after which phosphorylation at S112 began to decline. This suggests that at approximately 24 hpi, apoptosis activation may hinder viral replication and that the influenza virus manipulates BAD's regulatory role of anti-apoptotic factor Bcl-2 to block early activation of cell death. Although I cannot say this co-localization is due to a direct interaction of BAD and Bcl-2 with the present data, given the fact that BAD is known to specifically bind Bcl-2 (74), it is likely that BAD has some kind of an antagonistic role with Bcl-2 activity during influenza virus replication.

Overexpression of Bcl-2 showed inhibitory effects on influenza virus replication (53), but the involvement of Bcl-xL in influenza virus infection has, to my knowledge, not been reported. There are several reports that Bcl-xL expression inhibits virus-induced cell death or viral replication in other virus systems. For example, expression of Bcl-xL in rat progenitor oligodendrocytes is known to inhibit virus-induced cell death induced by coronaviruses (39), and recent studies have shown that expression of Bcl-xL led to the suppression of HIV-1 virus production (69, 70).

BAD is known to heterodimerize with Bcl-xL in mammalian cells to displace Bax and promote cell death (74). Bax has already been reported to play a significant role in promoting influenza virus replication (46). It is possible that influenza viruses hijack BAD in order to inhibit the interaction between Bcl-xL and Bax as a means to promote viral replication. Thus, Bcl-xL may also have anti-viral functions against influenza viruses, and its anti-viral action is subverted by BAD. However, I did not observe any changes in the co-localization of BAD and Bcl-xL during influenza virus infection compared to uninfected, but my observation is far from definitive and further studies with alternate assays are required. The possibility still remains that influenza viruses may manipulate BAD to appropriate Bcl-xL to inhibit apoptosis at early stages of viral replication. More detailed analysis of BAD interaction between Bcl-2 or Bcl-xL is required to sufficiently claim that BAD's role during viral infection involves its regulation of its anti-apoptotic partners.

BAD's ability to associate and inhibit anti-apoptotic factors is regulated by phosphorylation at three residues: S112, S136, and S155 (46). A tiered phosphorylation and model of BAD inactivation has been proposed based on evidence that showed

phosphorylation at S155 requires priming phosphorylation on S136 and S112 residues (46, 52). Sites S136, and especially S155, are reported to be more potent sites required for inactivating BAD activity (52, 191). Moreover, complete BAD inactivation requires phosphorylation of at least two serine residues (52). I determined that influenza viruses induce BAD phosphorylation of S112 and S136 sequentially from mid to late viral replication cycle. S112 phosphorylation occurred before S136, and both phosphorylation process gradually tapered off after 48 hpi. A study has shown that viral NS1 protein interacts with Akt, which results in enhanced Akt activity (195). S136 phosphorylation occurs via Akt in the PI3K signaling pathway (51, 67). I noticed an increase in NS1 protein production at 18 hpi to 20 hpi, whereas phosphorylation at S136 occurred from 20 hpi onward. This suggests that NS1 interaction with Akt may lead to the phosphorylation of BAD at residue S136. More studies are required to identify other cellular and/or viral factors that lead to the phosphorylation of BAD, and how these temporal interactions regulate BAD activity and modulate apoptosis during influenza virus replication.

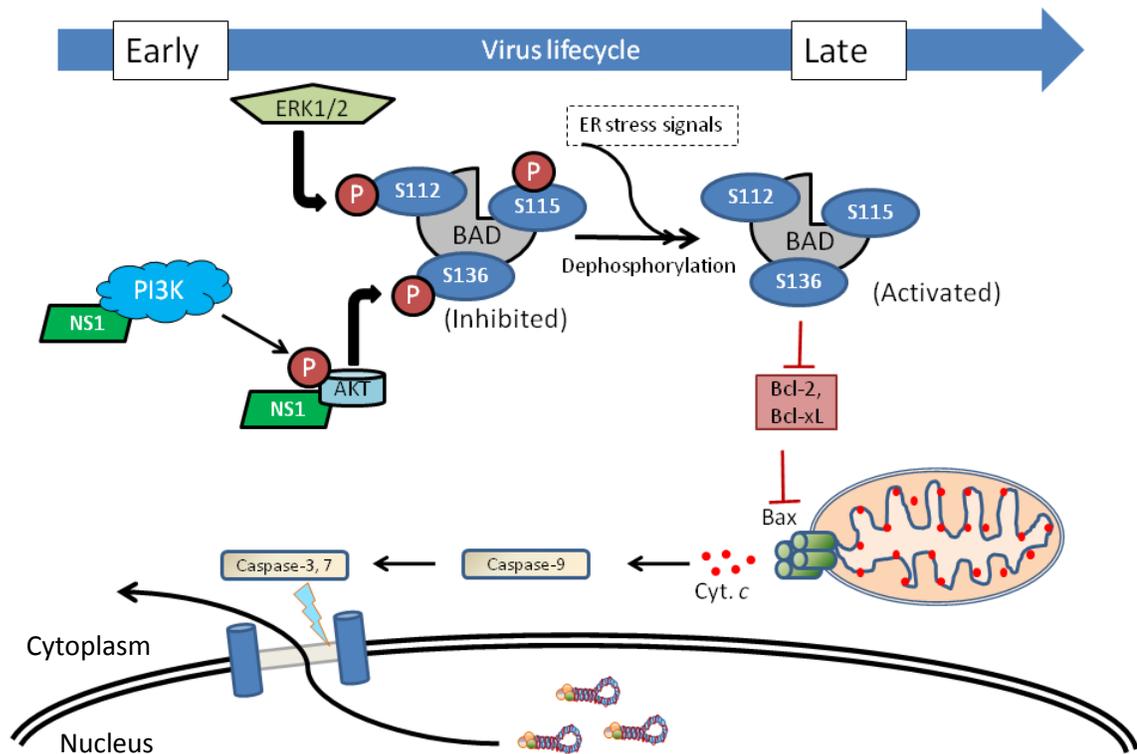
Interestingly, I also noticed the appearance of a truncated, or cleaved form of BAD starting at 48 hpi, with appearance of distinct cleavage at 72 hpi. BAD is cleaved by a number of caspases including caspase-3 and caspase-7 (40). The truncated form of BAD is reported to be a more potent inducer of cytochrome c release and apoptosis than the full-length form due to its higher affinity for Bcl-xL and the mitochondria membrane (40, 281). The N-terminally truncated BAD is poorly phosphorylated and has reduced affinity for 14-3-3, a cellular factor that inhibits BAD through direct association (52). It is proposed that caspase cleavage of BAD initiates a mitochondrial amplification loop during apoptosis, similar to what has been observed with BID cleavage (160). It is

possible that influenza viruses use BAD to moderate the degree of activation of the apoptotic pathway to enhance their replication but actively delays the complete onset of apoptosis that will lead to irreversible cell death. Thus, the virus moderates BAD activity by briefly inducing BAD phosphorylation and delays the appearance of the potent truncated form. Eventually, the death signaling factors will overwhelm the virus' control and cell death will occur.

In the absence of BAD, I observed a suppression of cytochrome c release from the mitochondria, which is an early process of the intrinsic apoptotic cascade. Cytochrome c release will lead to the eventual downstream cleavage and activation of caspase-3 and caspase-7. Although the specific role of caspase-7 during influenza replication remains uncertain, it has been shown that caspase-3 cleavage is essential for the translocation of vRNP from the nucleus to the cytosol (357), and inhibition of caspase-3-dependent apoptosis by anti-apoptotic Bcl-2 protein negatively effects influenza virus replication (199, 233, 268). I show here that in BAD deficient cells, virus-induced cleavage of both caspase-3 and caspase-7 was inhibited. It is likely that the absence of BAD permitted the anti-apoptotic activity of Bcl-2 and Bcl-xL that resulted in blockage of cytochrome c release, and thus suppresses activation of downstream apoptotic factors such as the caspases. A reduction in caspase-3 activity would be a contributing factor to the inhibition of viral replication discussed above. The failure to activate caspase-3 in BAD KD cells was further supported by a reduction in PARP cleavage, which is a substrate of caspase-3 (143). Caspase-7 was considered to be functionally redundant with caspase-3 but recent studies have suggested that caspase-7 may be involved in the inflammatory response as well as apoptosis (152). Further studies are needed to clarify caspase-7's role

during influenza virus replication. Nevertheless, my data showed that caspase-7 is activated upon viral replication and suggests a potentially significant role this protease may play during influenza infection.

Collectively, I propose a model for the role of BAD during influenza virus replication (Figure 41). The intrinsic apoptosis pathway involves the dysregulation of the mitochondria, which results in the release of Cyt. c that goes on to activate caspase-9 and effector caspases-3, and -7. Studies have shown that the activated caspase-3 cleaves nucleoporin proteins to allow for greater exportation of vRNPs late in the virus life cycle – inhibition of caspase-3 activation resulted in retention of the vRNPs in the nucleus (357). Caspase-3 activation through the intrinsic pathway is blocked by anti-apoptotic proteins Bcl-2 and Bcl-xL. Bcl-2 overexpression was shown to inhibit influenza virus replication (233). Moreover, studies have also shown that the activation of PI3K, AKT, and ERK1/2 promotes virus replication, and that NS1 actually directly interacts with these proteins early in the virus lifecycle (62, 174, 195). What is missing is how all these may tie-in together and, based on my work, I suggest that the key regulatory factor is BAD, which is known to be phosphorylated by AKT (51, 96) and ERK1/2 (175), and temporarily inhibited early to mid-virus infection as my data suggested. PI3K directly activates AKT (286). As infection progress to a later stage, ER stress signals overcome pro-survival signals and result in the activation (dephosphorylation) of BAD, which goes on to inhibit Bcl-2 and Bcl-xL and thus complete the circuit.



**Figure 41. Proposed model for the involvement of BAD in promoting influenza virus replication.** PI3K activation by NS1 leads to phosphorylation and activation of AKT, whose activity is also enhanced by association with NS1. ERK1/2 phosphorylates S112 on BAD whereas AKT phosphorylates S136 on BAD leading to inhibition of BAD pro-apoptotic activity early to mid-viral lifecycle. Late viral lifecycle induces ER stress signals that result in dephosphorylation of BAD, which actively blocks anti-apoptotic factors Bcl-2 and Bcl-xL, subsequently leading to the activation of caspase-3. Activated caspase-3 cleaves nucleoporins to enhance the release of vRNPs and promote efficient influenza virus replication.

### 5.3. MxB

Overexpression studies have identified MxA as an antiviral factor against both RNA and DNA viruses (72, 83, 102, 135, 147, 153, 296) but overexpression of human MxB showed no inhibitory effect (296). It is possible that MxB is a proponent of influenza virus replication and, thus, overexpression of MxB would likely mask this function. I demonstrate here that a deficiency in MxB resulted in significant reduction in influenza virus production, which suggests that MxB supports, rather than inhibits, efficient viral production. Moreover, my data strongly indicate that MxB knockdown in human lung epithelial A549 cells inhibited influenza A virus replication of multiple H and N types, and the role played by MxB is virus-type independent.

Absence of MxB also protected infected cells from virus-induced CPE and cell death. I showed that MxB knockdown cells remained completely viable in the presence of an influenza virus challenge, whereas the majority of the control cells showed gross cytopathic morphology and cell death. It is likely that the observed reduced viral yield resulted in the corresponding reduction in virus-induced cell death and CPE in MxB knockdown cells. However, despite reduction in virion production, viral protein production was not blocked. This suggests that cell death and CPE development during influenza virus replication requires certain aspects late in the virus lifecycle and not simply the activity of viral proteins. My assessment of virion particle release was crudely done with western blot; a more specific assay is required to provide a precise and accurate picture of virion particle to infectious particle production.

Surprisingly, MxA expression was elevated in infected MxB depleted cells. The specific mechanism for this increase in MxA expression remains unknown but it may

possibly explain the inhibition of viral replication observed in MxB knockdown cells. MxA proteins are reported to form punctate granula in the cytoplasm (302) and have the capacity to physically interact with viral NP proteins (134). The physical sequestration of viral NP by the elevated level of MxA in MxB knockdown cells may explain the punctate granular accumulations of viral NP observed in cytoplasm of infected MxB depleted cells. Although this retention of NP by MxA should result in reduced viral particle production, it is possible that the sheer amount of NP produced during viral replication overwhelms the anti-viral nature of endogenous MxA. On the other hand, MxA elevation may just be a consequence of host anti-viral activation. Perhaps the aberrant localization of NS1 due to a depletion in MxB prevented the viral protein from properly inhibiting anti-viral activity of IFN signaling. Further studies are warranted to delineate the specific nature of this relationship.

Mx proteins have been shown to have significant homology to dynamins, which are enzymes involved in intracellular protein trafficking (297). Unlike MxA, MxB has a putative nuclear targeting signal and localizes to the nuclear membrane (203). It has also been demonstrated to be involved in the nucleocytoplasmic trafficking of proteins (129, 202). The unusual “threadlike” formation of the NS1 protein suggests a possible impairment in viral protein trafficking, which may involve MxB activity. Given that results suggest the infectivity of progeny virions are affected in MxB KD cells, aberrant viral protein trafficking can result in inadequate or inappropriate post-translational modifications of viral proteins, which does not necessarily affect assembly and release of virion particles; however, this may potentially affect infection of neighboring cells.

## **5.4. TNFSF12-13, TNFSF13**

Influenza virus infection has been shown to up-regulate TNFSF13 expression in lung tissues (95). TNFSF13 and the fusion protein TNFSF12-13 belong to the TNF ligand superfamily (49, 258). Intergenic splicing between exon 6 of TNFSF12, another member of the TNF superfamily, and exon 2 of TNFSF13 leads to the production of the fusion protein TNFSF12-13, which displays the same receptor specificity as TNFSF13 (258). My study provides evidence to show that TNFSF12-13 and TNFSF13 play significant roles in influenza virus replication. Importantly, knockdown of these proteins inhibited virus-induced cell death and CPE development. Of significance is that TNFSF13 activity was reported to act via the NF- $\kappa$ B pathway (65). It is possible that TNFSF13 may support influenza replication via activation of the NF- $\kappa$ B transcription factor. NF- $\kappa$ B has been suggested to be exploited by influenza virus to promote viral replication via activation of pro-apoptotic factors, TRAIL and FasL (65). This suggests TNFSF13 ligand may support influenza replication via activation of NF- $\kappa$ B, which may lead to the activation of pro-apoptotic factors. The knockdown of both TNFSF13 and TNFSF12-13 lead to a reduction in influenza replication and virus-mediated cell death, coupled with the fact that both molecules share the same receptor specificity, suggests a common role for these two proteins in influenza replication perhaps via the same signal transduction pathway. Further characterization of the biological function of TNFSF12-13 is required to determine whether this may be the case.

## **5.5. USP47**

The involvement of DUB with influenza virus infection was recently shown with an A20 DUB knockout mouse model. Lethal challenges of these A20 knockout mice

resulted in protection against influenza virus infection (180), which suggests that specific DUB proteins actively promote influenza virus replication. Here I report the involvement of another DUB protein, USP47. Knockdown of this gene in my screen and subsequent validations also resulted in significant inhibition of virus replication and reduction in virus-induced cell death.

USP47 is a deubiquitinase, whose specific role in cellular processes remains largely unknown (259). A20 is reported to be a regulator of inflammation and cytokine production via NF- $\kappa$ B. Bioinformatics study of the positive hits in my screen also highlighted the significance of this signaling cascade; thus, it is possible that USP47 also has a specific regulatory role, either directly or indirectly, involved with NF- $\kappa$ B signaling. Nevertheless, other signaling cascades may also be targeted by USP47. For example, USP47 has been reported to play a function in scattering responses in A549 cells (26). The specific molecular mechanism underlying this role has not been determined, and whether this activity of USP47 is linked to its role in influenza infection is not certain. Two other recent studies reported that USP47 interacts with the E3 ubiquitin ligase, SCF <sup>$\beta$ -Trcp</sup> (249), and regulates the steady-state levels of DNA polymerase  $\beta$  (246). Interestingly, both these studies reported that knockdown of USP47 in HEK293T cells (249) and HeLa cells (246) led to a decrease in cell survival and proliferation. However, my work with stably transduced shRNA KD of USP47 and that of Buus et. al. (26) showed that knockdown of this protein in A549 cells have no effect on cell viability and proliferation. This suggests that USP47 function may be cell-type dependent. This may prove to be significant in regards to the types of cells that are permissive to influenza virus infection.

## Part VI | Future Directions

### 6.1. RNAi Screen

The fact that other RNAi screens have an overlap of 146 genes from the total 1256 potential targets (sum of 2 independent replicate screens), but only 3 genes from the 138 genes simultaneously identified in both of my screen replicates, suggests that cutoff parameter for positive hits may actually result in a greater number of false negatives. At the same time, it is possible that these 146 genes may be false positives from other screens but given the independent replications and validations, the likelihood this is the case is low. Thus, further follow-up such as the inclusion of more or larger siRNA arrays to reassess the remaining 1118 potential targets is warranted. However, as with large data sets, it will be difficult to provide individual analysis and confirmation for each positive hit. Therefore, focus should be given to genes involved in the major networks identified such as NF- $\kappa$ B signaling, ubiquitylation and deubiquitylation, and apoptosis. There are already mounting evidence that strongly suggests critical roles for these host signaling pathways in influenza virus infection, both promoting and antagonizing viral replication. Despite greater focus, much remains to be understood about the specific host-virus interactions that are critical for influenza virus replication and pathogenicity. Moreover, molecular details continue to be absent.

### 6.2. BAD

It is logical to propose that early activation of apoptosis is detrimental to virus propagation but activation of cell death late in the replication cycle enhances influenza virus production. As mentioned earlier, BAD is an important regulator of anti-apoptotic

partners Bcl-2 and Bcl-xL, and its activity is moderated by phosphorylation at 3 key sites (S112, S136, and S155) (46). My discovery that influenza viruses induce a temporal phosphorylation of BAD as well as unphosphorylation from mid to late viral replication suggests that BAD is a vital focal point for regulating apoptosis induction during infection. More studies are required to identify specific cellular and/or viral factors that phosphorylates BAD, and how these sequential phosphorylation events regulate BAD activity and modulate apoptosis during influenza virus replication. A proposed experiment will be to create mutations at these phosphorylation sites and then express these BAD mutants in BAD KD cells to determine the effect BAD phosphorylation has on influenza replication. Several combinations of phosphorylation mutants should be developed. Examples of such mutants include: (1) mutants that are constitutively phosphorylated only at individual sites S112, S136 or S155; (2) a combination of constitutively phosphorylated sites such as at S112 and S136 but not at S155, or at S112 and S155 but not at S136; (3) null mutants that cannot be phosphorylated at any of these sites; (4) and mutants that are constitutively phosphorylated at all 3 sites. The mutants can be expressed in BAD knockout cells for accurate assessment of these mutations on viral replication because knockout cells will lack endogenous BAD background noise. Furthermore, a timed transfection of these BAD mutants can be carried out to determine whether early or late phosphorylation of BAD has any detrimental or beneficial effects on influenza virus replication. Moreover, viral NS1 protein enhances Akt activity (195), and Akt is known to induce S136 phosphorylation (51, 67). Thus, Akt knockdown should also be carried out to determine whether BAD phosphorylation during influenza virus infection is Akt-dependent, and if NS1 association of Akt is the mechanism that lead to

BAD phosphorylation. Additionally, virus NS1-deletion mutants can be developed using reverse genetics and used to determine whether the presence of NS1 is required for Akt-dependent phosphorylation of BAD.

BAD cleavage late during influenza virus replication was a novel observation I report in my study. BAD is cleaved by a number of caspases (40) and the truncated form of BAD is a potent inducer of apoptosis (40, 281). Given the relatively late appearance of truncated BAD during influenza virus replication suggests this small protein is indicative of full apoptosis activation that irreversibly lead to cell death. The role of the truncated BAD can be verified with plasmids that express only the truncated version of BAD or a full-length BAD with a mutated cleavage site. In order to avoid endogenous BAD background interference, the constructs should be transfected into BAD KD cells. Transfection of these constructs can be carried out at different time points post-infection to determine whether early truncation of BAD will affect viral replication; in this case, an inducible expression system may be more suitable and easier to perform.

I also report here that influenza virus infection induces caspase-7 cleavage and activation. Caspase-7 activity during influenza virus replication has not been reported before, so very little is understood about its role(s) during viral infection. Given the well-studied role of caspase-7 in apoptosis, it is possible that caspase-7 has a similar function as caspase-3 in influenza virus replication. However, further studies are required to confirm this. Presently, I am unaware of any inhibitors that selectively inhibits caspase-7 activity without affecting caspase-3 activity as well. However, siRNA or shRNA knockdown of caspase-7 can easily circumvent this problem. Specific knockdown of this caspase in the presence of a viral infection will provide further support for caspase-7's role

in influenza virus replication and induction of cell death. Other alternative assay include creating an expression construct to overexpress caspase-7, express a full-length version with a mutated cleavage site, or express only the cleaved (active) caspase-7 protein. These assays will provide a clearer picture of caspase-7 function during viral infection.

BAD regulation of anti-apoptotic factors Bcl-2 and Bcl-xL during influenza virus infection requires further scrutiny. Despite my observation that BAD and Bcl-xL localization did not change, the possibility still remains that influenza viruses may manipulate BAD to appropriate Bcl-xL and Bcl-2 to inhibit apoptosis at early stages of viral replication. More detailed analysis of BAD interaction with Bcl-2 and/or Bcl-xL is required to sufficiently claim that BAD's role during viral infection involves its regulation of its anti-apoptotic partners. An example will be to look at changes in BAD co-localization with Bcl-2 or Bcl-xL over time by immunofluorescence, which may provide a clearer picture given that significant caspase activation did not occur until at earlier 30 hpi. Also, co-immunoprecipitation (co-IP) can be carried out on BAD and Bcl-2 or Bcl-xL to determine whether there is any interaction occurring between the proteins during influenza infection. Along with co-IP, fluorescence resonance energy transfer (FRET) can be used to provide further evidence for BAD and its association with its anti-apoptotic targets. FRET also provides information on spatial arrangement of these associations beyond the limits of conventional optical microscopy. Additionally, overexpression of Bcl-xL should be carried out to assess the specific role this protein plays in influenza virus infection, whether it will antagonize viral replication similar to Bcl-2. Importantly, studies with BAD phosphorylation mutants can also be extended to determine whether BAD phosphorylation at the specific times post-infection reported in

my study affects BAD association with Bcl-2 and/or Bcl-xL. This will illuminate the molecular mechanism undertaken by BAD to promote influenza virus replication and virus-induced cell death.

It is also necessary to identify any direct or indirect interaction between BAD and viral protein(s), or other host factors, to resolve whether the involvement of BAD is an intentional manipulation of the pathway or a consequence of activation of other signaling networks during infection. Additionally, the focus on BAD activity can be further extended to other biological systems and other viruses such as HIV induction of CD4<sup>+</sup> T-cell death.

### **6.3. MxB**

My study suggests that MxB KD affects influenza virus infectivity. I looked at virion particle production by accessing viral proteins obtained from virions pelleted from infected supernatants and determined that total particle produced in control and MxB KD cells were comparable. However, this assessment of progeny virion production was crudely determined by western blot. Quantitation of virus particle production by electron microscopy, coupled with plaque assay, will provide a more precise and accurate picture of virion particle to infectious particle production.

MxA expression was elevated in infected MxB depleted cells; however, MxA elevation may just be a consequence of host anti-viral activation. MxA and MxB double knockdown should be carried out to determine whether the knockdown of MxA will inhibit NP from forming the observed punctate pattern in MxB single knockdown cells. This will resolve the issue of whether the aberrant localization of NP was due to MxA interaction. Moreover, if MxA/MxB double knockdown results in an enhancement of

viral replication will suggest that viral inhibition observed in MxB KD cells was due to the anti-viral nature of MxA, which further suggests that MxB activity antagonizes MxA function or MxB activity is required for proper function of other host or viral proteins. An example is that the aberrant localization of NS1 can interfere with the viral protein's ability to properly inhibit the activation of IFN, which regulates MxA expression. Alternatively, aberrant viral protein trafficking in MxB KD cells can result in inadequate or inappropriate post-translational modifications of viral proteins, which does not necessarily affect assembly and release of virion particles; however, this may potentially affect viral infectivity. For example, viral NP protein is known to require ubiquitylation for proper function (165). Thus, additional studies are needed to identify post-translational modification(s) of other viral proteins, especially in the presence of deficient levels of MxB.

#### **6.4. TNFSF12-13 and TNFSF13**

The knockdown of both TNFSF13 and TNFSF12-13 both lead to a reduction in influenza replication and virus-mediated cell death, coupled with the fact that both molecules share the same receptor specificity, suggests a common role for these two proteins in influenza replication perhaps via the same signal transduction pathway. TNFSF13 activity was reported to act via the NF- $\kappa$ B pathway (65). It is possible that TNFSF13 may support influenza replication via activation of the NF- $\kappa$ B transcription factor. Since very little is known about TNFSF12-13 and TNFSF13 in the context of influenza virus replication, initial characterization of these 2 proteins are required. For example, western blots should be done to determine whether influenza virus infection result in an increase of both or one of these host proteins. Additionally, experiments are

needed to decide whether TNFSF12-13 and/or TNFSF13 localization or TNFSF13 secretion is altered by influenza virus replication. These are factors that affect the proper function of TNFSF12-13 and TNFSF13.

Moreover, assays should be carried out to verify if NF- $\kappa$ B activation is inhibited or reduced in the absence of either or both TNFSF12-13 or TNFSF13 during influenza virus infection. Specific targets of the NF- $\kappa$ B signaling pathway should be focused on such as phosphorylation of p65 or p50 subunits, or the degradation of IKK, which are all indicative of NF- $\kappa$ B activation.

## **6.5. USP47**

DUB are involved in the regulation of many important signaling pathways but there are several reports that the RIG-I pathway is tightly regulated by both DUB and Ub enzymes such as A20 (180) and TRIM25 (76), respectively. Thus, a reasonable initial approach is to look at USP47 activity on RIG-I signaling. RIG-I activation can be monitored by analyzing downstream factors such as TRAF3 and TRAF6, which mediate NF- $\kappa$ B and IRF3 action upon RIG-I stimulation (87, 138, 231). The identification of potential interacting host and/or viral factors by IP and mass spectroscopy will narrow down the focus to more specific pathways. I have actually initiated this assay but significant optimization is required to obtain satisfactory results. My initial attempt to look at ubiquitination levels in viral proteins and whole cell lysates in USP47 KD cells by IP with anti-Ub and anti-USP47 antibodies did not provide any definitive results. It was possible that too much background noise might have obscured any possible differences between KD cells and control. Further optimization and possibly better antibodies are required. It is also possible that knockdown of USP47 did not have major impacts on

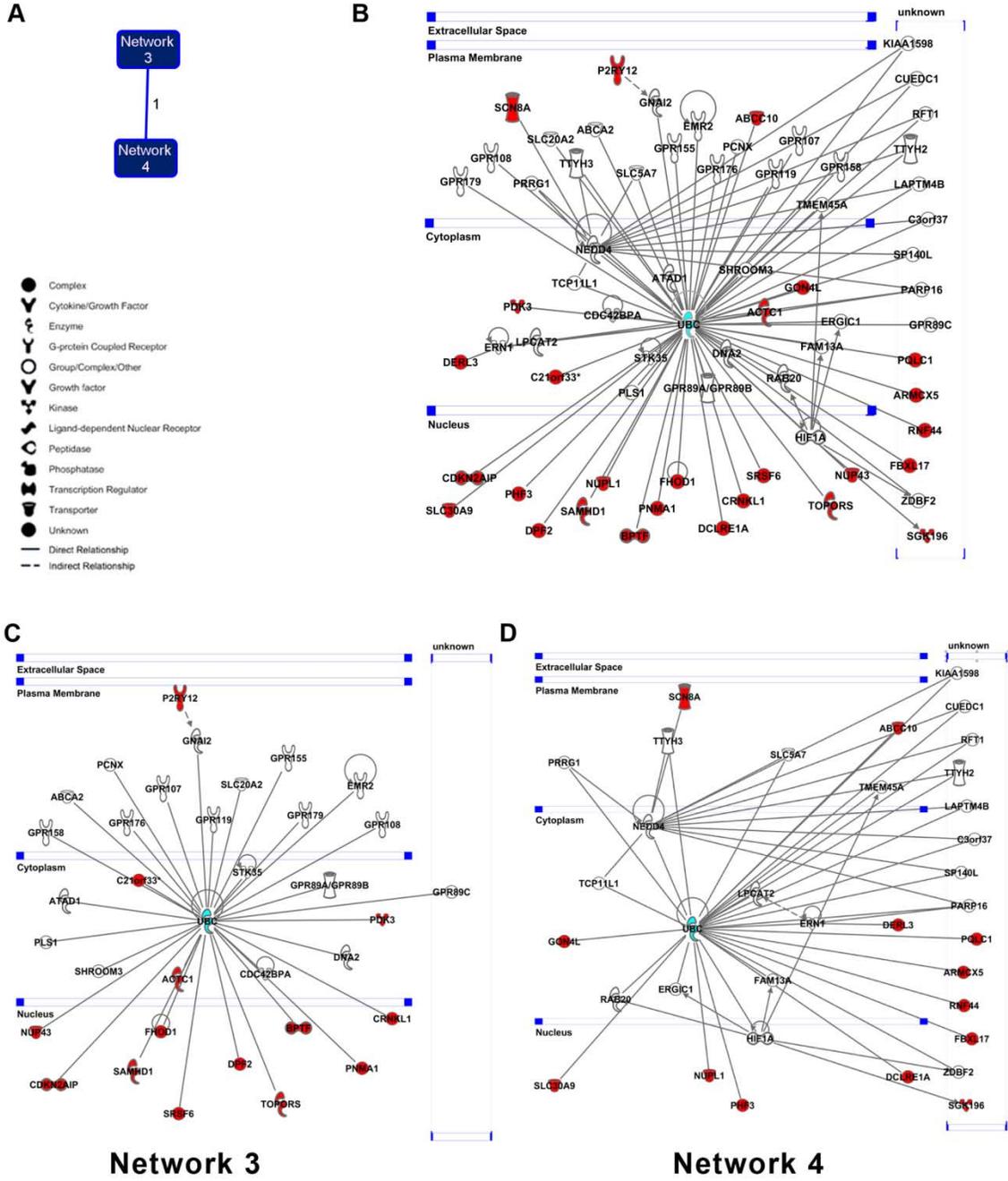
global ubiquitylation in host cells or viral proteins. Also, given the dynamic nature of Ub and DUB, a time course may be required to detect any effects.

Overall, my study with human genome-wide screen for host factors involved in influenza A virus infection has provided greater insight into the complex web of host-viral interaction and has further identified a subset of host factors shown to play significant roles in virus-induced cell death, with or without having effects on influenza replication, an aspect not highlighted in previous screens. I have provided evidence for novel association of 5 specific proteins in promoting influenza virus replication and induction of cell death. I have shown that BAD is an important cellular factor required for influenza virus-induction of the apoptotic signaling cascade that is essential for efficient viral replication. My data suggest that BAD supports influenza virus replication through its innate role as a potent regulator of the mitochondria-dependent apoptotic pathway and as an antagonist of anti-apoptotic factors such as Bcl-2 and Bcl-xL. To my knowledge, effects of MxB knockdown in the context of an influenza virus infection has not been reported before. This also is the first report where MxB is implicated to be directly involved in the support of influenza virus replication. The involvement of TNFSF12-13 and TNFSF13 may provide further evidence that host factors which act through the NF- $\kappa$ B signaling cascade play significant roles in different stages of viral infection. I have also provided evidence for USP47 involvement in influenza virus replication, which further underscores the importance ubiquitylation and deubiquitylation have in viral diseases.

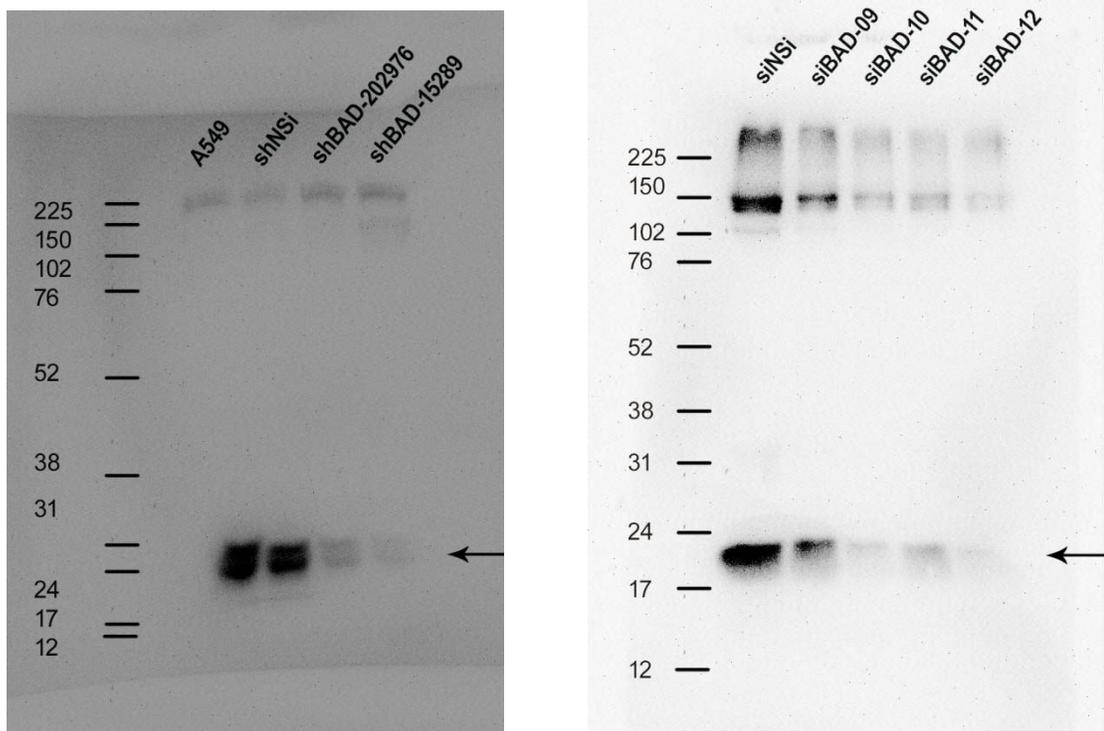
My study presents evidence to further emphasize the importance of understanding the intricate relationship apoptosis has in promoting influenza virus propagation, and how

its regulation may be a key to controlling influenza virus infection and prevention of host tissue damage. These genes may prove to be novel candidates for therapeutic development that not only inhibit viral replication but also reduce virus-induced tissue damage during infection.

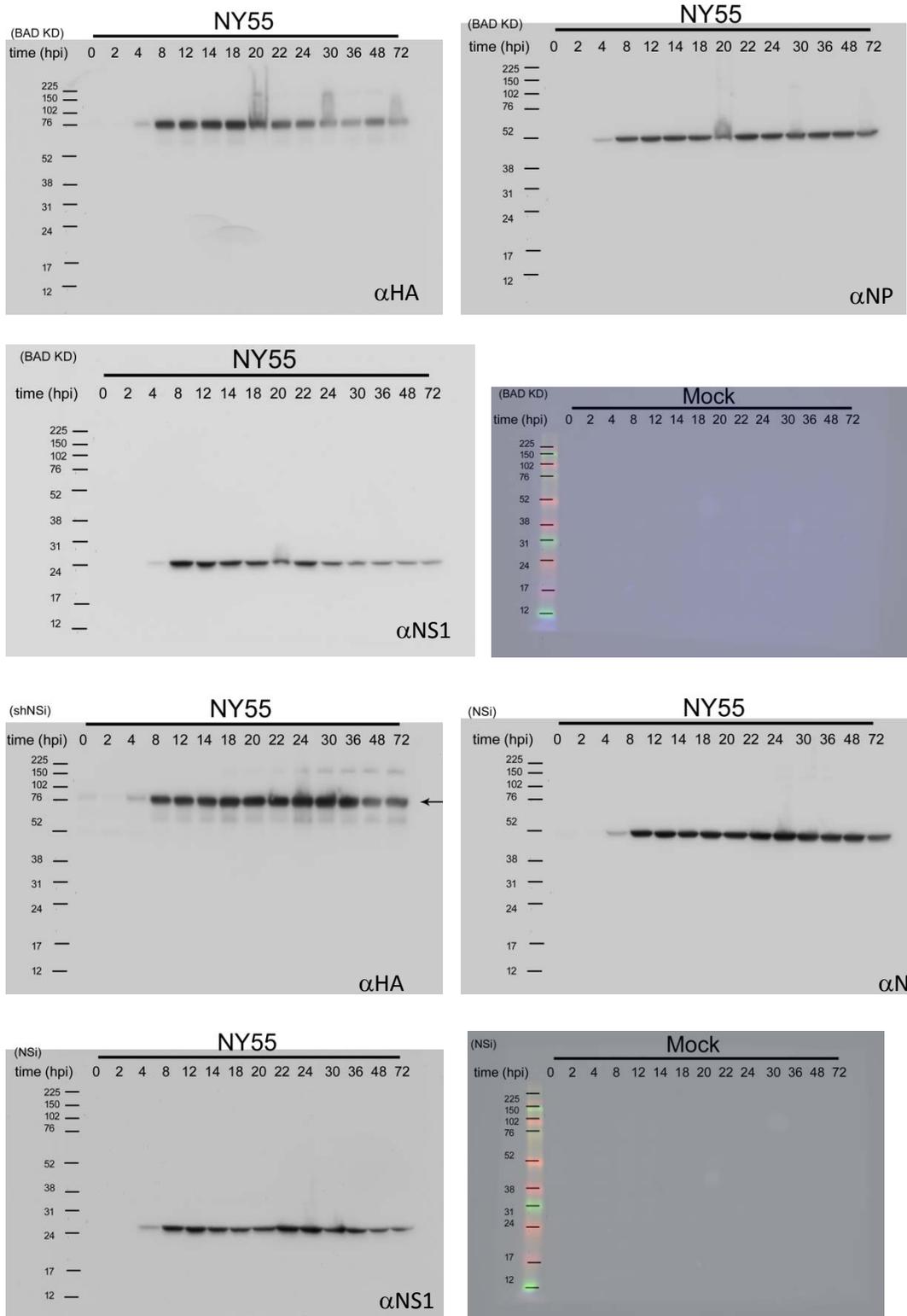
# APPENDIX



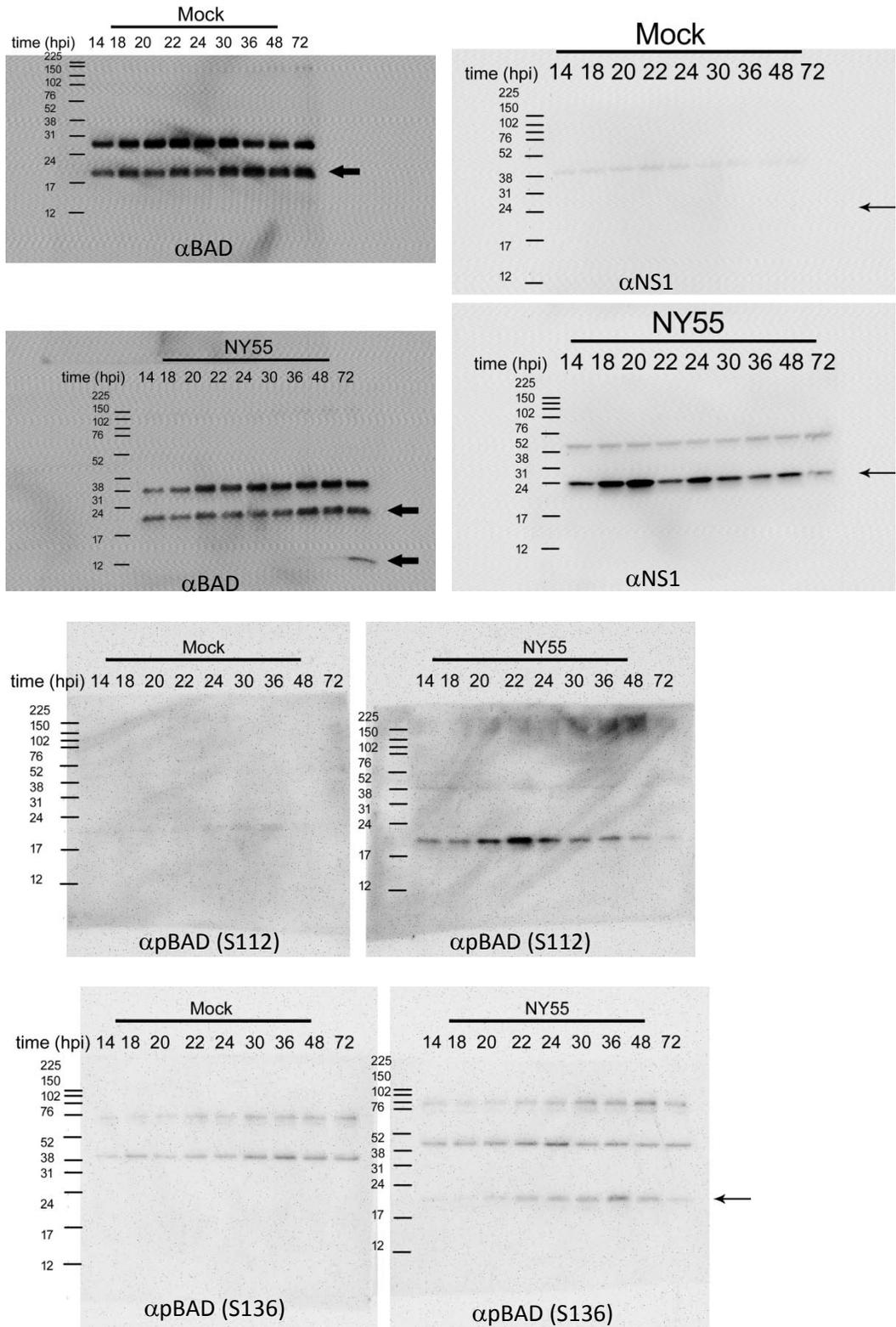
**Figure A1.** 97 genes overlapping with other published influenza RNAi screens were mapped to a single major protein complex. Interacting networks were determined with Ingenuity Pathway Analysis for the 97 genes identified in all 6 influenza RNAi screens and my screen. Red are genes identified in my screen, white are unidentified genes, and cyan highlights major protein complex. (A) Shows connecting Networks 3 and 4 with number of common genes indicated along connector line. (B) Merge of both networks in (A). (C and D) shows Network 3 and 4, respectively.



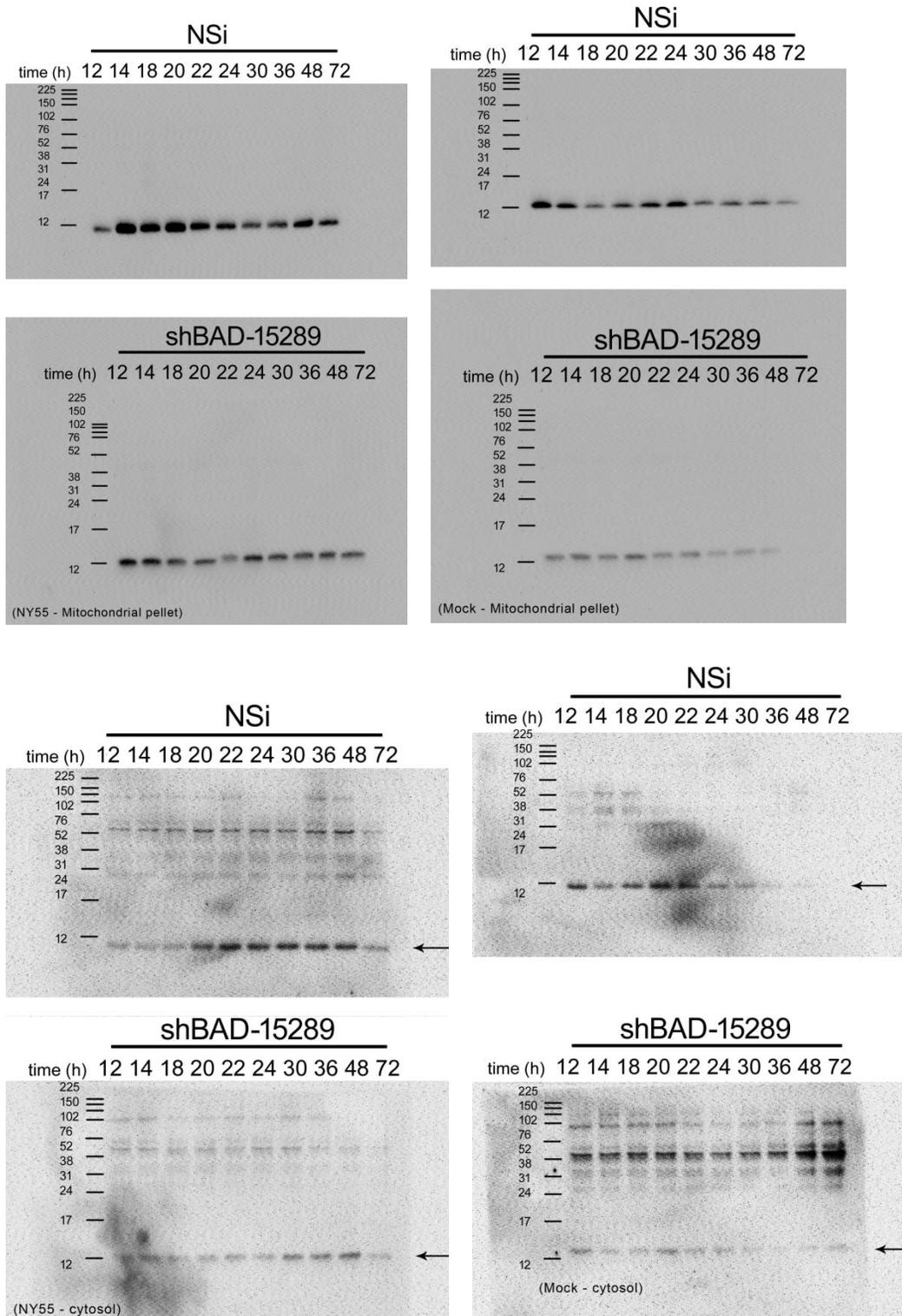
**Figure A2. Efficiency of BAD knockdown in A549 cells.** Full Western blot for Figure 21C (Left) and Figure 21D (Right).



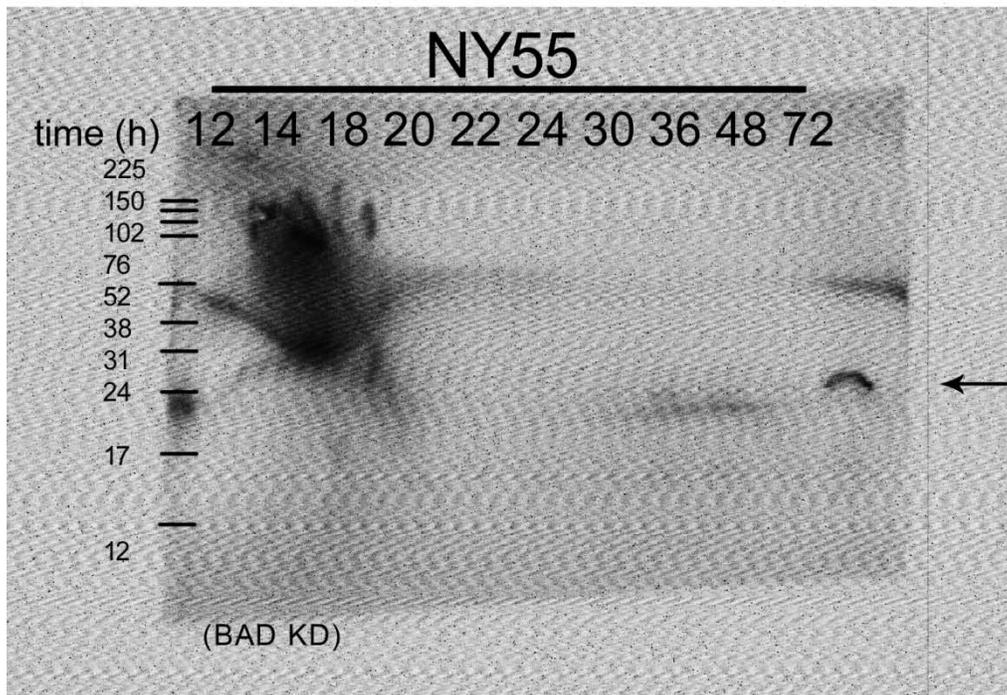
**Figure A3. Reduction of influenza virus protein in BAD knockdown cells. Full Western blots for Figure 23A.**



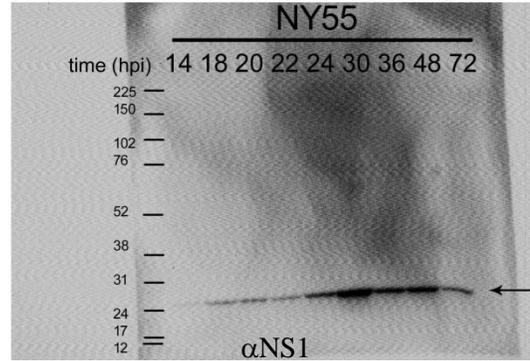
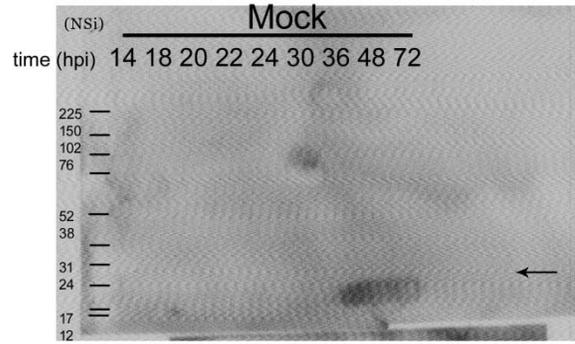
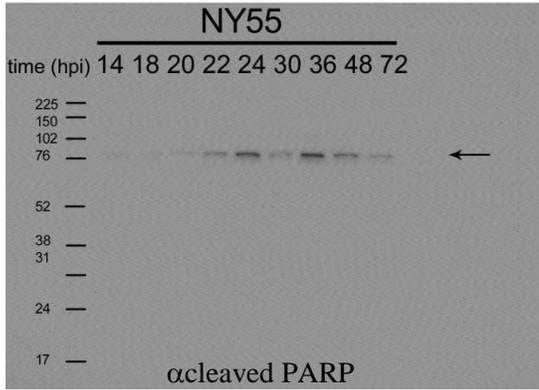
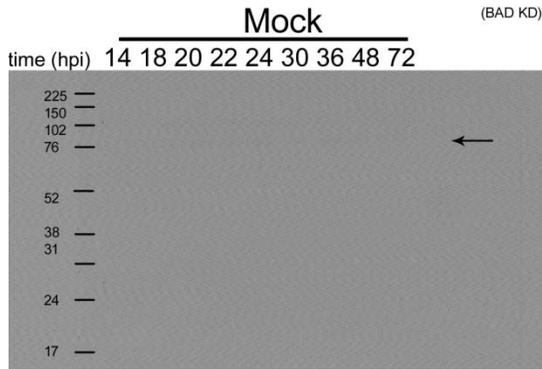
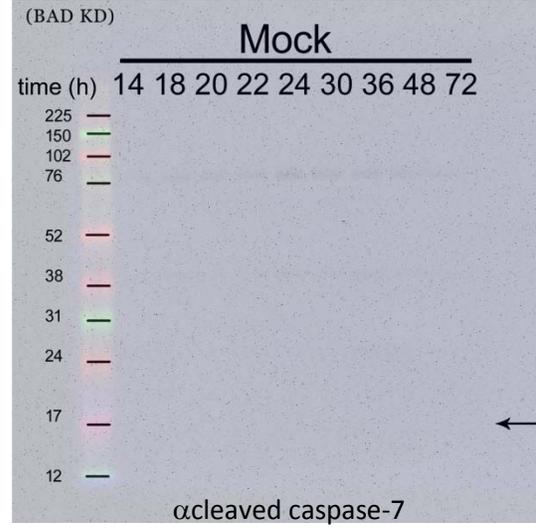
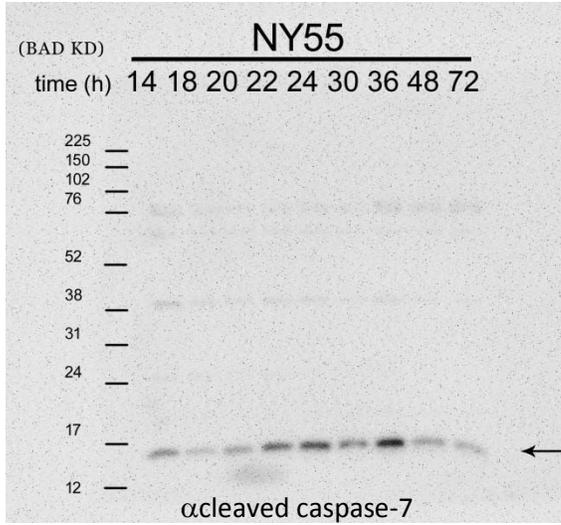
**Figure A4. Influenza virus induces BAD phosphorylation and cleavage.** Full Western blots for Figure 24.

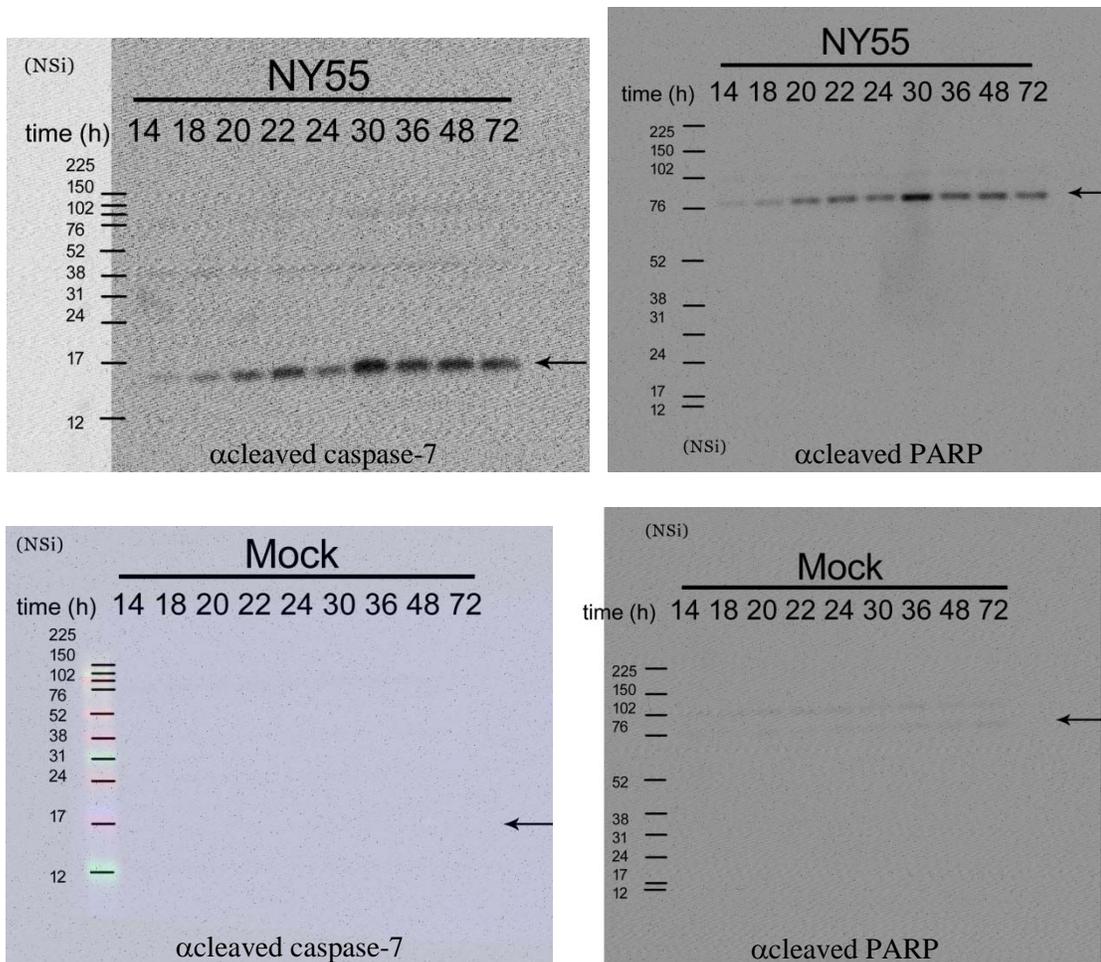


**Figure A5. Deficiency in BAD inhibits virus-induced Cytochrome c release.** Full Western blots for Figure 25.

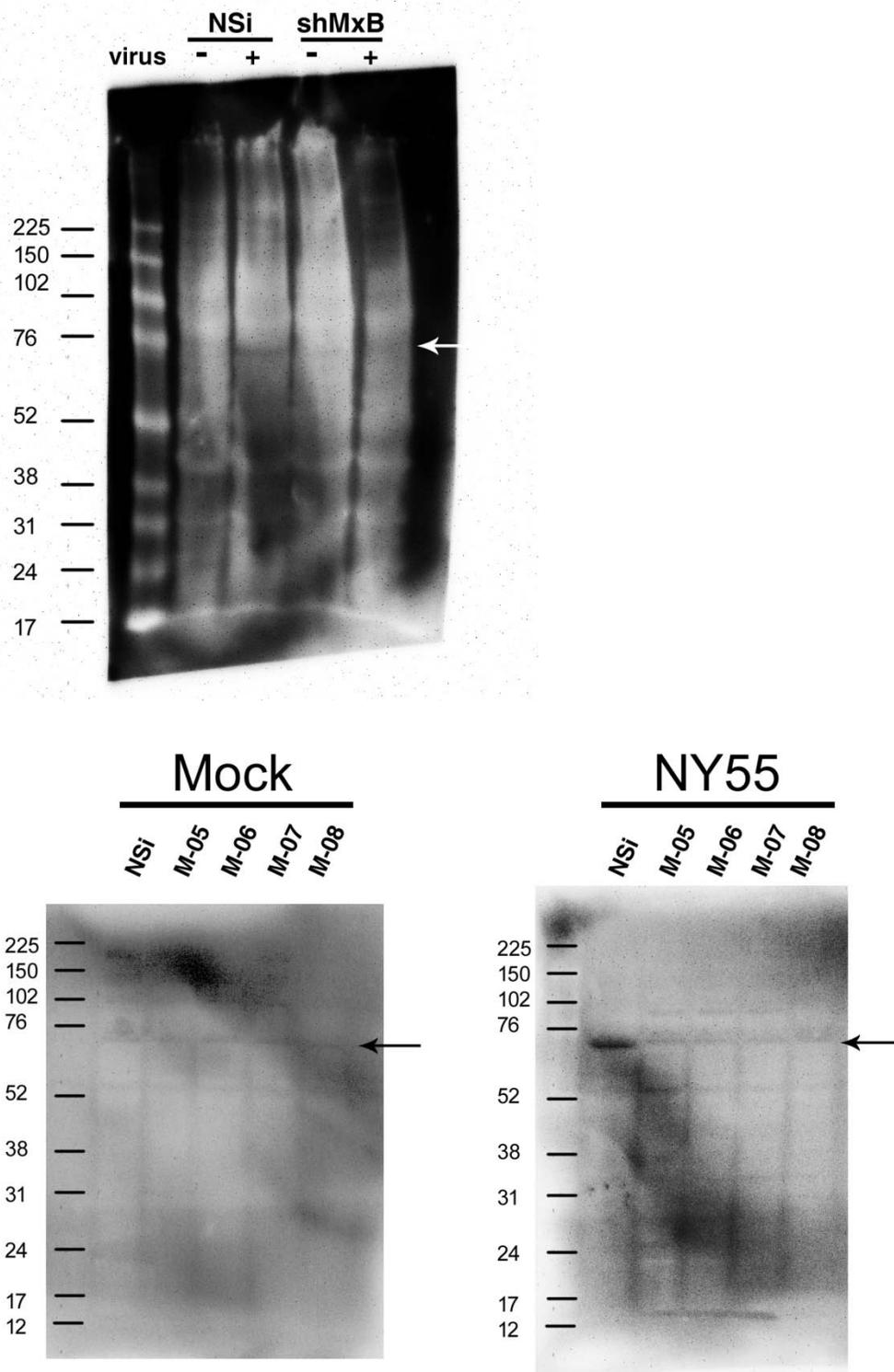


**Figure A6. BAD is required for influenza virus activation of caspase-3.** Full Western blot for Figure 26.

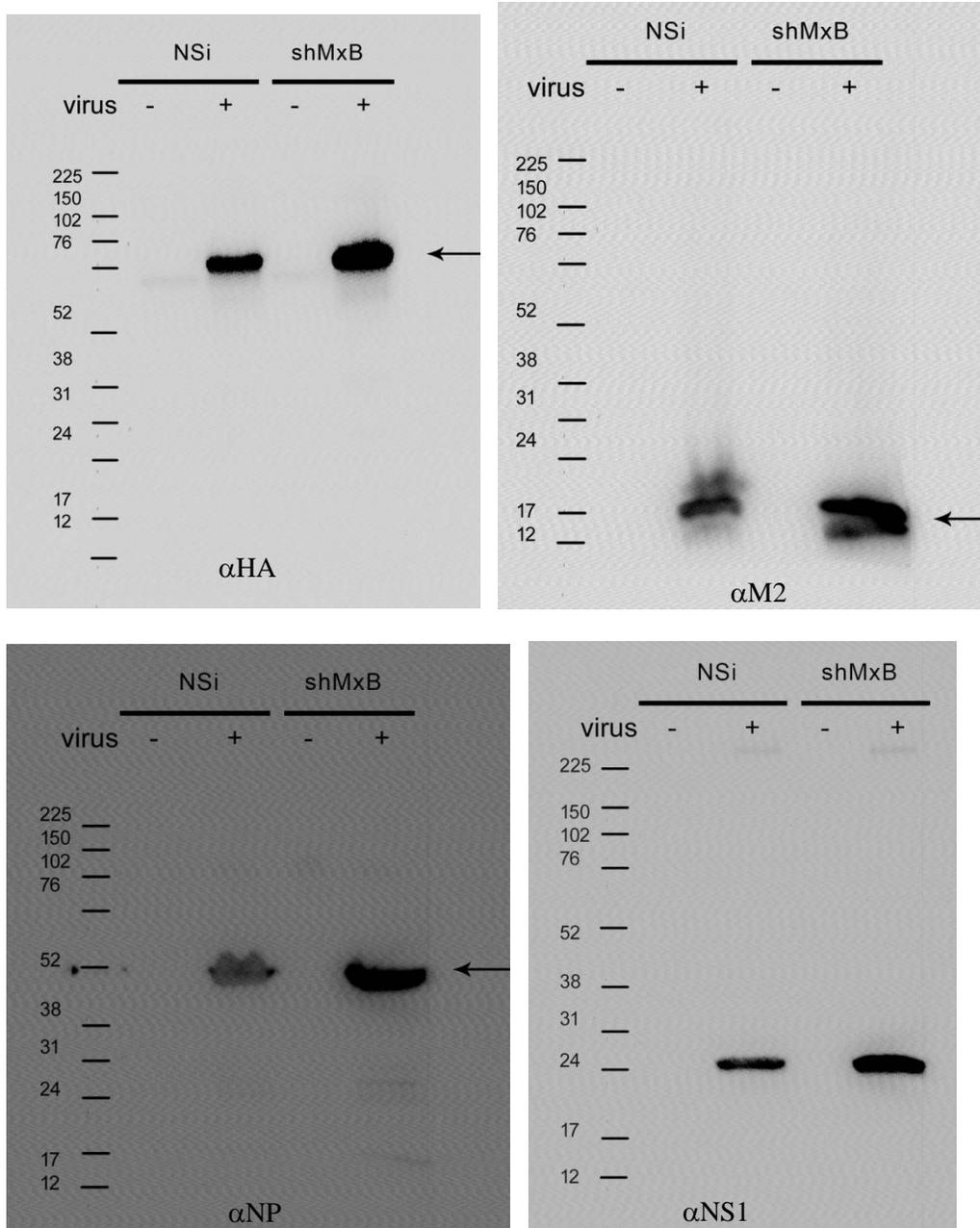




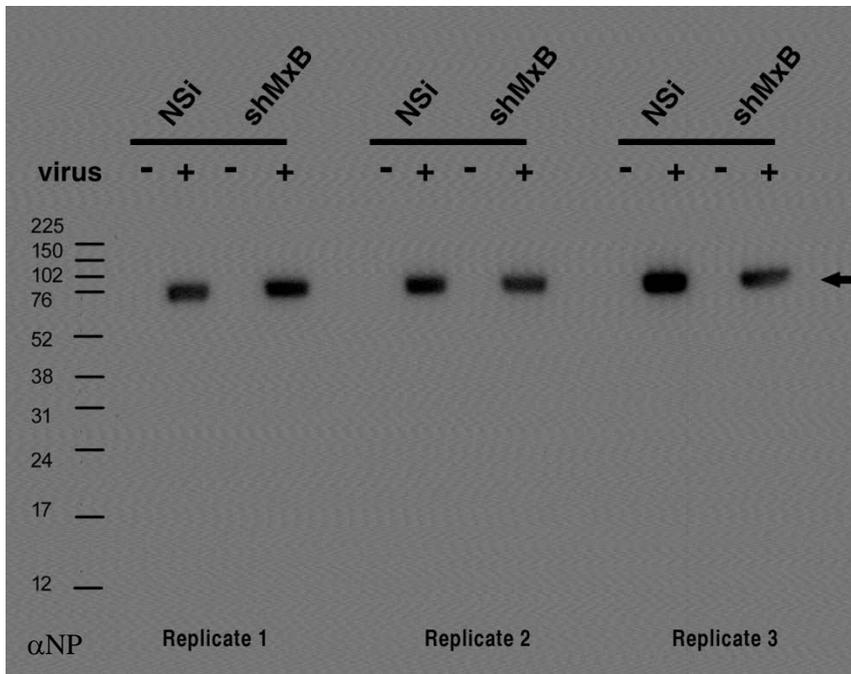
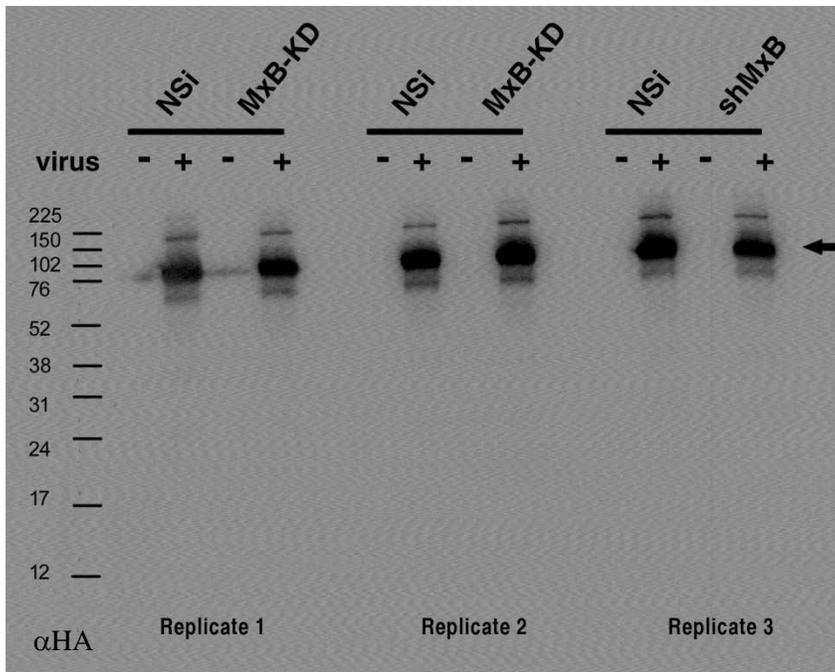
**Figure A7. Cleavage of caspase-7 and PARP is inhibited in influenza virus-infected BAD knockdown cells.** Full Western blot for Figure 27.



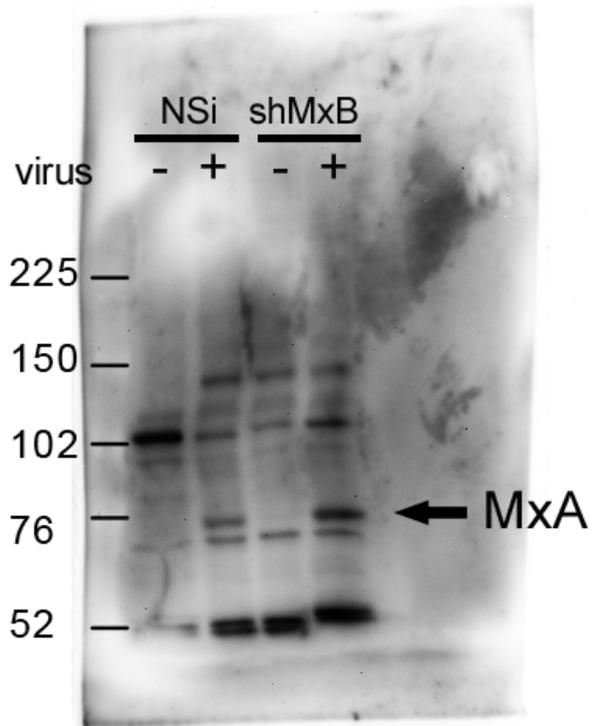
**Figure A8. Efficiency of MxB knockdown in A549 cells.** Full Western blot for Figure 30.



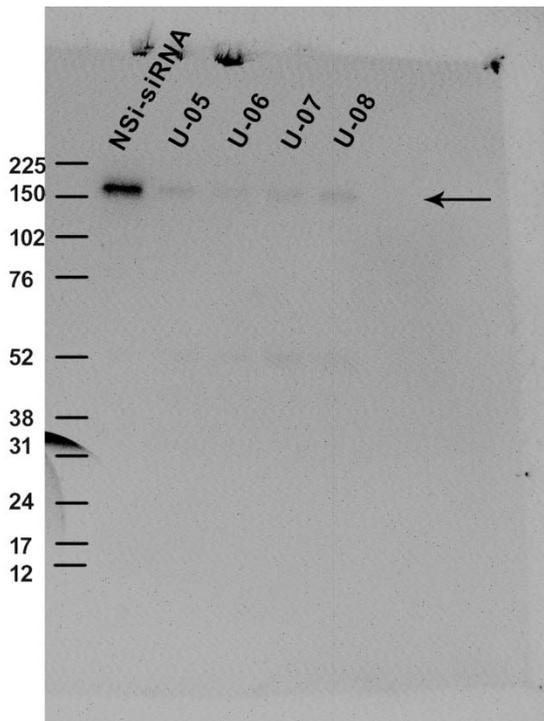
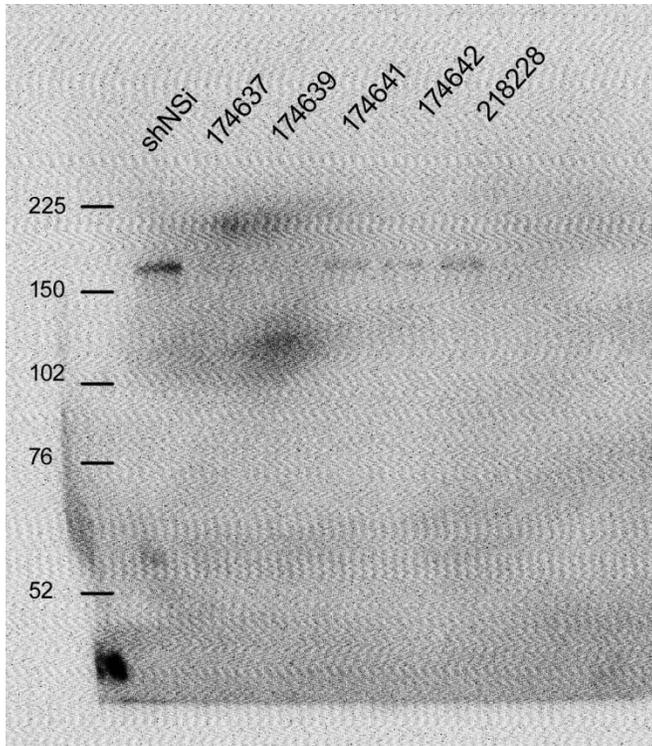
**Figure A9. Influenza virus protein production is unperturbed in MxB knockdown cells.** Full Western blot for Figure 33A.



**Figure A10. Protein of virion particles released into supernatant.** Full Western blot for Figure 33C.



**Figure A11. MxB knockdown did not affect induction of MxA expression.** Full Western blot for Figure 35B.



**Figure A12. Efficiency of USP47 knockdown.** Full Western blot for Figure 40. shRNA knockdown (Top), siRNA knockdown (bottom).

**Table A1. List of potential candidates identified in Replicate 1 RNAi screen.** All shRNA species identified are listed here. Some genes will have multiple shRNA species and are thus listed more than once.

Count	Oligo ID	Sense Sequence	Gene IDs*	Gene Symbols*
1	V2HS_94280	GACAAAGCAAACCTTCAACA	97	ACYP1
1	V2HS_172658	GGCATCATGTCTACTACA	109	ADCY3
1	V2HS_154118	CAAATCAGTTTGTTCCTTT	120	ADD3
1	V2HS_112012	CCTAAAGAAGGCTTATGAA	185	AGTR1
1	V2HS_48937	ATGCCTGTCCCATCAAGTA	230	ALDOC
1	V2HS_65427	CACATTGTCCGATTTATCA	269	AMHR2
1	V2HS_48801	GAGAATGTTCGAGAAAGAA	466	ATF1
1	V2HS_236634	GATATAACAACCTGTCATAT	483	ATP1B3
322584	V2HS_243025	CAGTGACCTTCGCTCCACA	572	BAD
2	V2HS_48576	GCCATAGTTTGTTAATCTC	648	BMI1
1	V2HS_280394	GATCGATTATGTGACTTAA	672	BRCA1
1	V2HS_15171	CACTCTATTTCCAGAGATA	683	BST1
20	V2HS_150110	CACCAGAAGTCCTGAAACT	685	BTC
1	V2HS_89106	CCTACAATGTGGAAAGCTA	732	C8B
2	V2HS_112215	GAAACCATTTGACATATTT	776	CACNA1D
1	V2HS_89057	CAAATAAACCGGAACAACA	779	CACNA1S
1	V2HS_220152	CTGTGAGGTTTCAGTTATTA	795	S100G
1	V2HS_77334	CTGCTGATTACCTGTCTTA	801	CALM1
2	V2HS_62791	GCTATAGTGCAGTAGAAAT	889	KRIT1
1	V2HS_262156	GAAATGGATTCTATTTATT	1032	CDKN2D
1	V2HS_14802	CTGATGATTTGATTCTGGA	1047	CLGN
7	V2HS_64314	CCCTCTATGATGCAACCTA	1072	CFL1
1	V2HS_113012	CGAACAAGCTCAGAAAGAA	1119	CHKA
1	V2HS_43989	GTGTGAACCCTGTTATCTA	1237	CCR8
1	V2HS_227008	CTGAGATTTCTGAAATGAA	1258	CNGB1
1	V2HS_150872	CCATGAAGCTCTTAGACAA	1374	CPT1A
1	V2HS_24094	CTCCCTATGTCCACCTTA	1605	DAG1

1	V2HS_151120	CCAAGTGAAGCATGGCATA	1635	DCTD
1	V2HS_95248	GTCCTTCCCTGGCTTGTTA	1718	DHCR24
2	V2HS_64794	CTAATTACTGGCTTCAATA	1783	DYNC1LI2
1	V2HS_227586	CATCTCATCCTCTGTAATA	1812	DRD1
10	V2HS_12037	CTCCAGAGGATGTTCAATA	1956	EGFR
2	V2HS_113654	CCTGATTTGTCAGGTAAGA	1967	EIF2B1
1	V2HS_17977	CAAGTTAAGAGAACTTTA	1993	ELAVL2
1	V2HS_151422	CTTTGAACTTGACCAAGTA	2028	ENPEP
1	V2HS_43353	CGATCATGATCATCACAGA	2041	EPHA1
1	V2HS_19871	GAATTGCACTCTTGGAAGA	2081	ERN1
1	V2HS_17544	GCCCATACCTGAGCTTACA	2103	ESRRB
1	V2HS_36498	CAAATGGTCTGGTTGTATA	2107	ETF1
1	V2HS_17547	CGTGGACTCTGAAACATAA	2110	ETFDH
1	V2HS_278084	CACTAGACCCTAGATTCTT	2220	FCN2
1	V2HS_262709	CAGTGACTACTGTGAGAGT	2237	FEN1
1	V2HS_263179	CTTTATAACTGACTTTATT	2247	FGF2
1	V2HS_17345	CGTTTAAATGACACACTTT	2262	GPC5
1	V2HS_113896	GCCGGAAGTTGTATGGTTA	2321	FLT1
2	V2HS_117615	CTTCTTGGCCTGGTTATAT	2519	FUCA2
1	V2HS_83216	GGACTTGGGAGATTCTAAA	2548	GAA
1	V2HS_160793	CTAATCGTCTGCTTCGAAT	2565	GABRG1
1	V2HS_42837	GAGAATAGCTTATGAGTTA	2620	GAS2
1	V2HS_246393	GAGTAAATTGTACAGTCAA	2620	GAS2
1	V2HS_72441	CAGTTAATCCTCTCATTTT	2691	GHRH
1	V2HS_173108	GCTTCATTGTGTATAGTTA	2764	GMFB
1	V2HS_21058	CTGACCATGTGAGGTTATC	2785	GNG3
1	V2HS_42239	ATCTTTAAATGAATTATTA	2824	GPM6B
1	V2HS_38354	CCCCTTCTTCCCCTCTA	2864	FFAR1
1	V2HS_38082	CCTTTAGCCTCATCAATGG	2925	GRPR
1	V2HS_35843	CGCACAGCTTCTTTGATGA	2931	GSK3A
1	V2HS_222494	GCAGATTTATCAACAGTCA	2974	GUCY1B2
1	V2HS_37947	GTGTCCGAGCTCATTACTA	3008	HIST1H1E
1	V2HS_37920	GACATTCTAATGCATATTT	3029	HAGH
1	V2HS_16783	GGCTATCAGTCCTCCCAGA	3068	HDGF

1	V2HS_84939	ATGTGTTATTTGTCACAAA	3084	NRG1
1	V2HS_239229	GGCACTACATAAATGTGAA	3174	HNF4G
1	V2HS_218617	GGCACTACATAAATGTGAA	3174	HNF4G
1	V2HS_30292	GCCAAGAATGCATATCATT	3267	AGFG1
1	V2HS_12569	CGTGCCATGACAAAGGATA	3312	HSPA8
1	V2HS_133292	CAAATACTTTGTCGGTATA	3467	IFNW1
2	V2HS_111503	GGAGACCTGTAATCATATA	3552	IL1A
1	V2HS_238059	GTTAAGAATTTGGTAAATT	3567	IL5
1	V2HS_48776	AGAACCATTCTACTTCAT	3607	FO XK2
1	V2HS_258435	GCTCCTTCGGCATGGTGTA	3643	INSR
1	V2HS_64703	CACCGTTTGAGATCTACTT	3664	IRF6
1	V2HS_188478	CGCCTAGAACCCAGTCTAA	3665	IRF7
1	V2HS_65055	CGACAGGCCTGCTGGGTAA	3770	KCNJ14
1	V2HS_133694	CTGCCCGAGTGCTACAAGA	3783	KCNN4
2	V2HS_133864	CAAGTACAAAGATAGTTAT	3904	LAIR2
1	V2HS_235059	CGGGATGACACTAGGATAA	4045	LSAMP
4	V2HS_134075	GACAGGACCGAGTCCCTAA	4046	LSP1
1	V2HS_256708	CCAAACTCTTGACTGAATA	4128	MAOA
1	V2HS_128339	CTCCTATACCCAATGACCT	4128	MAOA
1	V2HS_151557	CTAAGCAGGGCTTGTGAAT	4133	MAP2
9	V2HS_151656	CTGTCTATAAGAGAATTAT	4193	MDM2
1	V2HS_250765	CTGATGACATTCCAAATAT	4301	MLLT4
1	V2HS_131202	CAGTCTCCAGTCCCAATAA	4306	NR3C2
1	V2HS_239534	CAGTCTCCAGTCCCAATAA	4306	NR3C2
1	V2HS_151944	CTGGAAAGCATATACTATA	4478	MSN
1	V2HS_50290	CATTTAAAGTGACAAAAGT	4482	MSRA
122	V2HS_152026	CTCATCACACATATCTGTA	4599	MX1
83	V2HS_152028	CTGCCAGGCTTTGTGAATT	4599	MX1
15717	V2HS_152031	GACAAGATGTTCTTTCTAA	4600	MX2
1	V2HS_198044	CAGCCTCCCTTTGATGATT	4610	MYCL1
1	V2HS_20570	GCAATGTGGTCATCAATCT	4704	NDUFA9
1	V2HS_34048	CGAGTTACAGTATCAGCAA	4731	NDUFV3
2	V2HS_155453	CTAATGAGCTGTACAACAA	4838	NODAL
1	V2HS_63991	CACATCTTATGCTAACTGT	4878	NPPA

1	V2HS_152416	CAGAGCCTGTTTGTAGAAA	4947	OAZ2
1	V2HS_20237	CATTCTATATTCCAAGAAA	4958	OMD
1	V2HS_152707	CTGAGTGCTTTACCTTTAA	5105	PCK1
1	V2HS_221662	GTTTCAATGCATACAATAA	5176	SERPINF1
1	V2HS_169933	GATGGAAGCATTTCAAGAA	5236	PGM1
1	V2HS_97047	GAGACAAGATAGCAACGTT	5238	PGM3
1	V2HS_254293	CAGAAAGGCAGAAAGTTAA	5429	POLH
1	V2HS_239281	GATCAAGTGACATTGCTAA	5465	PPARA
1	V2HS_19644	GATCAAGTGACATTGCTAA	5465	PPARA
1	V2HS_239522	GCGGAGATCTCCAGTGATA	5468	PPARG
1	V2HS_239492	CTTAATGATGGGAGAAGAT	5468	PPARG
1	V2HS_203345	GCGGAGATCTCCAGTGATA	5468	PPARG
1	V2HS_47637	CGGCTACAAATAACCTATA	5521	PPP2R2B
1	V2HS_57694	GCCATACCCTTGATGAATT	5562	PRKAA1
1	V2HS_84652	GTCCTACCTTTGTATCTAA	5590	PRKCZ
1	V2HS_170489	GACCCAACATCCTTCATCA	5596	MAPK4
1	V2HS_275787	CTTCCAAATTTGAATGGAT	5602	MAPK10
1	V2HS_57318	CTCTCCCTCTGCATGTGTC	5630	PRPH
1	V2HS_19387	GATACATGATGCAGTTTAA	5649	RELN
1	V2HS_170731	GTTCTTACAACCGGTTTCA	5692	PSMB4
33	V2HS_173277	CCGGACAGGCTTCTTTCCA	5697	PYY
3	V2HS_57273	CAGTGAAAGTCCATCTATT	5783	PTPN13
1	V2HS_91546	CTAATTGACTCCACTGGAT	5795	PTPRJ
2	V2HS_197938	GTGCTTGGATCTGGAACAA	5891	RAGE
1	V2HS_171265	CCAATGGGAGGAAACGGAA	5919	RARRES2

1	V2HS_203362	CACAGTACTTGCTTGACAA	5924	RASGRF2
1	V2HS_171278	CGGATAAGACCGTAGCTTT	5931	RBBP7
1	V2HS_84878	AGACAATTATGACAACTGA	5935	RBM3
1	V2HS_32635	CGGATCATCTTGTAGCATG	6000	RGS7
1	V2HS_94799	CAATCTTAGCTTTGAAGAA	6013	RLN1
1	V2HS_56970	CCTGTGTTACAGCAATAA	6039	RNASE6
1	V2HS_32105	CACCATCTTGTGTACATCT	6119	RPA3
2	V2HS_233132	CATTCAGCCTCGTCTCTTT	6324	SCN1B
2	V2HS_261801	CAGTTCATTGAGTACTGTA	6334	SCN8A
1	V2HS_70582	CCTCTTCTATGCTTTGGAA	6357	CCL13
2	V2HS_173322	GAAGGTCACTCTGGAATAT	6389	SDHA
1	V2HS_56944	CTGATTAAATGTATGAGAA	6432	SFRS7
1	V2HS_93943	GCCTAGTGTTTCATCCTGTA	6530	SLC6A2
1	V2HS_56682	GCTCCCACGTCCAGGCTTA	6535	SLC6A8
1	V2HS_279379	GAATCACCATTGCAATACA	6580	SLC22A1
2	V2HS_153135	CATATTGGAGCAGAACATT	6594	SMARCA1
1	V2HS_153528	CCAATCCGTGGCTGATACT	6737	TRIM21
1	V2HS_33937	CCCAGAACTGCAATGTAT	6809	STX3
1	V2HS_56307	GTGCCTGTCAGACACTTAA	6940	ZNF354A
1	V2HS_243560	GACCAATCCTGAGACTTGT	7084	TK2
1	V2HS_171415	CTAAGAATCTGCTTAGTAA	7122	CLDN5
1	V2HS_275988	CTCTCCATAGAGGAGAGGA	7170	TPM3
1	V2HS_97879	CTACTTGAATGCTGTGGAA	7223	TRPC4
1	V2HS_171633	GTTCCCGACTTGAAATGAA	7273	TTN
1	V2HS_171665	GATTTAAAGGTCCTAGGAA	7289	TULP3
2	V2HS_171910	GGTTTATAATTGGGATGTA	7411	VBP1

1	V2HS_70167	GCCATAGCAAGATTGCTTA	7412	VCAM1
1	V2HS_26383	CTGGTAAGAATGTTTGAAA	7442	TRPV1
1	V2HS_171974	CAGATTACACTGGATGAAT	7447	VSNL1
1	V2HS_219592	CTCCTGATTATTCAGAATA	7528	YY1
1	V2HS_223424	CTCTTTCTTCCTCCTCTAT	7534	YWHAZ
1	V2HS_275414	CATCTTAACTCTACCAGCT	7693	ZNF134
1	V2HS_253983	CATACAAGATGCATTCCCA	7748	ZNF195
1	V2HS_179763	CCTGCTCCATCGGCACTAA	7760	ZNF213
1	V2HS_172375	CTAATTATAGGAATGCCTA	7803	PTP4A1
3	V2HS_209244	CCCTACTTCTCCGTTACTA	7980	TFPI2
2	V2HS_239520	CAATTATAGTTGCTTTGTA	8013	NR4A3
1	V2HS_172476	CTCCAAGTCTGTACCTCAA	8091	HMGA2
1	V2HS_21905	ACCACTGTCATTAGATTAA	8195	MKKS
1	V2HS_41535	GAAGCTCACGTGGACCAGA	8209	C21orf33
1	V2HS_246612	CAACTCTGCCCTTAGGTAT	8314	BAP1
1	V2HS_33954	CATCATAAGGCCAAGGGAA	8335	HIST1H2AB
1	V2HS_33402	CAGCATCTCGACTTCCCAA	8367	HIST1H4E
1	V2HS_284652	CAAATTTTCGAGTTAAAGTA	8406	SRPX
1	V2HS_33057	CCCTTTGCGTTATATTGTA	8408	ULK1
1	V2HS_262017	GTGTCAAAGACATTCCTA	8445	DYRK2
1	V2HS_32297	CACTATAAATGGCATCAAA	8462	KLF11
2	V2HS_240795	CTAGAAATGTCCTTAATAA	8509	NDST2
1	V2HS_239890	CTTTAATCACCTCTTTGCA	8528	DDO
1	V2HS_27346	CTTATTGTTCCGAATGTAA	8546	AP3B1
1	V2HS_185075	CGAGAAGATTGCTCATATA	8570	KHSRP
1	V2HS_178847	CAGTAGAATTTCTACTAAT	8632	DNAH17
2	V2HS_240695	CCTTCAATTTAGAAGAGAT	8635	RNASET2
1	V2HS_17163	GTAATACGTGAGGAATTTA	8744	TNFSF9
4	V2HS_16878	CACGTTTACTTATGTATAA	8780	RIOK3

3	V2HS_53220	GCCGTATGATTTTCAGTAGA	8834	TMEM11
1	V2HS_100766	CACCTGAAGCTCTTCAAGA	8844	KSR1
1	V2HS_100765	CAGTTTATCTTTCCAGACA	8844	KSR1
1	V2HS_203077	CACCTGAAGCTCTTCAAGA	8844	KSR1
1	V2HS_53091	CAAGTTTATTTCTATCTAT	8850	KAT2B
1	V2HS_52981	CAGGTACTIONACTCTGGTTT	8858	PROZ
1	V2HS_52984	GGTACTCACTIONCTGGTTTAA	8858	PROZ
1	V2HS_33545	GTGTTCACTIONCATAGCAGA	8973	CHRNA6
2	V2HS_47077	CTGTGTACTIONTCACTGGAAA	8975	USP13
2	V2HS_41402	GCAGGAGAAAGAGTTCAAAA	9022	CLIC3
1	V2HS_46777	CTCAGCAGATTACCACATT	9038	TAAR5
1	V2HS_37166	GCTCTGCAGTGACTACGTT	9133	CCNB2
1	V2HS_17815	GAAAGAATATGAACTGAGA	9202	ZMYM4
1	V2HS_36236	GATCTCATAGTGGAAGTTA	9223	MAGI1
1	V2HS_36251	GCAGCTGTCTCATATACAA	9228	DLGAP2
1	V2HS_71511	CACAGGAAGTGTTCAAGTAA	9240	PNMA1
1	V2HS_36178	GATTTCTCTTTCAAGCCA	9244	CRLF1
2	V2HS_262199	CAGTGGATGGGATGCATAA	9319	TRIP13
1	V2HS_118852	GTCAAATGTGGCAGTAGCA	9366	RAB9P1
1	V2HS_35328	CTTCTAATCTTAAACTTCA	9372	ZFYVE9
1	V2HS_35331	GTCAGAATGCTACTCAAAT	9372	ZFYVE9
1	V2HS_68945	CAATCAATAATGAAGGAAA	9391	CIAO1
1	V2HS_59255	CAGTCCAATTTCAAGAATA	9573	GDF3
1	V2HS_67332	GCTACAAACTGTTTGAGGA	9577	BRE
1	V2HS_62928	GAAACAATATGAAGCATAT	9589	WTAP
1	V2HS_248374	CCAACATATTAAGGATAA	9601	PDIA4
1	V2HS_27545	GAGAGCAAAGCCTTATTTA	9630	GNA14
1	V2HS_60346	GTCTAATTGTTGTTTCATA	9655	SOCS5
1	V2HS_79414	GGGCTATTTCCATCGGTAT	9672	SDC3
14	V2HS_257969	CCTTATTTGACTTCAAAGA	9753	ZSCAN12
6	V2HS_95200	GAATCGGCATTCCAAGTCA	9791	PTDSS1
1	V2HS_95318	CTGAATTATTGGCAGTATA	9815	GIT2
1	V2HS_95601	CACAGCTATGGAGAAATCA	9883	POM121

1	V2HS_49608	CTCACTTCCTGCTAGCTCA	9937	DCLRE1A
1	V2HS_68818	CAAGTTCACTCTGTTAGTT	10001	MED6
1	V2HS_29085	GTTATTGAACGACTCATT	10059	DNM1L
2	V2HS_81171	CTCCTATGAACCCAACCAA	10097	ACTR2
1	V2HS_64102	CTGTGTGTCGGGCTTATTA	10113	PREB
2	V2HS_196634	ACCAGTTCGTTATATTAGA	10152	ABI2
1	V2HS_250342	GCTTGAATATTATCCCTGT	10175	CNIH
1	V2HS_250172	CTGTGTCTGTAGAAGGTTA	10239	AP3S2
1	V2HS_196625	CAAGTTTCCCTCACTGTAA	10261	IGSF6
1	V2HS_209917	GGGAACAAATGAGACACAT	10280	OPRS1
1	V2HS_70447	GAGGTTCAACCTTACTGAA	10333	TLR6
1	V2HS_248106	GTCTGGATCAATATATAAT	10367	CBARA1
1	V2HS_65589	CAGCTTAATTTCTGTGAAT	10402	ST3GAL6
1	V2HS_65377	GAGAATATGCAATTAGCAT	10451	VAV3
2	V2HS_198872	CCTGGAGAATGACAACCTC	10467	ZNHIT1
1	V2HS_199108	GCTAACAAAGTTCATCACCA	10481	HOXB13
1	V2HS_198718	CTTCTACTGCCTACTTTAA	10562	OLFM4
3	V2HS_181700	CAGTTTCTTTTCTTTGGAT	10642	IGF2BP1
35	V2HS_202193	GGACCAGCTTTATCTGGAA	10714	POLD3
1	V2HS_47915	CTTGACTTTGGTTTCTTTA	10740	RFPL1S
1	V2HS_90530	CTGAAGAACGCCTGGAACA	10867	TSPAN9
178	V2HS_84222	CCAATATTGTGTTTGGATA	10892	MALT1
1	V2HS_258384	CTGATGATCCCAACTCAGA	10959	TMED2
1	V2HS_213752	ATATTTGACTATTAAAGGA	10973	ASCC3
1	V2HS_77730	CTCCACACCTGGTCTGGGA	11025	LILRB3
2	V2HS_77677	CTCCCAGCTCTGTTCTTAA	11035	RIPK3
1	V2HS_20746	CTGTGTATCTGATGACCAA	11043	MID2
1	V2HS_253462	CACCATCTCCTATCTCAGA	11075	STMN2
1	V2HS_284301	GGAAATAACTAATTAACA	11105	PRDM7
1	V2HS_261877	CACCTAGGACCCAGGAAAT	11142	PKIG

1	V2HS_196645	CATATTAAGTGGTTAATTA	11143	MYST2
1	V2HS_250790	GAGACAAATCACACAGAAT	11190	CEP250
1	V2HS_84980	CACATGAGGAGAAAGGTTT	11214	AKAP13
2	V2HS_12645	GACCTCATTGCAATCTTA	11236	RNF139
1	V2HS_198518	CATCCGATTTCTTCACTAA	11261	CHP
1	V2HS_53788	CTCATTAAGTAAATGGAAT	11280	SCN11A
23	V2HS_198493	GGGACTCTATTTATTCTGA	11322	TMC6
1	V2HS_203428	CTAACACCCTCTATCGGAT	23016	EXOSC7
1	V2HS_194798	GTCTGATGTTCAAGACATT	23162	MAPK8IP3
1	V2HS_29029	GAATTTATGTCTTACCACA	23208	SYT11
1	V2HS_263004	CCTCTACAATATTGACTAT	23219	FBXO28
1	V2HS_254695	GGCTTCAGTTATTTAAAGT	23219	FBXO28
1	V2HS_202888	CTCACATAATCAAACCTTA	23235	SNF1LK2
1	V2HS_100836	CTCACATAATCAAACCTTA	23235	SNF1LK2
1	V2HS_249221	GTGTTACAGTTACACTAA	23250	ATP11A
2	V2HS_28133	CATTTTCATCTTCTTTCATA	23251	KIAA1024
1	V2HS_51187	CCAATTATCTGTTTGAAT	23291	FBXW11
1	V2HS_51416	CACACTCATGCGTCATTCA	23324	MAN2B2
1	V2HS_60730	GATGTGCATTGTTTCTTGT	23326	USP22
1	V2HS_80461	CCTCTGTAATGAGGATCAT	23327	NEDD4L
1	V2HS_80186	CGAACAAAGCCAGAAGAGA	23336	SYNM
1	V2HS_178541	GGTAGAGTGATCTGAATTA	23367	LARP1
1	V2HS_28646	CTGAGGAACGTATTGAAA	23456	ABCB10
1	V2HS_261985	GGCCCTATGTGGGCATTCA	23509	POFUT1
1	V2HS_50370	ATCTGTAGTCCATGAGTTA	23531	MMD
1	V2HS_244561	GCTAGGTTTAAAGCATTCA	23538	OR52A1
1	V2HS_213902	CTTGGTGTCTGGGTATATA	23588	KLHDC2
2	V2HS_43517	GTCTTACTCCATTAATCA	23626	SPO11
2	V2HS_15306	GACAAGATTCCTTATATAA	23637	RABGAP1
6	V2HS_260346	CTTTCTATTTGTTAAGTAT	23677	SH3BP4

1	V2HS_168896	CATGTTCTGTTTCAGGAGTA	23760	PITPNB
1	V2HS_116697	CAGAGATACACAGTCGGAA	23762	OSBP2
1	V2HS_70568	CTGTTTGTATTGAGACTGA	23767	FLRT3
1	V2HS_34364	CTCTCCTGATGCATTTGTA	25791	NGEF
1	V2HS_200727	GCCACTATCACATATGCTT	25825	BACE2
1	V2HS_72903	GTGTACATGCTTCAAGAGA	25855	BRMS1
1	V2HS_96523	GTTCTTCATCCATTAGTTA	25897	RNF19A
1	V2HS_96539	CGTGGTGCAATAAGGAACT	25901	CCDC28A
1	V2HS_222249	GAATGAAAGTGCCAAAGAA	25960	GPR124
1	V2HS_96810	GAAATAACATCTTCAAGAA	25996	REXO2
1	V2HS_130208	CATTATTTGGTGCTCCTAA	26043	UBXN7
1	V2HS_43090	CAACCCAGGTCATATCTAA	26277	TINF2
3	V2HS_137577	CTCATTTACACCTTAAGAA	26692	OR2W1
1	V2HS_203342	CCTTCAAATTAATACTGAA	27039	PKD2L2
1	V2HS_207130	GTATCTACATGGATACATT	27069	GHITM
2	V2HS_197616	CTACTGATGTTATGAAACA	27130	INVS
1	V2HS_252634	CTATGTGCGCAAGTTTGTA	27243	CHMP2A
1	V2HS_207077	CGACTGATCTTCTTCCTAA	27284	SULT1B1
1	V2HS_197059	CGAGGTATTTTCAGTTACTA	27297	CRCP
1	V2HS_208097	GAAATACATTGATCTCTAT	27299	ADAMDEC1
1	V2HS_65030	CAAAGTACCTGTTCAGAAA	28568	TRBV19
2	V2HS_60266	CACTGAGAGTGGATCCGAA	28957	MRPS28
1	V2HS_281542	GCTTTCCATTATAATATA	28984	C13orf15
1	V2HS_195026	CTGTTATAAGGATTGCTAA	28994	PRO0471
3	V2HS_53474	AGCAGTTGTTGTTACCAGA	29063	ZCCHC4
1	V2HS_53401	CTGACTCACGGTGTTATGA	29072	SETD2
1	V2HS_53336	AAGTCTGTCCTTATGGGAA	29075	HSPC072

2	V2HS_199249	ACCAGAGTCAGGACAAGAA	29085	PHPT1
1	V2HS_71587	CGTGCACCCAGGCTCTCTA	29109	FHOD1
1	V2HS_71123	GCATCGTGCTCTTGTTTAA	29116	MYLIP
1	V2HS_180228	CACCCTCTTAAATTCCTAA	29799	YPEL1
8	V2HS_86338	GACTCCATGGGTGGGATAA	29843	SENPI
1	V2HS_249571	GAGACCATTGGGAAATAAT	29890	RBM15B
1	V2HS_66009	GGGAGAACATCTGATGTTA	29902	C12orf24
1	V2HS_65479	GGCTTTCTCTGAATTGGTA	29920	PYCR2
1	V2HS_58511	GGTGATTTCTTATTATTAT	29967	LRP12
2	V2HS_116704	CATCTTCAGACATCTCACA	29982	NRBF2
1	V2HS_58157	GCTTTGATGTGAATATTGA	29994	BAZ2B
1	V2HS_57250	GTGGCAGGATTTAAATTCA	30844	EHD4
1	V2HS_59242	CAATTTATCCCTGAGATGA	50617	ATP6V0A4
1	V2HS_115563	CAGATACCCTGAAGCATAA	50805	IRX4
1	V2HS_114879	GGAAGATCTTCCATACCAT	51046	ST8SIA3
1	V2HS_134295	CATATCCCGAATTAGGTAA	51059	FAM135B
1	V2HS_97188	CTCATCTTTAGTTTCAGAT	51110	LACTB2
1	V2HS_134769	CTGCAAATCTCAGTTCAAA	51265	CDKL3
1	V2HS_135128	CCACATGTGTCAGACCATA	51298	THEG
1	V2HS_135174	GTACCTGTGTCTTATCTAT	51304	ZDHHC3
1	V2HS_135350	CATATCAGGACATTGGTTA	51324	SPG21
1	V2HS_135403	CCGTGTTAGTGAATAAAGT	51333	ZNF771
1	V2HS_254665	GGGTACTACACTTTATCTT	51341	ZBTB7A
1	V2HS_114812	CTTCTTCTGATCATTTCAA	51352	WIT1
3	V2HS_134241	CATTCGTACTIONTAGGATTCA	51365	PLA1A
1	V2HS_235522	CAGAAACTAAATTAACGAA	51540	SCLY
1	V2HS_237510	GAGTCAGGCTCATGTACAA	51617	HMP19

1	V2HS_97082	GAAAGACTACTATTATTCT	51626	DYNC2LI1
1	V2HS_97697	CTACTTACTTGAGATTCAT	51668	HSPB11
1	V2HS_37666	GAATTTGCATCTTAATTCA	54073	C21orf41
1	V2HS_247253	CCTGTTGTGGCTTACCTAA	54101	RIPK4
1	V2HS_19934	GATAATCTCTGTGTCTTAT	54629	FAM63B
1	V2HS_219708	CACTTGATATGAAACAAAT	54664	TMEM106B
1	V2HS_154959	CTCTATAAATGCTCTGGAT	54798	DCHS2
1	V2HS_155059	GGGAAATCTCCAAGTCAGA	54814	QPCTL
1	V2HS_138350	CAGGTGAGAGCTGGAGAAT	54856	GON4L
1	V2HS_173472	CACAAATCATGCTTGTTAA	54863	C9orf167
1	V2HS_228678	CGTCTTGGGATCACAATGA	54865	GPATCH4
1	V2HS_173612	CCAGGAAGAAGGTTCTCAA	54884	RETSAT
2	V2HS_219003	CCATCCCTACTAATAGGAT	54884	RETSAT
1	V2HS_173630	CCTTATATGCCACGATGTA	54886	RP11-35N6.1
1	V2HS_173729	CACAGTAGTCTAAGATTAT	54900	LAX1
1	V2HS_173728	CTGAGATGAGCAGTGA ACT	54900	LAX1
4	V2HS_174183	CACATGAGGAGATGTCAGT	54964	C1orf56
1	V2HS_174321	GGCTGTCTGCTTTATGAAA	54986	ULK4
1	V2HS_219851	CCTAGAAGAAGATTACGTA	55023	PHIP
1	V2HS_174636	CTGCTCAAAGGATACCAAA	55030	FBXO34
6	V2HS_174640	CTTATAAGATGATGGATTT	55031	USP47
1	V2HS_174706	CAAATACACTTTGGAGATA	55039	TRMT12
1	V2HS_155504	CTTCTGTGAATACAGGTTA	55122	AKIRIN2
1	V2HS_155888	GTCAACATGTGCAGCATCA	55165	CEP55
1	V2HS_155987	GACTCTTACAGCAATAATA	55180	LINS1
1	V2HS_156023	CAGTTATTGTGGAGTCATT	55188	RIC8B
1	V2HS_156036	GTGTTTCAGAAATATTTCA	55190	NUDT11

1	V2HS_175209	GCTGCATGAACGTTTCGAA	55326	AGPAT5
2	V2HS_175261	CACAGAAATCAATGGTGAT	55332	DRAM
1	V2HS_175324	GTCTGATGGTCTTTGTTA	55339	WDR33
1	V2HS_175391	GTTCTAAAGTCAGTAAGAA	55347	ABHD10
1	V2HS_176062	CCGATACTCGGGAGAAGAA	55421	C17orf85
1	V2HS_190919	GCAGAAATAGCCATTACAA	55478	PRO2012
1	V2HS_26658	CAGAGCTGATGTTCCGTAA	55526	DHTKD1
1	V2HS_256074	CCACTCCTCTGTGTCTCTA	55588	MED29
1	V2HS_50579	CTGCTAAGATTTAAGTACA	55592	GOLGA2L1
1	V2HS_154917	CACTGATTCTAGACAACAA	55602	CDKN2AIP
1	V2HS_155164	CTCTCAATTAGGAAACATA	55613	MTMR8
1	V2HS_156763	CTTAAATGACCATGAAGAA	55768	NGLY1
1	V2HS_277783	CCTCTCCTTGCCATCATGA	55793	FAM63A
1	V2HS_221836	CTGATTGTTTAATTAGAAT	55847	CISD1
1	V2HS_175814	CAAACATAATTTGGGATTTA	55858	TMEM165
1	V2HS_241995	CTGAACATAGTGTGGATAT	55907	CMAS
1	V2HS_154549	CTTGTTATGCCTCAAGCAA	55922	NKRF
1	V2HS_253305	CAGTTGAGTTTCACTATGA	56000	NXF3
1	V2HS_195963	CAGAGAAGAATTTATTCAA	56062	KLHL4
1	V2HS_21847	GACTTGGTGTCTGAATATC	56604	TUBB4Q
1	V2HS_52431	GAGATATTTCCACAGGATA	56832	IFNK
2	V2HS_51574	CACAGATTAGATTATTCTA	56918	C2orf83
1	V2HS_51357	GACATCTTGTCATTTGAGG	56931	DUS3L
1	V2HS_45254	ACTAAGATCTCTTCTAACA	56988	C8orf17
1	V2HS_39942	CGAATGGAGCTGTTAGAAA	57035	C1orf63
1	V2HS_35766	GTGTTCTTATAGTTATTTA	57150	C6orf162
1	V2HS_58527	CTGAGAAACTGTAGCATAT	57338	JPH3
2	V2HS_53708	GTGTTATTGTGCCTTTGGA	57461	ISY1
1	V2HS_46786	CTCTGGATTTAGAGATATA	57475	PLEKHH1
1	V2HS_210033	ACAATGCATTCCTTCTTAT	57537	SORCS2
1	V2HS_217697	GGCCTTGGCTACTACCAGT	57561	ARRDC3
1	V2HS_225003	CAATGTATGCTTTAGAAGT	57567	ZNF319
1	V2HS_204676	GACATGGAAGAAGTTTGTA	57577	KIAA1407
1	V2HS_253197	CATCTGCACAGAGAAGATA	57620	STIM2
1	V2HS_200672	CAACTGGTGTCTACATGA	57699	CPNE5

2	V2HS_57109	GTGTTGACAATATATTGAT	57709	SLC7A14
1	V2HS_66078	GAACATAGGTCTTGGAATA	57817	HAMP
3	V2HS_277241	CAAGAAATATGAGGTATAT	58491	ZNF71
1	V2HS_81245	GAGTTACTGTTTGACAAGT	60412	EXOC4
1	V2HS_80856	GTGCATTACTTTGAATCAA	60489	APOBEC3G
1	V2HS_206581	GGTATCTTGATGTATATAT	64089	SNX16
1	V2HS_217747	CAAGCAATGTTATATTCAT	64092	SAMSN1
1	V2HS_212116	CTAAGGAATCCATCGGACA	64096	GFRA4
1	V2HS_200756	CACAGGACTATTTGGATAA	64105	CENPK
1	V2HS_257282	GTCATCACGGAATACCAGA	64221	ROBO3
1	V2HS_235752	CAGATCCTGTTTGGAATTA	64232	MS4A5
1	V2HS_158584	CACTTACTGATGTGAAGAA	64518	TEKT3
1	V2HS_98457	CAGCTGAGCTCCAAGGAGA	64787	EPS8L2
1	V2HS_111347	GGGATACTCTGGCAAGACT	64839	FBXL17
1	V2HS_202913	GGAGCCAAGCCTTACAGAT	64857	PLEKHG2
1	V2HS_78473	GGGTCTACATGTTTCCAAT	64857	PLEKHG2
2	V2HS_82196	GTCTTTATATCTGACTGTA	65264	UBE2Z
3	V2HS_238923	GTCTGAGAGAGTGACCTAT	65268	WNK2
1	V2HS_237729	CTCACACCAGAAATATTAT	65989	DLK2
1	V2HS_230623	CGTGTTACATCTCCATTTA	79019	CENPM
1	V2HS_230793	CCCATCAATATTCATTTCA	79038	ZFYVE21
1	V2HS_99003	CTATTCATCATTTC AATT	79072	FASTKD3
1	V2HS_225356	GGCCTTGAGTGTCCACATT	79095	C9orf16
1	V2HS_19490	GAACTTGCTAAATCATAT	79192	IRX1
1	V2HS_160034	CAGATTCAAAGAAGCATT A	79339	OR51B4
1	V2HS_136158	CTAAGGAATAGTGAGTTTA	79571	GCC1
1	V2HS_136349	CTCAGCACGTGTATTGAAA	79616	CCNJL

1	V2HS_157109	CTGGTCACCTGTGTGACAT	79714	CCDC51
1	V2HS_261980	CGCATTGCAGACATTCCAA	79753	SNIP1
1	V2HS_187951	GGAAACCAACTACGATGTA	79799	UGT2A3
1	V2HS_157641	GAAACTTGGTGATCAGTAT	79815	NPAL2
1	V2HS_157884	CATGCAGAAGAGAGATTAA	79860	FLJ21369
1	V2HS_158021	CTCAGTGAAGAATTGGAGT	79884	MAP9
1	V2HS_158122	CGTAAGGATGTAGATGTTT	79902	NUP85
1	V2HS_176763	CAAAGATGCTGCTCTTAGT	79963	ABCA11P
1	V2HS_176767	CTGATGAGCTTGCTCCACT	79966	SCD5
1	V2HS_218489	CATAATAGTGACATTACAA	79972	FLJ14327
1	V2HS_278078	GCAATTCATAGTATATTTA	79991	OBFC1
1	V2HS_270074	CACCTGTCTTTGTCTCAGT	80003	PCNXL2
1	V2HS_176950	CAAGCAAGGAGAAATCGAT	80008	TMEM156
1	V2HS_176969	CATAACTGATGCTCTGTGA	80011	NIP30
1	V2HS_218218	GCCGTGACCCTGATAATAA	80023	NRSN2
1	V2HS_219702	CAGCATGTATATAACATAA	80042	FLJ12078
10	V2HS_136866	CTCCTTTACAGCTGCAGAT	80148	PQLC1
1	V2HS_237511	CTTAGGCAATGTTACAATT	80215	C21orf96
1	V2HS_274775	CTCATGACAAGTACATGTT	80218	NAT13
1	V2HS_236360	CGTGGAGATTCAAGTGGAA	80233	C17orf70
1	V2HS_137283	CAGAGATTAAGAAGTTGAA	80254	CEP63
1	V2HS_99057	CATGAGTAATGCTATCGTT	80765	STARD5
1	V2HS_116681	CAAGCAATAAGATTTATGT	81542	TXNDC1
1	V2HS_137633	CTGGAAAGCTTACTGTGAA	81608	FIP1L1
1	V2HS_116132	GTCAGACAGCCATCAGAAT	81631	MAP1LC3B
1	V2HS_194333	GCTATACATCTAGAATATA	81871	KRTAP4-6
1	V2HS_68508	CAAGAATGATCTTTATCCA	83540	NUF2
1	V2HS_168732	GCTCAAGAGAGCTGTAGAA	83598	LUZPP1
1	V2HS_158666	GCCATTAATACAGAGAAGA	83853	ROPN1L
1	V2HS_263859	CAGTTAATTATTCAGTTAT	84002	B3GNT5
1	V2HS_138100	CTGATCATGGCCCTGATGA	84197	SGK196
1	V2HS_138148	CCAATCCAACTTTAAGGA	84210	ANKRD20A1
2	V2HS_138215	CTGAGAAAGTACAGGAATT	84224	NBPF3
1	V2HS_138436	CATGTTTGCTGATTATAGA	84267	C9orf64

1	V2HS_138449	GA CTG TCA CTAC CT TAAGA	84269	CHCHD5
1	V2HS_237384	CTCGTAAACTTTATAAACA	84280	BTBD10
1	V2HS_138514	CAGTTAATGTCAAAGAGGA	84281	MGC13057
1	V2HS_272320	GGACTTAAACCTTACCTCT	84285	EIF1AD
1	V2HS_138784	GAGCAACTAAGTGAATTTA	84328	LZIC
1	V2HS_159055	GGAGATGTATCAACAGAAT	84539	MCHR2
2	V2HS_159305	CAGCTTTGCTAGAGAATGA	84654	SPZ1
1	V2HS_177395	GGATGATCTCTGTCTTTAT	84843	MGC15705
1	V2HS_177667	CTTTATTCCTCAAGGCAAT	84899	TMTC4
2	V2HS_177987	CATTATCGATCATGTCTAT	84959	UBASH3B
1	V2HS_276463	GGCACCATAACAAGCTCTAT	84975	MFSD5
1	V2HS_178077	GCCTACATTGTATGTATAA	84976	DISP1
8	V2HS_35865	CACTTCCAGGACTTTGATA	85452	KIAA1751
1	V2HS_70495	GCCTATCCGTGTATATGGA	89781	HPS4
1	V2HS_275690	GTTTCTACCTCACCGTGTA	89845	ABCC10
1	V2HS_178411	CCAAGGATGAAGAACTCA	89886	SLAMF9
1	V2HS_160243	GACCATCCATTAAAGACAA	90134	KCNH7
1	V2HS_268641	CTGGGCTTCTTTCTCTCAT	90416	C15orf57
1	V2HS_250202	GACCAAGAAGGAACTATCT	90987	ZNF251
1	V2HS_47739	CTGGGATTCAGCTTCTTCT	91319	DERL3
1	V2HS_244021	GTTCAACGATGTAAAGATA	91937	TIMD4
1	V2HS_31515	GA CT TGTATGTTGACTTCAA	92293	TMEM132C
1	V2HS_48070	CCTATGTATTTCTGTCTAT	93145	OLFM2
1	V2HS_159641	CATGCATACTGTATGCCAT	94015	TTYH2
1	V2HS_21997	CCCGGAATAGCTCAGCAAA	94236	HEJ1
1	V2HS_69795	ACCATAATGATCGAATCTT	113444	C1orf212
8	V2HS_118395	CAATGAGTCATTTATAACA	113540	CMTM1
1	V2HS_67425	AAGTGAAAGCCAAAGCTAA	114548	NLRP3
2	V2HS_75193	GAGATATTTCCACACAATA	114786	XKR4
1	V2HS_100984	GGCAACACGTCAACATTCT	115992	RNF166
1	V2HS_92084	CCTACCAAATTGTATTTGT	116442	RAB39B

1	V2HS_19886	CACTTAAGAGAGTTTGTTA	118924	C10orf4
1	V2HS_161524	CTGGTTATGGATATTCATA	120526	DNAJC24
1	V2HS_161523	GCTGGTTATGGATATTCAT	120526	DNAJC24
2	V2HS_216584	CTGCCTAAATTGTTTATAA	122553	TRAPPC6B
1	V2HS_195102	CCTAGTAGAGGAAAGCTTA	123228	SENP8
1	V2HS_180090	CGAAAGAGTTGTGTTTAAA	123720	WHDC1
1	V2HS_19472	CTGAGGTTATCCAAGGAGA	123970	C16orf78
1	V2HS_87088	GAGTCGTTCTGAGATTTA	126321	C19orf28
2	V2HS_18852	GAATTATTTGGCCTTCAGT	127495	LRRC39
1	V2HS_18774	GAATCAAAGTGTTCTCTCA	127731	VWA5B1
1	V2HS_241242	GCTTCACTGTCTCTCATT	128061	C1orf131
1	V2HS_73619	ACTTGAATTTGACCTTGAA	128861	C20orf71
1	V2HS_18108	GAATTTATGAAAGGGTTAA	133690	CAPSL
1	V2HS_70452	CTACATATGTGGTAAGGAA	133923	ZNF474
3	V2HS_19177	CAGATGATGTTAATCCAGT	135154	C6orf57
1	V2HS_21161	GTCCCTAATCGGAAGGTGA	138009	WDR21C
1	V2HS_29175	CCTTTGTAGTGTTGTTTAT	138065	RNF183
1	V2HS_29179	CTGACTAGCCCAAATAGCA	138065	RNF183
1	V2HS_180404	CATAGCAATTGGTCATTAT	139212	CXorf41
2	V2HS_245699	GATGAGTACTGGACCTTGA	140576	S100A16
1	V2HS_71756	CATATTTAGTTGTACAGTA	140707	BRI3BP
1	V2HS_70416	GAAGGACATTGCTGCCTAT	140735	DYNLL2
1	V2HS_29354	GTGGTTCTTCATTAAGTAA	140775	SMCR8
1	V2HS_73242	GTCACCAAAGGTATCAATA	140836	BANF2
1	V2HS_20946	CTGAGTACATCAATGGATA	143162	FRMPD2
1	V2HS_205676	GAGGCAGACCTATGACCAA	144097	C11orf84
1	V2HS_216455	CTGGTAATGATTTAAATGT	144363	LYRM5
1	V2HS_20353	AAGAGAAGAATATGGCTAA	145508	C14orf145
1	V2HS_250892	CTGGGCATCTTCTACTAAA	145873	MESP2
1	V2HS_31953	CTGATTACCTCTTGCTAAT	145899	LOC145899
1	V2HS_162690	CAACCCATCTATTTCCAGA	146850	PIK3R6
1	V2HS_181271	GCTTCACTTTCATTCTGTA	147381	CBLN2
6	V2HS_100172	CACCCATGTTTCATTCTGAA	147409	DSG4
1	V2HS_22838	GCCACTTCATCTTCCATGA	147495	APCDD1
1	V2HS_88057	CTCTTCATAGTACAGTCAA	148418	SAMD13

1	V2HS_101498	CTCTGAAACCATGCCTTAA	149157	LOC149157
1	V2HS_51382	CTCTGGATAGCATTACTTC	150280	HORMAD2
2	V2HS_45550	CGTCTACAGTCTGAAATAA	153241	CEP120
1	V2HS_100554	CCTGAGATTTGTGTTGTCA	153571	C5orf38
2	V2HS_37526	CGGGCTAGGTTGAAAGGAA	154860	LOC154860
1	V2HS_119984	GAGAAACAGAGATTCTAAA	155054	ZNF425
1	V2HS_140402	CACCTACAAATATTCCTTA	157740	LOC157740
1	V2HS_22601	CCATGTAAGATGTATAAGA	157753	TMEM74
1	V2HS_217748	CTGCTGTAGAGTTACTATA	159989	CCDC67
3	V2HS_49229	CCCTCAACCAGCTACTAGA	160492	IFLTD1
1	V2HS_36102	ATGAGAATCGATCATTAAT	160857	CCDC122
1	V2HS_18607	ACTATATGTTTCGCTTTATA	161003	STOML3
1	V2HS_66763	CACTCTAAAGCTACAACCA	162417	NAGS
2	V2HS_44743	GAATTGAAGTGTATTTATA	163051	ZNF709
1	V2HS_44617	CGGAGGAACTCTGTTAGAA	163882	C1orf71
1	V2HS_67431	GCTCTCTTAATATTTCTTA	164781	WDR69
1	V2HS_35964	CTATACAATTGGAAACAGA	168090	C6orf118
2	V2HS_86298	GAGGTCTTGATCTTAATGA	168400	DDX53
1	V2HS_33382	GACCCAATCTGCACATTCA	169026	SLC30A8
1	V2HS_59127	GGGACAATCCTTTCATTAA	170572	HTR3C
1	V2HS_216756	CAGTTGCTGTAATGACAGA	170712	COX7B2
1	V2HS_191402	CGCAAGTGTAGATGAGTAT	170959	ZNF431
1	V2HS_229211	GGCTCCTCACCCAGGATGA	200205	C1orf69
1	V2HS_270462	CATCCTTGGTTTACTCA	200504	GKN2
2	V2HS_286526	CTCTCCTCCCTGGGACAAT	201254	STRA13
1	V2HS_43217	AGCTGTTTATGTGATAGCA	201780	SLC10A4
1	V2HS_59679	CTCCACTCTTTAGTTTATA	202333	CMYA5
1	V2HS_64947	CAGATTCAGCAGTGGATTA	219402	MTIF3
1	V2HS_100048	GCCAGATATTCCAGCTGCA	219699	UNC5B
1	V2HS_29882	GTATGTATTCCACATTA	219833	C11orf45
1	V2HS_29850	CTGTCATACTCCAATGTAA	219970	GLYATL2
2	V2HS_83574	ACACCAAGGCCATTTCAAT	220032	GDPD4
1	V2HS_51047	CCCATCATTTGGAAAGGGA	220070	C11orf76
1	V2HS_38511	ACATGGACCTTATGTAATT	221718	C6orf218
1	V2HS_57804	CTCATGATGCTGGATATGA	245802	MS4A6E
1	V2HS_168697	GAAACATTCTCAACAGGGA	246126	CYorf15A
1	V2HS_191825	CTTACCTTAGCATAACATGT	253128	C12orf33

1	V2HS_38014	GGGAGTATCAAATATGAGA	253260	RICTOR
2	V2HS_245169	CTACTTTGGTGCTAAATAA	255394	TCP11L2
1	V2HS_34851	CTGAGAAATGAAGAGGTAA	256144	OR4C3
1	V2HS_244266	CTCGTTTGCTGGCAAATCA	256536	TCERG1L
1	V2HS_37347	CTCCTTCAATCTAGAGGAA	257397	MAP3K7IP3
1	V2HS_207032	CATTTACTTTGTGTCTGTA	259291	TAS2R45
1	V2HS_210046	CATTTACTTTCTGTATCTA	259294	TAS2R48
1	V2HS_182357	CATCCCTCACTCTTACTTA	283089	LOC283089
1	V2HS_218984	CATGTTTGTTAAACAAAGA	283237	TTC9C
1	V2HS_178679	GGATTGTGAAGCTTTATAA	283310	C12orf64
1	V2HS_215077	CACTTTATTTATAGGCTCT	283383	GPR133
1	V2HS_142608	GCACCTACAATGTTCAAGA	283440	LOC283440
1	V2HS_163790	GAGTCCCTGGGAAACTAT	283592	C14orf86
2	V2HS_163850	CTCCCTAAGTTCATTTGTA	283658	LOC283658
1	V2HS_103498	GCGATGATGACCGTAATGA	284440	MGC39821
1	V2HS_103584	GTACTGAAAGTGTACTGAA	284546	C1orf185
1	V2HS_183121	GGATTCCAGGAATCATTTA	284578	LOC284578
1	V2HS_218185	GAGCCTCTGGTAAAGAGAT	284600	LOC284600
1	V2HS_223527	GTACACGAAGGATTGCATA	284898	LOC284898
3	V2HS_25904	CAATTATGGTGACTGCTTA	285286	LOC285286
2	V2HS_25400	GAGAGGCTGGGCATTATTT	285375	LOC285375
1	V2HS_207212	GTGGGAATTTCTGATGAAT	285555	C4orf37
1	V2HS_174748	CTTACATCAGCAATAAGCA	285590	SH3PXD2B
1	V2HS_179086	CCAAATATCAGATAAACTT	285780	RP3-398D13.1
1	V2HS_276242	CCAGGGCCATTTCCCTAAT	285857	LOC285857
1	V2HS_272775	GAAATTATGTAGTTAGAAT	286006	C7orf53
1	V2HS_286550	GAGGATATGGATTCTCAAT	337971	KRTAP19-4
1	V2HS_286775	ATGGTGTTATGAAAGATCT	337979	KRTAP22-1
1	V2HS_50142	GCAGTCACATCCAACCTTA	338339	CLEC4D
1	V2HS_217902	CTAAGCTTCGAAAGTTTAT	338862	LOC338862
2	V2HS_218363	CTAACTATAGCTTGTCTTA	338949	TMEM202
1	V2HS_100429	CTCTGCACTCACTTTCTAT	339327	ZNF546
1	V2HS_166569	CTATTCATATCATTTTATT	339622	LOC339622
3	V2HS_216560	GAGTTCACTTGGGATCAGA	339766	HEATR7B1

1	V2HS_100539	GCATTGAAGGTTTATATAT	340481	ZDHHC21
1	V2HS_210719	CCCATATTGTTCCAGCAAA	345275	HSD17B13
1	V2HS_77606	CAGAATTACTAAATTCTAA	348751	LOC348751
1	V2HS_157037	CTGTAAGAACCATAGACAA	348995	NUP43
1	V2HS_286494	GACATGTTATGAAATCTAT	353299	RGSL1
1	V2HS_286401	CAAGTTTCTTACAACAATT	353322	ANKRD37
1	V2HS_267231	CAGCCATTCTGAATTACAA	374443	LOC374443
1	V2HS_15550	GCCTTGGAATATTTACATA	375449	MAST4
1	V2HS_77698	CTCTTTAACTCAGTAGTTA	388403	YPEL2
1	V2HS_103615	GAGACTCTGCTTCACGAGT	388581	FAM132A
1	V2HS_84701	CTGATCTGTTCTACATATT	388667	C1orf137
1	V2HS_145157	CTGAGACAGATAACCTTGA	389396	C6orf140
1	V2HS_166261	GCCTTCCTGTTTACCTACT	389690	FLJ43860
1	V2HS_63568	CTGAAGCACTGTTACCACA	390597	LETM1P1
1	V2HS_207320	GTTCTGATGTCCTCATCAA	390883	OR7G3
1	V2HS_163384	CAGATGCCTTCAGAGAAGA	391578	LOC391578
1	V2HS_195929	GAGCCTGTCACATATTGGA	399694	SHC4
2	V2HS_38902	CAAATTCTCTAGTAGCAGA	399978	hCG_2032978
1	V2HS_285941	CAATGCCAAACACAATTAA	399990	FLJ44874
1	V2HS_259583	CACCGCAAAGTCTAAATAA	400823	FAM177B
1	V2HS_285809	CTATGATAATCGTTAGTCA	404201	C4orf12
1	V2HS_75780	ACCATTCTGAGTTTATTAA	431707	LHX8
1	V2HS_47700	CCCTCTAGGGAAATTCTTA	440823	MIAT
1	V2HS_29824	CCTGTGTACTIONCTCGGAGA	440867	FLJ16124
1	V2HS_20688	CGTTTAACCGGTACAGAAT	441086	LOC441086
1	V2HS_188312	CATGTTCTCACAGTTATTA	441246	hCG_1983332
1	V2HS_183530	CACCTGGCTGAACATAACT	642852	LOC642852
1	V2HS_55833	AGTACATTTGGGTTTCTAA	644314	MT1IP
1	V2HS_105257	CAGTCAACATTACAACCTGA	645693	LOC645693
2	V2HS_279205	CTGAGAAATAAGTCTATAA	645843	TMEM14E
1	V2HS_103662	CAGTTCTTTCTCTAGGCAA	646976	LOC646976
1	V2HS_123801	GAGAAAGCACGAATACTAA	649489	LOC649489
1	V2HS_165267	CTGAGATTGCTCACAATGT	728572	LOC728572
2	V2HS_63684	CAACTTGAGTCCTTGGAGA	728586	hCG_1981531
1	V2HS_169644	CATGTTATTGGAGCTTGAT	728773	PABPCP2
1	V2HS_181335	CAGAGTATTTATCTTCCTT	728853	LOC728853
17	V2HS_29650	GTGCTTTATTCAAATCTAA	729324	hCG_1986447
1	V2HS_25169	GGATGTAAGTGTTACATCT	730139	LOC730139

2	V2HS_276853	GTTAACACCACTTCCCTCT	100129126	hCG_1812962
1	V2HS_62616	AGGTCTGGCTCTAACCATA	100129702	LOC100129702
1	V2HS_40271	CGCAACACCGCATGGAACA	100130077	LOC100130077
1	V2HS_63323	CAGACGACAGGAATGACTT	100130298	LOC100130298
1	V2HS_53768	CCAGTGATGATATTCATAA	100130401	LOC100130401
2	V2HS_221308	CTCGCCTTCCTTATTTCAA	100131213	C10orf41
1	V2HS_246262	CTAGTCAGAGAATCATAGA	100131510	LOC100131510
1	V2HS_222555	CTGTTGCAAACAATCATT	100131794	LOC100131794
1	V2HS_144183	CTCTGGGAATGTCACATT	100130940, 100132460, 100133153	LOC100130940, LOC100132460, LOC100133153
2	V2HS_200103	GTGCCATTCTCCTCTCTGT	10626, 147166	TRIM16, TRIM16L
1	V2HS_73896	GTGATTCTCCTCATTCCAA	115123, 100133609	LOC100133609, MARCH3
1	V2HS_28473	CCATCTAAGGACTTGATTT	127833, 100127887	LOC100127887, SYT2
2	V2HS_104228	GCCCAATTCCTACAAGGAA	128102, 391081	LOC128102, LOC391081
1	V2HS_180258	CACCGTGTGACATAGACCA	150763, 643219	LOC150763, LOC643219
1	V2HS_61273	CTCATAGAAAGTCACTTTA	1649, 4141	DDIT3, MARS
1	V2HS_188999	GACAGAAGAAATACAGCAA	200030, 728841	NBPF8, RP11- 94I2.2
1	V2HS_188580	CTAATGATGCACCAGATTA	2186, 646043	BPTF, LOC646043
1	V2HS_65532	GTTGTTTATTCTTAGTAGT	23192, 727737	ATG4B, LOC727737
1	V2HS_130382	GCAAGATGAGGGTGAATGT	23246, 727967	BOP1, LOC727967
1	V2HS_282894	GCCATTCTGAAGATAATAA	26609, 51480, 51481, 425054	VCX, VCX2, VCX3A, VCX3B
1	V2HS_23667	GATTCACCTTCAGTAGCTA	28439, 652070, 652494, 100132941, 100133739, 100134256	IGHV3-30, LOC100132941, LOC100133739, LOC100134256, LOC652494, SCFV

1	V2HS_188946	CTTGGAATTGCCAGATAAA	285855, 643507, 728620, 729123, 100129743	LOC100129743, LOC643507, LOC728620, LOC729123, RPL7L1
1	V2HS_220255	CTCCTACATGTTTATATTA	286333, 100128385	C9orf109, C9orf110
1	V2HS_70516	GTGTTTCCTCCGTTCCCTA	29128, 728688	hCG_23738, UHRF1
1	V2HS_132083	GAAATATGCTCAAGCCATA	2969, 2970, 100093631	GTF2I, GTF2IP1, LOC100093631
1	V2HS_193059	CGGTGTAATGTAGACTTGT	29883, 100128883	CNOT7, LOC100128883
1	V2HS_86051	CAGGCATTGGCATGACCAA	3326, 3327, 391634, 664618	HSP90AB1, HSP90AB2P, HSP90AB3P, HSP90AB4P
1	V2HS_190348	GATATCCCTGCCTAGATCT	349196, 100133099	LOC100133099, LOC349196
2	V2HS_229810	GTTACTGTCTTTGGAGATA	374462, 100129165	LOC100129165, PTPRQ
1	V2HS_276358	GAGAAGCTTTATGGGCCAA	388692, 644634	LOC388692, LOC644634
1	V2HS_183055	GCTCTAGTGGGTTCTTTCA	5081, 284530	LOC284530, PAX7
1	V2HS_179229	CACCTACTCATGCACCTAA	51132, 64115, 344905, 376940, 100133315	ATP13A5, C10orf54, LOC100133315, RNF12, ZC3H6
1	V2HS_181050	CTCTTCTCCCTACCTGCTT	5426, 100128843	LOC100128843, POLE
1	V2HS_174197	GTCGGAGCCAAAGCATTTA	54967, 650024	CXorf48, LOC650024
1	V2HS_43473	CAAACCTAGTTACCTAGAA	5768, 200058	FLJ23867, QSOX1
1	V2HS_237760	GTATGAAGCATTACCACTT	6157, 389435	hCG_21078, RPL27A
120	V2HS_93855	CTTAGTAAAGGACTTATCA	6230, 100131196	LOC100131196, RPS25
1	V2HS_179237	GCCATCATCCTAGTCCTCA	64145, 220323	OAF, ZFYVE20

1	V2HS_217436	CATTATGAAAGAGAAATGA	6672, 645292, 729858, 100132863	HMGIL6, LOC100132863, LOC729858, SP100
1	V2HS_284104	CAGTCGCCTTTGGAAATAA	84187, 100130886	LOC100130886, TMEM164
1	V2HS_159404	GGCTGCTTGTTTCTGTGAA	84673, 100101118	LOC100101118, TTY8
34	V2HS_17317	GAGACTCTATTCCGATGTA	8741, 407977	TNFSF12- TNFSF13, TNFSF13
11	V2HS_17314	GATATTCTGAGTGTCATAA	8741, 407977	TNFSF12- TNFSF13, TNFSF13
41	V2HS_17313	GCCGCCCTCTGCTAGGGAA	8741, 407977	TNFSF12- TNFSF13, TNFSF13
7	V2HS_17318	CTCCAGAGATGTAGCTATT	8741, 407977	TNFSF12- TNFSF13, TNFSF13
31	V2HS_17316	GGTGCCTTCGCAGTCAAAT	8741, 407977	TNFSF12- TNFSF13, TNFSF13
1	V2HS_37264	CAGCTCTTCTCAGAAAGAA	90326, 100129138	LOC100129138, THAP3
1	V2HS_46734	CTCAGCGATGGGAAATGAA	9040, 606551, 100133517	LOC100133517, UBE2M, UBE2MP1
1	V2HS_23046	GGACCAGTAGCAAAGGAGT	9349, 646949	LOC646949, RPL23
1	V2HS_22091	CACGGAATGCTGGTCATTC	-	-
1	V2HS_122678	CCAGTGGAGAACATGGAAA	-	-
28	V2HS_145727	GCCTCAGGACAAAGATGAA	-	-
1	V2HS_125389	CAGGTCTGCAAATACCAGA	-	-
24	V2HS_19513	CTACTCAATGCTTGGCCCT	-	-
2	V2HS_111082	CACAAGACGGGAAATCCAA	-	-
1	V2HS_39398	GAGTTGTTCGTGATAGAAA	-	-
1	V2HS_52653	CATCTCAGTAATGCTAAGT	-	-
40	V2HS_193088	CTAAATAAACGTTATCGAT	-	-
22	V2HS_169189	CAAACCTTGCATTAATAAT	-	-
10	V2HS_182066	GGAACAGAACGCCAGAATA	-	-
18	V2HS_79588	CAGAGACAATCCAAAGCCT	-	-
3	V2HS_109366	CAGAATTATTTAAACAGAA	-	-
3	V2HS_108699	CTCACCCAGTGAAGAGAAA	-	-
1	V2HS_142164	CTGATGTCTGGGCCCAATA	-	-
2	V2HS_256884	CACAAAGAATCAACCTGAT	-	-

1	V2HS_53386	CCCGCTGGGACCTTGCTAA	-	-
1	V2HS_89666	GTCTTAATTTGAATAATGT	-	-
1	V2HS_165492	CTTGTCCCATAAGTACAAT	-	-
1	V2HS_186813	CATTATAAGGCTTATGATT	-	-
1	V2HS_145885	GAATGAACAACCTGGTGTA	-	-
1	V2HS_89289	GGAAGAAGATGAAACACAC	-	-
1	V2HS_120806	CTCATCTGCCCTCTCACTA	-	-
1	V2HS_15504	GAGATTACAGGACCTAAGA	-	-
1	V2HS_127367	CTTAGAGATTTCTCAACA	-	-
1	V2HS_52706	CTAATGACCAAGTTAGTTA	-	-
1	V2HS_65880	GTCCTATTCTGCATGCATT	-	-
1	V2HS_25043	GCCATTGGATGCAGTCTTT	-	-
3	V2HS_217568	CAACAGCTCTGCAGTTGGA	-	-
1	V2HS_91534	AGAACCATGTAAGGGTAAA	-	-
1	V2HS_146271	CAAGAACCTTTGCTGTAA	-	-
1	V2HS_107757	CAATCACAACGAGACAACA	-	-
1	V2HS_250042	CAATCAGAGAGGAGACCCT	-	-
1	V2HS_31615	CACAGAACCTCAATTGGAT	-	-
1	V2HS_121207	CACAGGGTCTTTGATGAGA	-	-
1	V2HS_147063	CAGACACATGTGGATCTGT	-	-
1	V2HS_241584	CATTAAAGCTTCGTAAATA	-	-
1	V2HS_124718	CCCTCCTCGTTTCTCCATA	-	-
1	V2HS_23175	CCCTGCCAAGGAATCATT	-	-
1	V2HS_129204	CGAGCAGTCTAGAGCAGGA	-	-
1	V2HS_34104	CGCTGGAAATCAACTCAAT	-	-
1	V2HS_122101	CGGACAGAGGTGACATTTA	-	-
1	V2HS_204240	CTATGGATGGCATATGGAA	-	-
1	V2HS_79147	CTGCTGGATTGTTATGAGG	-	-
1	V2HS_243248	CTGTAATAACTTAGCTATT	-	-
1	V2HS_215556	CTGTTGGCATGTATCTTAA	-	-
1	V2HS_219564	GAAGAAGTATGAGAATTAA	-	-
1	V2HS_231328	GAAGCACATTGTATTTGAT	-	-
1	V2HS_204070	GAGTACCTATCTGGTGGTA	-	-
1	V2HS_148105	GATAAGAAGCTGTGAAGAA	-	-
1	V2HS_80097	GCCAGTCACTTCTCACAAT	-	-
1	V2HS_145685	GCCAGTTTAGCAGTTTCAA	-	-
1	V2HS_149821	GCTTAAACATTTATGGAAT	-	-
1	V2HS_233616	GTCTTATCTAGTTTAATTA	-	-
1	V2HS_224677	GTGCTAGCCTCATTCAAGGA	-	-
1	V2HS_213004	GTTGTATTGCTTGTTCAA	-	-
1	V2HS_142348	CATGTAGAGCTGTTGGTCA	-	-
1	V2HS_251570	GGAATTAACCCGAAAGTTT	-	-
2	V2HS_124154	GACAGCACCAAGAACAAGA	-	-
2	V2HS_155511	CACATCCATTCTACTAACA	-	-

1	V2HS_246078	CCCGGACAAATGGAATTTA	-	-
1	V2HS_54310	CACACACGATTGCAACTTA	-	-
1	V2HS_247631	CCCTATTCCTCTGAAGTT	-	-
1	V2HS_180278	GACTTCTTTGTATGGACTA	-	-
1	V2HS_217042	GAGCCAAAGGAATAAGGAA	-	-
1	V2HS_49825	CCTTGTACTTCTTGTTAGA	-	-
1	V2HS_129926	CAGAGTACGAGACTTCTGA	-	-
1	V2HS_106284	CTCACCAGATCCAATTCCT	-	-
1	V2HS_106299	CTCTTGTTATCATCAGAAA	-	-
1	V2HS_106308	CTTGGTGAAGATTCATAGT	-	-
1	V2HS_106193	CACAAACGCTGAGAGACAA	-	-
1	V2HS_84055	CTGAGGACTTTGATGATGA	-	-
1	V2HS_144903	GGATGACACTGAAAGGTGT	-	-
1	V2HS_148338	CCGCTGATGTTGCTTGAAT	-	-
1	V2HS_148508	CAAATCAAGAGGAGATCAA	-	-
1	V2HS_76595	CCTAGCACTTGCCATGTGA	-	-
1	V2HS_169348	CTATATCTCTCTATGGGAA	-	-
2	V2HS_236504	CAAATAAGATCCAAGATCA	-	-
1	V2HS_120960	GCTTTCTGTTCTCACAGAT	-	-
1	V2HS_269067	TTGTGTCAGCATATGTGAT	-	-
1	V2HS_178691	CAAATTAGTAAGAGACCAA	-	-
1	V2HS_107731	CAGCCATCATCTTTCCACA	-	-
1	V2HS_162781	GAATGCAGGTACTIONTACATA	-	-
1	V2HS_207677	CATGCAATTATTTGGACAA	-	-
1	V2HS_145538	CCAGAACCCTTATGACTAA	-	-
1	V2HS_181529	CCACCTCCACTCATAGCTT	-	-
1	V2HS_167571	CCTATAGCTTCATTATGCA	-	-
1	V2HS_184430	GACTCGTTCCTGAGCTTA	-	-
1	V2HS_91185	GTACCATCATATTTGAAAT	-	-
1	V2HS_274489	GACAAGAAGTGTGATAGAA	-	-
2	V2HS_276361	CAATCTTCCTAATCCACAT	-	-
1	V2HS_22640	GCAATGGACTCAACCTATT	-	-
1	V2HS_168319	GGACTCACGCCCTCCTACT	-	-
1	V2HS_184599	CCTTCATCAGCATAATTCA	-	-
1	V2HS_117073	GACTGTAAAGTTGTAGGTA	-	-
2	V2HS_244118	CAAATGATGCTGCAATGTA	-	-
1	V2HS_42355	CCTCAAAGCAGATCATCCA	-	-
1	V2HS_45679	CTAAGCTCATGAAACACAA	-	-
1	V2HS_231873	GTGAGTACTTCTTCTTAGA	-	-
1	V2HS_44335	ATATTCAGCAGGTTACTAA	-	-
1	V2HS_104782	CAGAGAGAATCTCTGTTGA	-	-
1	V2HS_85180	GATTCGTATTGAAATGCAT	-	-
1	V2HS_44015	CTCTGGAGATCTGATTTTC	-	-
2	V2HS_37229	GTCTGATAGTTCCATTTA	-	-

1	V2HS_240662	CAGACTCTTACAAATGAAT	-	-
1	V2HS_22893	CTGGCTGCCTGCTACATAT	-	-
1	V2HS_74133	CAGTTCAACGGGAGATGAA	-	-
1	V2HS_21710	GATATTAGCTTAAACTATG	-	-
1	V2HS_181505	GTCTGCATGTAGTTCTAAA	-	-
2	V2HS_163907	GTGGGTTCCCTAAATAACAA	-	-
1	V2HS_32180	CACTGAAGTCCCTGTCCTA	-	-
1	V2HS_90738	CATTGCATCTTACTTATTA	-	-
1	V2HS_217188	GCTAAACAAGGACAAATTA	-	-
1	V2HS_190585	GGCACATTCTTGACAGAGAA	-	-
1	V2HS_90801	GGATAAGATTCACTAGATA	-	-
1	V2HS_192716	CCATCAAGGGAATAGTCAA	-	-
1	V2HS_125998	CTGAAGATGAACCAAAGCA	-	-
1	V2HS_125999	CTTCGGAAGTGGAGGATGA	-	-
2	V2HS_29828	GCTTTGCTGGTTTCTATTA	-	-
2	V2HS_218150	GTGAGTTCCTTTTCAGAGA	-	-
1	V2HS_53517	CATCTATGATGAAAGCTCT	-	-
1	V2HS_136735	CAATTTACACATGGATGA	-	-
2	V2HS_56603	GAGTTATCTCAATACATAA	-	-
1	V2HS_101147	GAAATTCTGATGAAGTTAT	-	-
2	V2HS_56071	AAGTCAATAGAGTTACCTA	-	-
1	V2HS_244391	CAGAAACCCTATTAGAGCT	-	-
1	V2HS_46180	CCCTGCAATTTGTCCTGA	-	-
1	V2HS_46260	CTCTTACAATCAATGGGAT	-	-
1	V2HS_92028	CACAGTGCAGAGAGTGGAA	-	-
1	V2HS_236352	CAGGGTGGCAGAAATAGAA	-	-
1	V2HS_101400	GACATGGAGGAAGAAAGAA	-	-
1	V2HS_179613	CTTAGTATGAATTACTGCT	-	-
1	V2HS_252474	CACAGTGATGATCTTTGTA	-	-
1	V2HS_209142	CCAAGTATACTTAAAGCAA	-	-
1	V2HS_21547	GCTACATTATATATAGGAT	-	-
1	V2HS_180618	GTCCATATCCATTGTTGCA	-	-
1	V2HS_33362	CCCAGTTCAGTTATCTAA	-	-
1	V2HS_85643	AGTGGATTCTGTACTGGGA	-	-
1	V2HS_244348	CCATCACGCTATCAAGAAT	-	-
1	V2HS_184487	CCGCGTAGTAATTCCTAAT	-	-
1	V2HS_25956	CAGACCACAGGTCTCCTTA	-	-
1	V2HS_186594	CGCACAGCGATTTACATT	-	-
1	V2HS_250313	GCACAAAGGCCTTTGCACA	-	-
1	V2HS_55108	CAGTTCAAATATCTTGATA	-	-
1	V2HS_274959	CTCGTGCTGACTTGTAAGT	-	-
1	V2HS_235621	CTGGGACTCAGCATTAAATA	-	-
1	V2HS_146529	GAGAGAGATCAATTTCTGA	-	-
1	V2HS_16927	GCTCTTGATTCTCAATGAA	-	-

2	V2HS_65234	CTACGAACAGTGTAGGCAA	-	-
1	V2HS_141402	CTGGTGCTCACCTGAGTAT	-	-
1	V2HS_74979	GAACTCCCTGTATAACCACA	-	-
1	V2HS_220599	CAGGCAATTATAACCAGATT	-	-
1	V2HS_168133	CTGTCCGTGGCCCATTAGA	-	-
1	V2HS_276130	GGGTTTACTTACTTGGA	-	-
2	V2HS_163166	CAGGGAAAGCATTACTGCT	-	-
1	V2HS_142788	CTGATGAACTGAAGATTGA	-	-
1	V2HS_163167	GAAAGCATTACTGCTTTCA	-	-
1	V2HS_109255	GTGTATAATTTGGTTGCTA	-	-
1	V2HS_163161	CCAGCAGCTTTAGTAAGGA	-	-
1	V2HS_268023	GAAGGAATGGGAGTAATTA	-	-
1	V2HS_108975	CAGCTGCTCACACTGACTA	-	-
2	V2HS_109007	CAAGGTAGCATGCCTTTAT	-	-
1	V2HS_52677	CAACAGAAGCCCTCAGAAT	-	-
1	V2HS_244622	GGATTATCCAGCCATTCTA	-	-
1	V2HS_256866	CAATGCAAATGTAGGGTTA	-	-
2	V2HS_221340	CCTTAGCAGAGAGAATATT	-	-
1	V2HS_44257	CAAATTCTCTAAGTTTAAA	-	-
1	V2HS_251063	CCTTTTCCCTTGACATCCT	-	-
1	V2HS_147845	CTGTTACAGGGATGTGGTA	-	-
1	V2HS_147849	GCTCCTGACACAAGGAGAA	-	-
1	V2HS_130639	GTTGGGTTACTAAACTTTA	-	-
2	V2HS_44947	AGCTCTGCCTCCTCTCTAA	-	-
1	V2HS_105132	CGGAGGAGCGTAAGAAGAA	-	-
1	V2HS_40503	GAGAGACTAGCTTGTATTG	-	-
1	V2HS_40095	CCACTAGTAACTTACAGTT	-	-
1	V2HS_181516	CAGTACCAACTGCTTTGAA	-	-
1	V2HS_237211	GTGAGGGAGAGTAATTTGA	-	-
1	V2HS_43155	CTTCTGATGACTACAGATA	-	-
1	V2HS_43224	GATACTTATTCATCAACAA	-	-
1	V2HS_91013	CAGAGAATAGAATCCTCAT	-	-
1	V2HS_52579	CTAATCAACTGGATAGCTA	-	-
1	V2HS_26223	GTCAGAGCACTGATTGCTA	-	-
1	V2HS_26100	ATTAGAGTCCCTATTTCTA	-	-
1	V2HS_182486	CCGAGGAATGAGGAAGAGA	-	-
1	V2HS_79816	GTGGCAGTTACTAATTATG	-	-
1	V2HS_245934	CTACTGATGTCAGTCAGAT	-	-
1	V2HS_182414	GATCTGGTGGAAAGAGATA	-	-
1	V2HS_219926	CCGGGACAGAAGTGGGAAA	-	-
1	V2HS_32611	GGCACAATCACTCTTTGTA	-	-
1	V2HS_216692	CAGTTAATACTCTTGATA	-	-
1	V2HS_129691	CATCAAGGCTGAATATGCA	-	-
2	V2HS_37358	CTGGTGAAGTGTACATCA	-	-

1	V2HS_105984	CAGGCTAGGTGACAACCTA	-	-
1	V2HS_107961	CTGCTCCTATTCTGAGGGT	-	-
2	V2HS_59184	CATTATCTCTGCCATTGTA	-	-
1	V2HS_50896	CTGCCCATCTCACTTCATA	-	-
1	V2HS_50638	GAGGAAATGGCAATGGAGA	-	-
1	V2HS_217620	CTGATATTATGGATACCAA	-	-
1	V2HS_219766	CACTTTATGAACACTAGGA	-	-
1	V2HS_251244	GTAACATGGCTAATCAGGA	-	-
1	V2HS_217082	GTTATTGCTCTGTTTCAGA	-	-
1	V2HS_146766	ACTGGAAACTCTATCATGA	-	-
1	V2HS_146801	GGTTGCCTCTATTACGAGT	-	-
1	V2HS_147399	CAAAGAGGGAGATAGAACA	-	-
1	V2HS_210696	CAGCCTTCTATGAAACTCA	-	-
1	V2HS_256589	CAGTGCTTAATGAGAATAA	-	-
1	V2HS_14973	CATAGTATTTGCTACTGAA	-	-
1	V2HS_15255	CCACTCCTCACCGAGATGA	-	-
1	V2HS_212757	GAGTGAGTGCTATAAGCAA	-	-
1	V2HS_147422	GCTAAAGGAAGAAATGCAT	-	-
1	V2HS_36976	GCTATGTTGTCAAGACTTT	-	-
1	V2HS_236523	CTCTATCCTACAGAAGAAT	-	-
1	V2HS_147618	GCCTTTAGCTACACCTTTA	-	-
5	V2HS_203877	GGACAATTATGCTGGCTAT	-	-
1	V2HS_217354	CTGGCACTTGAATTAAGTA	-	-
1	V2HS_55503	GCTTTCCTCTACTTGTTCT	-	-
1	V2HS_124101	CACTCCTCTGTGTCCTCTT	-	-
1	V2HS_59316	CATGTACCTTCATTTGGAA	-	-
1	V2HS_274988	GGGCTATTTATTTTCAGGAA	-	-
2	V2HS_60948	CATATAGTGGATCCTGAGT	-	-
1	V2HS_162116	CATGTATCCTGGAGCAAGA	-	-
1	V2HS_146234	GTGACACCTTCATAAACTT	-	-
2	V2HS_55581	CTGTAGTACCCGTTGCTAT	-	-
1	V2HS_146242	GTGGACTTCTCTTTGAAGT	-	-
1	V2HS_21538	CATCATATTTGTTGTTGTA	-	-
1	V2HS_169420	CAGCAAACACTTAATAGTA	-	-
1	V2HS_169506	CTGTTTGACTTCATAATGT	-	-
1	V2HS_169535	CAATTCAGGCAGGCTTTA	-	-
1	V2HS_169581	CGACAAAGATGATCACATT	-	-
1	V2HS_169619	CGCTGTTTATCACAAGTTA	-	-
1	V2HS_105213	CAGAAGATTGCCACAAGA	-	-
1	V2HS_195642	GAAGCAAGACTAGAATCTA	-	-
2	V2HS_59847	CTATGAAACTGATCACTTC	-	-
1	V2HS_41240	ATGTCTCAGTTGAAGATTA	-	-
1	V2HS_143984	CTGAAGATGCTGATGACAA	-	-
2	V2HS_188887	CTGTTTCAGTACGAAGATGT	-	-

1	V2HS_165911	CTCATCAGGACCTCAAAGA	-	-
2	V2HS_189407	CAAACCACATATTGAGCTA	-	-
1	V2HS_224093	GTCATCTGGTCCCTGTTTA	-	-
2	V2HS_165401	GTCATTATTGGAGATAATA	-	-
1	V2HS_43247	CTGCTTCAGTTGAGAAGAA	-	-
2	V2HS_236276	GGCATGAACGATGAGAAGT	-	-
1	V2HS_107323	CTCATATTAGATGAATGTA	-	-
1	V2HS_180699	CATGTACAGCAGGAATTGA	-	-
1	V2HS_105271	CTCAGAAATCCATTGTATT	-	-
1	V2HS_61763	CGATTACAATTAGAGCTGA	-	-
1	V2HS_210448	GAGGTCACCACCCTTCCCA	-	-
2	V2HS_126832	CATCTTACTGGAGTCTGTA	-	-
2	V2HS_216964	CAATACACAGGACCCACTG	-	-
1	V2HS_67413	GAGATTTTCAGGAATCAGCA	-	-
1	V2HS_192571	CAGTTGAACTCATGGAGCT	-	-
1	V2HS_58361	CACCTGAGATCTAAGTTAA	-	-
2	V2HS_256336	CATCAAAGACATCAAAGAT	-	-
1	V2HS_211484	CACCTTCAGTTCTCGTAGT	-	-
1	V2HS_216803	ATGTTCCCTGTGTCACCTTA	-	-
1	V2HS_144985	CGCATCTGCTACTCGCTCT	-	-
1	V2HS_148275	CACTTCTCATCTACCTAGA	-	-
1	V2HS_56805	CTATGTGACCTACATCCTT	-	-
1	V2HS_106994	CTCTTCTTTGCAGAGAGAA	-	-
1	V2HS_92143	CATCCATATCCTGGACAT	-	-
1	V2HS_229177	CATCGCTCCATTAAGTTA	-	-
1	V2HS_166862	CGGAAATAATTTCTTTCA	-	-
1	V2HS_229438	GCCTCTAAATCGGAACAGA	-	-
1	V2HS_187611	CTGACAGTGCCTGAAAGGT	-	-
2	V2HS_275375	GAGGCTGAAACCTTTAAGA	-	-
2	V2HS_45663	CGCACTGCCTCTTTAAGAT	-	-
1	V2HS_162583	GAGCTATTTCTGACTGTAA	-	-
1	V2HS_220203	GAAATAACATGAGCTTGGA	-	-
1	V2HS_67012	GCCACAATCCAATATCAA	-	-
1	V2HS_213330	ATCCTGCTTTGTGCTGAGA	-	-
1	V2HS_23684	CCAACAATGTTAGAGAACA	-	-
1	V2HS_62311	CATTCTCTAGGACTTAATA	-	-
1	V2HS_168622	CAGAGAACACTAATTGTTT	-	-
1	V2HS_14965	CCCAATATAGCCAAACCAA	-	-
1	V2HS_43706	GAAATGTTCTTTCAAGATC	-	-
1	V2HS_143382	CAGCGCCACACAGGAAGAA	-	-
1	V2HS_147823	CTAAGGTGTCTCTGAGGAA	-	-
1	V2HS_75872	CCACTTGTGCCAATGCCAA	-	-
1	V2HS_149385	CAATTCCACAGTGATTTAA	-	-
2	V2HS_46109	CGGGCAATCTTCTAGTTGT	-	-

1	V2HS_52452	GGAATATTTGAAAGTTCCA	-	-
1	V2HS_90460	CTTCTCCTCTCATTAAAGAA	-	-
1	V2HS_260601	GACTGATGACGTGTCACAT	-	-
1	V2HS_147726	CAGTGAATATAGACAGAGA	-	-
1	V2HS_129603	CGAAAGGAACTGCTTTCAA	-	-
1	V2HS_276571	CTCGGCATAATGACCTTAT	-	-
1	V2HS_62971	ACACAGGGTGGAAATCAAA	-	-
1	V2HS_162503	CAGTCTGTCTCTTTAATTA	-	-
1	V2HS_162301	GCTTCTATTTCTGTTTATA	-	-
1	V2HS_220854	CCAGTTTGCTTCATTCTTA	-	-
1	V2HS_77299	CAAACAAATTGAATTAGAG	-	-
1	V2HS_62166	CAAACATATGGCCTGTCCAT	-	-
1	V2HS_44343	GCCAGAATTAGATCCTCAA	-	-
1	V2HS_267087	GGTGTCTGCTTAATGTTAA	-	-
1	V2HS_106421	CATCGAACAGGACCACAAA	-	-
1	V2HS_184791	CTGATGACCTTAAGGGTCA	-	-
1	V2HS_145592	CACTAAATCTGCTTGGAGA	-	-
1	V2HS_184474	CGACTTTGGGTCTCATATA	-	-
1	V2HS_44010	CCTATTTCTCAATGCAGA	-	-
1	V2HS_26033	CAGGGAAATTGTGCTGGAT	-	-
1	V2HS_216586	CCAGTAAGAGAGAAGGAAT	-	-
1	V2HS_246775	CAAGGAAGAGTTAGAGGAA	-	-
1	V2HS_276688	GGGCTCAGTCAACACATAT	-	-
1	V2HS_53961	ACACCAAGATCAAAGTCAA	-	-
1	V2HS_169020	CCATCAGTGTGACGACAGA	-	-
1	V2HS_169666	CCTTCTAAGCCCTCCTTAA	-	-
1	V2HS_147429	GCAAGCACACTACACACAA	-	-
1	V2HS_89382	CCTTACAAGCCTATTTCT	-	-
1	V2HS_146057	CTACTATTCCTATTCCTAT	-	-
1	V2HS_144507	CTAAGGGTGAATTTGAATA	-	-
1	V2HS_147136	CAGAGTCAAGGATGCCCAA	-	-
1	V2HS_22319	GATGCTAAGAAGTGCCTAA	-	-
1	V2HS_182422	GTGAAGGAGAAAATAAGGTA	-	-
1	V2HS_29124	GCAATTTATTGGTGATTG	-	-
1	V2HS_101982	CAGGGAACATCATTGGATA	-	-
1	V2HS_209302	GCTACATTATATATAGGAT	-	-
1	V2HS_54194	GAAATGAACCTGAACTTA	-	-
1	V2HS_105738	CCAACTCTGAGGAAGTACA	-	-
1	V2HS_58994	GAGAACCACTGTTAGCAAA	-	-
1	V2HS_106508	CTCAAGAGAGCCTCTTATA	-	-
4	V2HS_38347	GCTGTGTCATGTGAACCAA	-	-
1	V2HS_162007	CAAATACTACGACAAGGGA	-	-
1	V2HS_22391	GTGTCATTGTGAGGAATTA	-	-
1	V2HS_169428	GGTAAAGTGAGAACTATA	-	-

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1	V2HS_83893	GCTCCTATCACTGAGATTA	-	-
1	V2HS_141784	GTGCCAAGTACTGCATCTA	-	-

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\* a hyphen (-) indicates unannotated genes; no gene symbol or gene ID is associated.

**Table A2. List of Potential Candidates Identified in Replicate 2 RNAi Screen.** All shRNA species identified are listed here. Some genes will have multiple shRNA species and are thus listed more than once.

Count	Oligo ID	Sense Sequence	Gene IDs*	Gene Symbols*
1	V2HS_49201	CATGAAGTGTGACATTGAT	70	ACTC1
1	V2HS_89871	ATATTCCTATCCATAAAGA	175	AGA
1	V2HS_132571	CTCATCAGGTATTGCTGAA	325	APCS
1	V2HS_277661	CGACGAGAAAGAAATAAGA	467	ATF3
30	V2HS_15289	CTCACTACCAAATGTTAAT	572	BAD
20532	V2HS_243025	CAGTGACCTTCGCTCCACA	572	BAD
30	V2HS_262043	CTCACTACCAAATGTTAAT	572	BAD
1	V2HS_202976	GTGCTCACTACCAAATGTT	572	BAD
31	V2HS_201511	GAGTTTGTGGACTCCTTTA	572	BAD
1	V2HS_231021	GAGTGTTATTCTACTTGTA	574	BAGE
1	V2HS_112598	CTCGATACAGCATTGGGTT	658	BMPR1B
1	V2HS_254648	CACAAAGTGTGACCACATA	672	BRCA1
1	V2HS_255064	CCCTTTCACCCATACACAT	672	BRCA1
1	V2HS_241288	GTGTGGAATATCAAACAAA	673	BRAF
1	V2HS_62452	CACATTAAGATGAATGTAA	677	ZFP36L1
1	V2HS_150310	GTTTCTACCTCTTTGGATT	911	CD1C
1	V2HS_261171	CAGCTAATCCCTCTGAGAA	931	MS4A1
1	V2HS_150391	CTGTCATCAACTTATTATA	965	CD58

1	V2HS_172812	CTTCTGGTGATTGGTATAA	1015	CDH17
1	V2HS_262142	GGAATAACCTTCCATACAT	1030	CDKN2B
1	V2HS_263239	GTTATCAACTTGAATGTAA	1033	CDKN3
3	V2HS_150519	CAAGTGATTATTAGAGAAA	1054	CEBPG
1	V2HS_150521	CTGATTGGTGTTACATAAT	1054	CEBPG
1	V2HS_64314	CCCTCTATGATGCAACCTA	1072	CFL1
1	V2HS_88711	CTCTGGAATCCCTGAGATA	1184	CLCN5
2	V2HS_229079	GAATCAAGATCCTGAAATA	1244	ABCC2
1	V2HS_43930	ATGAATCTTTCATACATAT	1326	MAP3K8
1	V2HS_150805	CGCTGTGGAGCCCATTACA	1329	COX5B
1	V2HS_113198	CTGCCTGAAGTGCGAGAAA	1396	CRIP1
1	V2HS_222624	GACATATCCCAACAGAGGA	1431	CS
1	V2HS_112475	GGAAGTAGCTCTGATGAGA	1580	CYP4B1
1	V2HS_113362	CAAAGATCCTCAAGGATTT	1606	DGKA
1	V2HS_11924	CATGCTTTGTGAAATGAGA	1745	DLX1
1	V2HS_241479	CATATGATATGTAAACCAA	1787	TRDMT1
1	V2HS_11917	CATATGATATGTAAACCAA	1787	TRDMT1
1	V2HS_18542	GCAGCAATGCAATGTGGAA	1827	RCAN1
1	V2HS_183290	GTGGCACTATTGTAATGGA	1889	ECE1
1	V2HS_151315	CTGCGAACTTGTGTTTATA	1907	EDN2
1	V2HS_280198	CGAACTTGTGTTTATAATA	1907	EDN2

1	V2HS_118603	CTGATATTCTCAATAGTTA	1997	ELF1
1	V2HS_17739	CTATAGACTTGAACCTCCTA	2045	EPHA7
1	V2HS_17742	AGGAGACCTTCAACTTGTA	2047	EPHB1
1	V2HS_17604	CAGACATACCAGCCTTATA	2079	ERH
1	V2HS_73856	GAATATTGGCCTTTAATTA	2087	ERVK2
1	V2HS_151487	CAGCCAAGCCCAGCACATT	2140	EYA3
1	V2HS_131700	GTCACTTCCACGAGAGTTT	2167	FABP4
1	V2HS_74073	CTGAGAAGTTCAGTGTCTT	2178	FANCE
1	V2HS_172627	CGTTGAGAACAATCATCTA	2213	FCGR2B
1	V2HS_259301	GAACTATCCTTCCAGGGAA	2218	FKTN
1	V2HS_130671	CAGTTAAACTGATTAGTAT	2246	FGF1
2	V2HS_201766	GAAACACAAGAATATCATA	2263	FGFR2
1	V2HS_93401	CCTTCAAGGAAGTGGTATA	2488	FSHB
1	V2HS_61887	GTATTTATCTGCAAGGTTG	2516	NR5A1
1	V2HS_83278	CATCTTTCAGGGAAAGATA	2538	G6PC
1	V2HS_114003	CATTTCCACTGTGGATTTA	2549	GAB1
23	V2HS_43346	CTCATTTCCCTGGTATGACA	2597	GAPDH
1	V2HS_227073	CCAAGAAGTTTAAGGAATA	2625	GATA3
2	V2HS_111541	CTGTAGTCCTGAAGCATGT	2638	GC
1	V2HS_42587	CAAGTTATCTGTTTCCTGT	2674	GFRA1

2	V2HS_99485	GAAATGAGAGCCACCTAAT	2686	GGT7
1	V2HS_231897	CACTCTGAGTTCACCTAAGT	2707	GJB3
1	V2HS_82773	AGCACCTGACCAAAGGCTT	2815	GP9
1	V2HS_132008	GAGTTAAAGAGGAGAAGGA	2852	GPFR
1	V2HS_114652	CCAAAGGTTTAGTTGTTGT	2882	GPX7
1	V2HS_130913	GTTATATTCGGGCTGTAAA	2918	GRM8
1	V2HS_130967	CCAAGAATCTGGTTATGAT	2983	GUCY1B3
1	V2HS_178387	GTTGCAGGACTGTAATAAA	2999	GZMH
1	V2HS_84939	ATGTGTTATTTGTCACAAA	3084	NRG1
1	V2HS_63803	GGAGCTGTGTCTACTATTA	3187	HNRNPH1
1	V2HS_236188	CCAAATCCTTCATGTAATA	3433	IFIT2
1	V2HS_111791	CTTAGTGATTCATTCCATA	3454	IFNAR1
1	V2HS_18095	CTGCTACACTTATGACAGA	3512	IGJ
5	V2HS_263385	GGAAGCTATGCGAAATTAT	3516	RBPJ
1	V2HS_132302	CATATGAGCTTTATTCTTT	3547	IGSF1
1	V2HS_121826	GAGAGTATTCCATTCAGA	3556	IL1RAP
1	V2HS_111547	CACAGTCAATATTAGTAAT	3576	IL8
1	V2HS_133352	GGCTTTAAGTTATTTATGT	3605	IL17A
1	V2HS_179526	GATGAGATTTCCATACAGA	3704	ITPA

1	V2HS_234020	CAAATGATGTGCTTATTAA	3707	ITPKB
1	V2HS_233969	CCATTTATTAGGCAATAAT	3738	KCNA3
1	V2HS_92953	GTCCATCTCTGGAGGAGGA	3849	KRT2
1	V2HS_133996	GTCCAGATCTTATTACCTA	3983	ABLIM1
1	V2HS_76727	CAAAGGAATTGCTAGTAAT	3990	LIPC
1	V2HS_76739	CACCTGCGGTATTTGTGAA	4023	LPL
1	V2HS_134145	CTGCCTCATTTAGAGAGGA	4067	LYN
1	V2HS_151636	CATATATACGGGATCCTTT	4179	CD46
1	V2HS_11941	CTATTTAGGTCAGTACATA	4194	MDM4
1	V2HS_151737	CGAAGAATGCCGCTTTGAA	4247	MGAT2
1830	V2HS_152026	CTCATCACACATATCTGTA	4599	MX1
95	V2HS_152028	CTGCCAGGCTTTGTGAATT	4599	MX1
1057	V2HS_152031	GACAAGATGTTCTTTCTAA	4600	MX2
1	V2HS_48297	GCCTTTCCTTAAGGAACAA	4671	NAIP
1	V2HS_22056	AGAATATTAGTAAGATTTA	4709	NDUFB3
2	V2HS_152212	CGAGTGGTGGCCTACAATA	4756	NEO1
1	V2HS_152269	CTCTCGAAGGGCTCCAGTA	4811	NID1
1	V2HS_239227	CACATGATCGAGCAGAGGA	4929	NR4A2
1	V2HS_218392	CATGTTGTAATCGTGCAAA	4946	OAZ1
14	V2HS_58978	CTCTATCCTTCCAAATGAA	5156	PDGFRA

1	V2HS_36419	GTACTGATGCTGTCATTTA	5165	PKD3
1	V2HS_72606	GATGGAGATTCTTCTCAAA	5239	PGM5
1	V2HS_58679	CACAGGACTTCTATGAGAA	5260	PHKG1
1	V2HS_75457	CCAAGAAACAGATCAACGA	5265	SERPINA1
1	V2HS_19949	ACTTGTCAGTGAATATGTA	5274	SERPINI1
1	V2HS_58599	CTCCCTAGATTTAAAGTAG	5276	SERPINI2
1	V2HS_111458	CAAATATTGCGAAGATCTA	5332	PLCB4
1	V2HS_93085	CGATGGCAAGTATGTCTAT	5444	PON1
1	V2HS_35113	AGGAGCACCTTCTGTGGAA	5494	PPM1A
1	V2HS_262715	CTAGATGTATCTGTGACTA	5571	PRKAG1
2	V2HS_170426	CTGATTACCAGTCTGATTA	5573	PRKAR1A
1	V2HS_170511	CAGATGCTTTGTGGTATTA	5601	MAPK9
1	V2HS_170527	CAGCACATCTACAAGGAGA	5603	MAPK13
2	V2HS_238224	CTGAGTAGCCATACCCGGA	5639	PRRG2
1	V2HS_170655	CCCACCACTGCCCAAGTAA	5675	PSG6
1	V2HS_170830	CCACGGCCCTAAATAGGAA	5715	PSMD9
1	V2HS_201516	CAACCCTTCTCCTCTTGTA	5777	PTPN6
1	V2HS_57273	CAGTGAAAGTCCATCTATT	5783	PTPN13

1	V2HS_171000	GGAAGTCACGTATTTGAAT	5795	PTPRJ
1	V2HS_173285	CGTGGTTGGTTAGAATATA	5861	RAB1A
1	V2HS_171132	CCGTCACCCTTATTTATTA	5864	RAB3A
1	V2HS_252754	GTGTAAATATTAGTGCAGT	5901	RAN
1	V2HS_196660	GCTACTATGTAGACAAGTT	5922	RASA2
1	V2HS_57185	GCCACAAGGAGATGGATAA	5977	DPF2
1	V2HS_56994	GATATCACCTGAATTATTA	5999	RGS4
1	V2HS_94799	CAATCTTAGCTTTGAAGAA	6013	RLN1
1	V2HS_94823	GTGATTACACTATTGCAAA	6102	RP2
1	V2HS_131580	GTCCGGAACGTCTATAAGA	6137	RPL13
1	V2HS_31983	CCTGTACTGGCAAAGTAGA	6259	RYK
1	V2HS_31785	GAAACCATGATGTTTACAT	6281	S100A10
2	V2HS_84837	GTGTCAAGGCGTGAGAATA	6322	SCML1
1	V2HS_31241	CAGCAGACATTGTGCCATA	6362	CCL18
1	V2HS_20638	CGCAGTAGATCTCGAAGTA	6431	SFRS6
1	V2HS_202633	CTGAGAATCTGGGACCTCA	6468	FBXW4
1	V2HS_183181	GTCACTGATCATGGCATGA	6548	SLC9A1
1	V2HS_56559	CCTATACTACTAATGTTA	6578	SLCO2A1
1	V2HS_98079	GAAATGCACACTTATTCTT	6581	SLC22A3
1	V2HS_153448	GCCTTAGCTGTTGAAGGAA	6711	SPTBN1

2	V2HS_33937	CCCAGAAACTGCAATGTAT	6809	STX3
1	V2HS_153678	CAGAAATACTTCTCAGCAA	6832	SUPV3L1
1	V2HS_246279	CTAATTTAATATGTCAAAT	6902	TBCA
1	V2HS_68358	CCCACATTTCTGTCTTACA	6955	TRA@
1	V2HS_134362	GGTTGCTTCAGGAACTTAA	7041	TGFB1I1
1	V2HS_42282	GTCCTCAACTTATATGTAT	7188	TRAF5
1	V2HS_171637	CCATCTCGGTTCTTTAGAA	7273	TTN
1	V2HS_171680	CGCTGCAGCCGCTCCCAGA	7293	TNFRSF4
1	V2HS_94107	CGTGACCTATCAGTTATTA	7298	TYMS
1	V2HS_171959	GATTTAAACTCCAGCATTT	7434	VIPR2
1	V2HS_172003	CTGTAACTTCTCAAGAGGA	7464	CORO2A
1	V2HS_219656	CATTTTCAGAGGCTAATTTA	7584	ZNF35
1	V2HS_172316	GAGACCTTGGTTATTATGA	7752	ZNF200
1	V2HS_72036	CTCTTTAATTTCAACAACAT	7763	ZFAND5
3	V2HS_172401	CTCTTGATTCTCAGTCTAT	7857	SCG2
2	V2HS_172400	CTCCTATGTATGAAGAGAA	7857	SCG2
19	V2HS_172404	GCCAGGATGCTAGTTAAAT	7857	SCG2
1	V2HS_69569	GCCTTATAGTGCTTAAACA	7984	ARHGEF5

1	V2HS_18094	CTCAAATACCTCTAACCTA	8021	NUP214
1	V2HS_219863	CTGCCTATGAGGAATATAT	8309	ACOX2
1	V2HS_172614	GCAAGTGATTTGACAGGTA	8334	HIST1H2AC
1	V2HS_33954	CATCATAAGGCCAAGGGAA	8335	HIST1H2AB
1	V2HS_33176	GACATACGCTCCATTAGCA	8395	PIP5K1B
1	V2HS_28083	CAGCTATTCTAGTGATAGG	8470	SORBS2
2	V2HS_95563	CCTTTATGATGAATGCAAT	8476	CDC42BPA
1	V2HS_27788	GCTTGCAAGTCTTAGAGGA	8501	SLC43A1
1	V2HS_23977	CACATTTACTGAAATGTAA	8650	NUMB
1	V2HS_240952	CCACAAAGGTGCTGAGGAA	8659	ALDH4A1
1	V2HS_41438	CTCGTATATTGTTTCCCAA	8697	CDC23
1	V2HS_17422	ACCAACAGATGAATCTATA	8737	RIPK1
1	V2HS_17000	CAGAGAAGGCTGTACAGTT	8774	NAPG
1	V2HS_16654	CACCTAAGTCGAGACAGTA	8804	CREG1
1	V2HS_16578	GGACTAGGAACTGCATATA	8813	DPM1
1	V2HS_244216	CCATCTGAATTGTTTAAAT	8987	STBD1
2	V2HS_47054	CACATCATTGTCATTACTG	8996	NOL3
1	V2HS_49253	CACTACACAGCCTATTGTA	9101	USP8
1	V2HS_36415	GCTACAATAACAGAACGAT	9201	DCLK1
1	V2HS_69232	GGCAATTTATCTTGATGTG	9214	FAIM3

1	V2HS_36244	AGGAGATTCGATTTCTGAA	9227	LRAT
1	V2HS_28393	GATGTTATCAGACTCATTA	9262	STK17B
1	V2HS_28326	GCACTCAAATGGTAGGTTT	9306	SOCS6
1	V2HS_35885	GGGAAAGTGCTACACATTT	9311	ACCN3
1	V2HS_130541	CTGGCAAGCTTGAATCCAA	9320	TRIP12
1	V2HS_201562	CTGGCAAGCTTGAATCCAA	9320	TRIP12
1	V2HS_240414	CTAACAAACAGTGTATTTA	9330	GTF3C3
1	V2HS_35444	CTCAGAATATCCTTAACAA	9358	ITGBL1
1	V2HS_68743	GTA CTGACATCATTGATAA	9404	LPXN
1	V2HS_68447	ACCTTATATTCAACATTTA	9425	CDYL
2	V2HS_67978	CCCATCTTTCCTTAACGAA	9474	ATG5
1	V2HS_192631	CATTTATACTTGAGAAGAA	9534	ZNF254
1	V2HS_67386	GCTTCAGACTCATTATTAT	9575	CLOCK
1	V2HS_27585	GACTTTGCAGAAGAAGTAT	9615	GDA
1	V2HS_62630	CGGTTTAAGAGATGGCATT	9623	TCL1B
1	V2HS_18674	CATTCTATCTTTGCTCAAA	9643	MORF4L2
1	V2HS_18676	GCAGGGAAATGTTGATAAT	9643	MORF4L2
2	V2HS_74142	AACAGAGTCTGCATTGCAA	9729	KIAA0408
1	V2HS_73989	CTATGATTCTGTTACAGAT	9736	USP34
1	V2HS_95333	CATCTTGCCACTCAAGCAA	9818	NUPL1
2	V2HS_95535	CTCAGATGACCTCTTAATA	9867	PJA2

1	V2HS_270549	GGTAGATAATCTGGTAATA	9926	LPGAT1
1	V2HS_50064	CAAAGAACGTGATCGACAA	9968	MED12
1	V2HS_49943	CTAAGTTAATGTCTAAGAT	9976	CLEC2B
1	V2HS_239090	CAGGCTGAACCTGCAGATA	10018	BCL2L11
1	V2HS_197622	CATTAACAGTGGTGGGAAA	10146	G3BP1
1	V2HS_196634	ACCAGTTCGTTATATTAGA	10152	ABI2
1	V2HS_6081	CAAAGCATTTCATTGAGAA	10210	TOPORS
1	V2HS_97746	CACCCAGTGCCTGCATTT	10229	COQ7
1	V2HS_70310	GGTGTTAATTGAAAGTATA	10342	TFG
1	V2HS_70237	CAGCTTCAAAGAAAGCTAA	10345	TRDN
1	V2HS_69530	GTCAAATTCTTCTTCCACT	10392	NOD1
1	V2HS_13442	CTGATACAGAGATCTGTGA	10425	ARIH2
1	V2HS_65311	GTGAACTTCTTATCCAGAA	10455	PECI
1	V2HS_65277	GCCATAAACACTGAGATGT	10455	PECI
460	V2HS_168768	CAGACGTTATTTACCGTCA	10461	MERTK
371	V2HS_197158	CCTTCAGTGATCCAGTGAA	10461	MERTK
1178	V2HS_1643	CTGCATACTTACTTACTTT	10461	MERTK
1	V2HS_197795	GACCAAATGTTACAAGAAA	10463	SLC30A9
1	V2HS_5748	CTCCTAAAGGCAAGATGTA	10468	FST
1	V2HS_281883	CTATTTCCCTGCTGAAAGTA	10473	HMGN4
1	V2HS_196249	AGCTGTCTAGTTTCATTCA	10491	CRTAP

1	V2HS_199741	CGCAAATAGTCATCTCTCT	10600	USP16
1	V2HS_1434	CAGACTACATCAAACAGAA	10733	PLK4
1	V2HS_91172	CAGAAGCTTTGTCAAAGTA	10773	ZBTB6
1	V2HS_90273	CAGCCCGCGCTATATATTT	10882	C1QL1
145	V2HS_84222	CCAATATTGTGTTTGGATA	10892	MALT1
4	V2HS_78624	CAGAGTTGAGTTCTATGTA	10962	MLLT11
1	V2HS_258025	CTTCATTTTCATATAAACAT	10971	YWHAQ
1	V2HS_250824	GACATCATCCAGGATAATA	10982	MAPRE2
1	V2HS_198901	GGAGTTATGTACGGCAATT	11069	RAPGEF4
1	V2HS_284597	CTCCACCTTGTTAAATAAA	11118	BTN3A2
1	V2HS_13532	CCAACAACAGGAAGATATA	11124	FAF1
1	V2HS_2161	CAGTTGAACTGTTAGACAA	11183	MAP4K5
1	V2HS_95890	CTGGGCAGCCACTTCATAT	11259	FILIP1L
1	V2HS_198493	GGGACTCTATTTATTCTGA	11322	TMC6
1	V2HS_254693	ACAACATACTAGTTATGTT	22838	RNF44
1	V2HS_96062	CTGAGTCTGCTATAAGGAA	22858	ICK
1	V2HS_96106	GTGTTATCTTAACTTTGAA	22866	CNKSR2
1	V2HS_156032	CAACTCTAATGAGAATGAA	22950	SLC4A1AP
1	V2HS_201316	CTGCCAAATTTGCTGTTTA	23089	PEG10
1	V2HS_253914	CAGAGATAAACTGATTTA	23102	TBC1D2B
1	V2HS_36857	GGCTCTGTCCTATCTACAA	23145	SSPO
1	V2HS_268618	GTATGTATAGAGTTGTTAA	23349	KIAA1045

1	V2HS_200922	GAGAGATTCTTGGCATGTA	23408	SIRT5
1	V2HS_28870	GTATTACACCCTGAATAAA	23412	COMMD3
1	V2HS_87347	GCCAATAAGTCATTGGAGA	23469	PHF3
1	V2HS_49243	CTTGGAATATTCTGGTTTA	23538	OR52A1
1	V2HS_50417	CACTTTACGAATTAGATGA	23556	PIGN
93	V2HS_260346	CTTTCTATTTGTAAAGTAT	23677	SH3BP4
1	V2HS_73723	GTAATGAACTCCTACTTCA	25775	C22orf24
1	V2HS_73640	GAAATTAGTGGAGTCTGTA	25780	RASGRP3
1	V2HS_96523	GTTCTTCATCCATTAGTTA	25897	RNF19A
1	V2HS_96662	CTTCCTTTATGAGATAGTA	25939	SAMHD1
1	V2HS_84532	GAAATGAACGTGTGGTTAA	26034	PIP3-E
1	V2HS_234485	GCAAGAACAAGAATTAAGA	26043	UBXN7
1	V2HS_256914	GTCCTGATCAGATAGATAA	26049	FAM169A
1	V2HS_114355	CTCTGTCCAGGCAGCATT	26097	C1orf77
1	V2HS_15524	GTCACTGGTCCATATACAA	26269	FBXO8
1	V2HS_85381	CCCTCTCCATCTCTGAGAT	26532	OR10H3
1	V2HS_114602	GTCTAAACTGCAGAAGTTT	27077	B9D1
1	V2HS_250473	CTTGCTCTGCGATAGATTA	27241	BBS9
1	V2HS_91459	CCATGGAAAGGTTATCCAA	27327	TNRC6A
1	V2HS_59736	CAGATGAAACTTTGGAGAA	28976	ACAD9

1	V2HS_59418	CTCAGACATGGCTTCCTTA	28988	DBNL
1	V2HS_5699	GCCATTATACCCAGAATAA	29094	HSPC159
1	V2HS_53296	CGAAGATTGCCGTTTAAGA	49854	ZNF295
14	V2HS_97933	CACACAGGGACTGTGGAAT	49860	CRNN
1	V2HS_65924	GACTTTCTAAGCATAGATA	50616	IL22
1	V2HS_218343	CTTCTCACCTGCCAAGTAA	50807	ASAP1
4	V2HS_154198	GCATCTCTCGCTTCTGTCT	50833	TAS2R16
1	V2HS_114878	CTGAGCACAGGTATTCTTA	51046	ST8SIA3
1	V2HS_134406	CTCCCAGAGATTGATCATA	51067	YARS2
1	V2HS_154242	CTGTGATTTGTGCTAATTA	51091	SEPSECS
1	V2HS_134712	CAGATCTACTTCTGGAACA	51092	SIDT2
1	V2HS_263532	CAGTCAGTAGTGTAAGAAT	51209	RAB9B
1	V2HS_135449	CCCATGCACGGAAAGTGTA	51340	CRNKL1
1	V2HS_191570	CTCAGCCCTTAATACACAT	51351	ZNF117
8	V2HS_228512	GAGTGAGCATCAATAAAGA	51497	TH1L
1	V2HS_115939	CTCTTCATCATCCAGGATA	51524	TMEM138
1	V2HS_135068	CAGGGTAATTTATATGAAT	51559	NT5DC3
1	V2HS_115863	GTCATGAAATCCAGATATA	51678	MPP6
1	V2HS_188543	CGGTACATAGGAGGAGAGA	51716	CES4
1	V2HS_285202	GTGTTCTAACTATTATCAA	53340	SPA17

1	V2HS_130238	GTATATGATGGGTCAAATA	53844	COPG2IT1
1	V2HS_47749	GTGTAAATATGCTTATGTA	54065	FAM165B
1	V2HS_215410	GAGAGAAGATCTCACAATT	54439	RBM27
1	V2HS_50851	GGGATCATCTTAAGAAGGA	54456	MOV10L1
1	V2HS_154663	GTCTTATCATGTGACCATA	54763	ROPN1
1	V2HS_254793	CCTCAAGTGGATTATGTTA	54778	RNF111
1	V2HS_138350	CAGGTGAGAGCTGGAGAAT	54856	GON4L
1	V2HS_173926	CATGCTAGGAGTTATACAT	54928	IMPAD1
1	V2HS_174222	CTTTGATTGGTGTACAGTA	54969	C4orf27
1	V2HS_174503	CAGCAATACAACAAGTTAA	55013	CCDC109B
263	V2HS_174640	CTTATAAGATGATGGATTT	55031	USP47
5	V2HS_174639	CGCAATACATGCAAGATAA	55031	USP47
39	V2HS_218228	CAATGACTTGCTATTTGAA	55031	USP47
10	V2HS_174641	GGATTCCTTTGGATGATAT	55031	USP47
1110	V2HS_174637	GAATCTGTCTTGAAACCAA	55031	USP47
59	V2HS_174642	GATTTAGACTGGAATCCTA	55031	USP47
1	V2HS_174648	CAGCTCTCTTGAACCTTAT	55032	SLC35A5
1	V2HS_219670	GATTATGTCTCTGTTTAAT	55048	VPS37C

1	V2HS_234429	CAGTAGAAGTCTTAATTAA	55075	UACA
1	V2HS_155567	CACGGCATATCCAGATGAA	55129	ANO10
1	V2HS_104285	CTGTTGTCTGCTTCCATCA	55182	RNF220
1	V2HS_156000	GCCACTTTACTGGGATGCA	55184	C20orf12
1	V2HS_28641	CGCCCAGGGCTGATCTATA	55187	VPS13D
1	V2HS_218161	GCAAGCAATTTCTCAGATT	55329	MNS1
1	V2HS_175422	CTACATGGCTCCAGAAGTA	55351	STK32B
1	V2HS_176062	CCGATACTCGGGAGAAGAA	55421	C17orf85
1	V2HS_176086	GTGATAAAGATCTCAAGGT	55425	KIAA1704
1	V2HS_26555	CGCTTTCATTCCAAATACT	55536	CDCA7L
1	V2HS_156380	CTGTTATCCTTGTTTGTA	55743	CHFR
1	V2HS_156481	CCAAGAATGAACTGCACAA	55752	40067
1	V2HS_156557	CAGGGACTCTGATGCATAT	55761	TTC17
1	V2HS_175047	CTCCTCAGTTCTCTACCTA	55777	MBD5
1	V2HS_175559	CATTTATGAAGATGAAGAA	55813	UTP6
1	V2HS_221493	CATTGTGTGAGGTGAATAA	55814	BDP1
1	V2HS_29937	CAATGTATATCTCCATTTA	55916	NXT2
1	V2HS_286439	GCGTATCTACCATTTAAAT	56106	PCDHGA10

1	V2HS_99331	CATCTTACATTGATGAGAT	56158	TEX12
1	V2HS_34552	AGGCAGGGATGCTAGATTA	56302	TRPV5
1	V2HS_21438	CAAAGTGTATATGAAGTTA	56751	BARHL1
1	V2HS_59975	CACCACTATTCATAAAGTA	56833	SLAMF8
1	V2HS_117553	GAAACTGTCTCGTAATGAA	56894	AGPAT3
1	V2HS_229301	CATTATCCCAGGAAACTTA	56905	C15orf39
1	V2HS_50679	CCGGTTAAGAGATTCTTAT	56950	SMYD2
1	V2HS_45068	GGGACTATACGATTCCTAT	56994	CHPT1
1	V2HS_58877	AGATTTAGCTCATGGATAT	57045	TWSG1
1	V2HS_35766	GTGTTCTTATAGTTATTTA	57150	C6orf162
1	V2HS_58216	GTGCCCATCTGGATTATTA	57393	TMEM27
1	V2HS_46786	CTCTGGATTTAGAGATATA	57475	PLEKHH1
2	V2HS_111008	GACTACTTCTACACAATCA	57476	GRAMD1B
1	V2HS_217697	GGCCTTGGCTACTACCAGT	57561	ARRDC3
1	V2HS_200776	CCTAGTAGTTCACTACAAT	57695	USP37
1	V2HS_57109	GTGTTGACAATATATTGAT	57709	SLC7A14
1	V2HS_60715	CGATTCACTACTTGTTAAT	58487	CREBZF
1	V2HS_60151	GTGAACTGCTCATATGCTT	58524	DMRT3
1	V2HS_87592	GGCACAAATATTTCTTGAA	64174	DPEP2
1	V2HS_80713	GGCTTATTATCGTATTAGA	64418	TMEM168
1	V2HS_98555	CTTTGCAAGTCCTTCAGAA	64805	P2RY12

1	V2HS_116227	GCTTTAGTTTAGAGCCTAA	64860	ARMCX5
1	V2HS_116295	CATTCTTTCAGTTACATCT	64922	LRRC19
1	V2HS_227038	CTATTAGAGCTAAATAAAT	64946	CENPH
1	V2HS_116332	CTGCCCTTAAGGAAATTGT	64946	CENPH
30	V2HS_238923	GTCTGAGAGAGTGACCTAT	65268	WNK2
1	V2HS_116447	GATTAACCCTAAAGGCTTT	79086	C19orf42
1	V2HS_226747	GTATCCCTCATGTCTTAAT	79088	ZNF426
1	V2HS_136245	GACTTAATTGATTGGTATA	79589	RNF128
1	V2HS_136349	CTCAGCACGTGTATTGAAA	79616	CCNJL
1	V2HS_164820	GTGACAATGCTGATTTATA	79633	FAT4
1	V2HS_157614	CAAATTACATCCTTGCTGA	79810	PTCD2
1	V2HS_157951	CTTGAAGAATTACATCTAA	79871	RPAP2
2	V2HS_195593	CGAATTATGTGAGTAAATA	79875	THSD4
1	V2HS_158239	CTAAAGATAGAGAACATTT	79925	SPEF2
1	V2HS_137114	CACCTAATCATGTTTCTTT	80205	CHD9
1	V2HS_137348	CGTATTTAGCCCGTCACAT	80273	GRPEL1
1	V2HS_137570	CTCTGGAGCCTATGGCTTT	80380	PDCD1LG2
1	V2HS_158495	CATATAGGATCTTTGAGTA	80746	TSEN2
1	V2HS_44872	GATATAAAGTTGTAGAGAA	81035	COLEC12

1	V2HS_286010	CTGTCAAGGAGAACACTCT	81610	FAM83D
1	V2HS_137892	CTTCTAATTGATAAGGCAA	81856	ZNF611
1	V2HS_117394	CTCCCTTTCTGGGCTTCCA	83661	MS4A8B
1	V2HS_178405	CAGAGTTCAAGAAGCAATA	83992	CTTNBP2
1	V2HS_14653	GACAGACACCCAGAAGGAA	84133	ZNRF3
1	V2HS_237215	GCAATACTACTTTAATAGT	84236	RHBDD1
1	V2HS_234793	CTGGGAAATAGGAGGCTTA	84293	C10orf58
1	V2HS_100677	CTCCTCATGGATTCCAGAA	84456	L3MBTL3
1	V2HS_40025	CAGACATGATGATTGCAAA	84612	PARD6B
1	V2HS_159755	CTGTAAACACCATTGAAA	84765	ZNF577
1	V2HS_177189	CTGGCAAATATGAAGGTAA	84791	C1orf97
1	V2HS_275500	CATGAATAATCTTGAAAT	84899	TMTC4
1	V2HS_177752	CATCGAGTACGTTTCTTTA	84916	CIRH1A
1	V2HS_261809	GACAATATGCTTATTCTA	84930	MASTL
1	V2HS_138238	CTTCCTAACCTCTTAAGCA	84937	ZNRF1
1	V2HS_177895	CCTAGTAGCAATTAAGGAA	84942	WDR73
1	V2HS_158692	CTTTAAGAGTGGAAGTAT	85021	REPS1
1	V2HS_139121	CAGTCACCATCCCATGATA	85285	KRTAP4-1
1	V2HS_160001	CACATCCTAACCTGAGACA	85416	ZIC5
2	V2HS_149470	GAATGGATTTCTCCTCTAA	85464	SSH2

1	V2HS_159974	CATATGTGGAGCAAGAGAA	85481	PSKH2
1	V2HS_159971	CTCTTGGTGTGATCACATA	85481	PSKH2
1	V2HS_244577	CCATCTCACTCTAAAGATA	89777	SERPINB12
1	V2HS_70495	GCCTATCCGTGTATATGGA	89781	HPS4
1	V2HS_262084	CACTCTTCCCTTTCCTGA	90199	WFDC8
1	V2HS_35694	CCGCCAAGGTGTACTACAT	90288	C3orf25
1	V2HS_39634	GAGTGAGCATGTAACAAGA	90737	PAGE5
1	V2HS_215547	AATATTACTGGCAAAGTAA	90987	ZNF251
1	V2HS_70702	CAATCCCTGTGGAACAGGT	91450	LOC91450
1	V2HS_68638	GATTTAGTGTCTTTAGTTA	91464	ISX
1	V2HS_247493	GGGATACAGAGATAAACAA	91464	ISX
1	V2HS_57496	CCAGGCTGATCTGACTGTA	91526	ANKRD44
1	V2HS_267209	CAGGTAGTATTGTGCTTTA	92017	SNX29
1	V2HS_285951	GACACCTAGATGTTGTGAA	92565	FANK1
1	V2HS_36372	CTCACAATGATCATTTAAA	92691	TMEM169
1	V2HS_85938	CACAAATACTCCTTTAATA	96459	FNIP1
1	V2HS_69490	AGGATACAATCAGTGAGAA	114926	C8orf40
1	V2HS_12168	CAATGCATATCCTATGTAA	115207	KCTD12
1	V2HS_19927	GGATTAAGTAGGTGAGTTT	115265	DDIT4L
1	V2HS_265073	CAATATATGCTGTAGAGCA	116093	DIRC1
1	V2HS_88391	GTTCTAAGCATATCTTTGA	116840	CNTROB

1	V2HS_74988	CAGTTTATCTCTGAAACTA	117154	DACH2
1	V2HS_53980	CCCTTTCTGCTTAGCATAT	119774	OR52K2
1	V2HS_247460	GTAATCTCAGCATAATTAT	120065	OR5P2
1	V2HS_206410	GACCAAATCTGAGTGATAA	121006	FAM186A
1	V2HS_24930	ACAGCTACATCAGTCAGTA	123591	C15orf27
2	V2HS_24825	GGGACTCTCTGTATATGGA	124044	SPATA2L
1	V2HS_180154	CCACTAGAGCAGGTGACAA	124773	C17orf64
1	V2HS_16158	CTCACTCACTCATTGACAA	126868	C1orf161
1	V2HS_18851	ACCTTGATCTGAGTATGAA	127495	LRRC39
1	V2HS_250724	CTGCAGATGACCAGTACCA	129138	ANKRD54
1	V2HS_68841	GGTATGAAGTCTTTCCATA	130026	ICA1L
1	V2HS_180819	CAGAGAATTTGGTTACCAA	131054	LOC131054
1	V2HS_58888	CTCCTGAGATCCTAATGGA	131890	GRK7
1	V2HS_19354	GAAGCCATGTCCGATTATA	132671	SPATA18
1	V2HS_22665	AGGCTTAAATAAAGGACTA	133686	C5orf33
1	V2HS_26662	AGAAGCAGCTGAACAATTT	134218	DNAJC21
1	V2HS_21442	GTGAGAGTATGAATGAAGA	134266	GRPEL2
1	V2HS_44435	GCTGGAAACCGACTTATTC	134829	RLBP1L2
1	V2HS_21288	CTGTTTCTTAGATTACAAT	135138	PACRG
1	V2HS_139201	CTGAGTTAATTGGGTTGAA	135458	HUS1B
1	V2HS_17883	CAAACAATCTTATAGACAA	137994	LETM2

1	V2HS_180722	GAATCAATCTGATGAGAGT	139363	MAGEA13P
1	V2HS_27953	CTGCTAAATTTAGAGCAAT	139629	RP11-167P23.2
1	V2HS_277714	CACTCAGCCTTACCATCGT	140710	C20orf117
1	V2HS_225634	CAGTGAGGGCCAATAATTA	142685	ASB15
1	V2HS_254671	CAGTGAGGGCCAATAATTA	142685	ASB15
1	V2HS_20718	ACCTTAATATTTACAAGAA	144193	AMDHD1
1	V2HS_62875	GCAGATTTGTTGGAACAAA	144608	C12orf60
1	V2HS_32949	CAGAGATGATTCTAGATTA	145741	FAM148A
1	V2HS_21852	CAACCAAGTTTATCAGAAA	145858	C15orf32
1	V2HS_162645	CGCTGTAAACTGAATCTAT	146713	HRNBP3
1	V2HS_17277	GTCACTGTCAGATTAATCA	146857	SLFN13
1	V2HS_17047	CAAGTTACGATATATTCAA	148362	C1orf58
1	V2HS_18884	CATCTATTGACTATGAATA	149465	WDR65
1	V2HS_50752	CTGAGCCACTGTTTGAACA	149998	LIPI
1	V2HS_119892	CAGAATACAAGGTTTCAAT	152573	SHISA3
1	V2HS_49049	CAATTCATTTGTAATGCAT	152815	THAP6
1	V2HS_226072	GTCATGGTCAGATGGAATA	154865	IQUB
1	V2HS_188382	CCTAATTGGTGCTCAATAA	155060	ZNF783
1	V2HS_233445	CATCTACATTCTTTACTAA	157657	C8orf37
1	V2HS_36154	GACTTAAACAGTAAGACTA	158326	FREM1

1	V2HS_49646	CAGTTGATCATATTCAGAA	158506	ZNF645
1	V2HS_22617	GTAACACTACTATAGGAAT	158696	FLJ30672
1	V2HS_53699	GCCTGAGATCTTCACTGAA	161497	STRC
1	V2HS_180897	GACAATATCTATGAAGATA	163259	DENND2C
1	V2HS_44617	CGGAGGAACTCTGTTAGAA	163882	C1orf71
1	V2HS_160813	CTGAGAATGACAGTTGGAA	170384	FUT11
1	V2HS_99934	GTCTCACTTTGCTTCCTAT	171017	ZNF384
1	V2HS_41554	CCAAGGACACTAAAGACAA	171482	FAM9A
1	V2HS_43890	GAGCAATACTTCTTAGTAG	196792	FAM24B
1	V2HS_43197	ACACCTACCTCTTATTGGA	201456	FBXO15
1	V2HS_221598	CTCAAGAACTTACAGTTAA	201633	TIGIT
1	V2HS_51123	GCATTAATATCTAACATAA	203427	SLC25A43
1	V2HS_140662	CAAAGATGGGCTGATTCAA	203522	DDX26B
1	V2HS_181632	CACAGAATCCAAAGGATTA	206412	C6orf163
1	V2HS_145231	GAGATTCAAGGGAGATTAA	222584	FAM83B
1	V2HS_254712	CAAAGCCACGAATAACCTA	223082	ZNRF2
1	V2HS_190835	GTTATTTAACATTCTAATA	246705	C21orf94
1	V2HS_122018	GAGAACATGCAGACAGTTA	252946	CYorf16
1	V2HS_82212	GACTCCCTGTGTTAGCTAA	253264	LOC253264
1	V2HS_259579	CAGGAAACTGAATAATTAT	254100	LOC254100

1	V2HS_82999	CCCTGGTATTCAAGAGAAA	254312	LOC254312
1	V2HS_184674	CAAAGCGAGTTTATTTATA	256006	ANKRD31
1	V2HS_225962	CTAAAGACGTGGAGAGGTA	256643	CXorf23
1	V2HS_264918	CTATTAATAAGTTTGACTT	283267	LOC283267
1	V2HS_237873	GGATTATCCAGATACTACTA	283378	LOC283378
1	V2HS_57228	CCATCAGAGTCTCTGACAT	283450	C12orf51
1	V2HS_212263	CTAAGAATTTCCCAATAAT	283459	GATC
1	V2HS_178714	CTGTAATTATGGATGAAGA	283461	C12orf40
1	V2HS_235170	CCAAGAACTCATAAACTA	283508	LOC283508
1	V2HS_220876	CCTTCTCAGTGAAGTAAAT	284309	ZNF776
1	V2HS_103335	CTTACCCAGGCTTAGGCTT	284340	CXCL17
1	V2HS_15849	GATCCTGCTTGATTATTAA	284649	DKFZP564C196
1	V2HS_183469	CAGAGGAGATGACCATGAT	284798	LOC284798
1	V2HS_178983	GAGAAAGAAACCTCATAAA	285349	ZNF660
1	V2HS_57118	GAGATAACGTAGGAATATT	285464	CRIPAK
1	V2HS_21604	GTTAGACACTATGTTCAAA	285593	LOC285593
1	V2HS_222787	CAGGACAAGTGTAAGTGA	285605	DTWD2
1	V2HS_141654	GATTTCTGACGAATTGATA	285908	LOC285908
1	V2HS_76473	GTTAGACTCACAAATAATA	286336	FAM78A
1	V2HS_149596	CTCATTAATAACCTGATT	326332	FLJ31813
1	V2HS_14455	CTATATGTGGACCATTAAT	339745	SPOPL

1	V2HS_166835	CTCTGAGCTGCCAGGATAT	339902	hCG_1813818
1	V2HS_164990	CATCTTAAATCAGAAGATA	339967	TMPRSS11A
1	V2HS_256141	GACTCTGGCCTTAAGACCA	340508	LOC340508
1	V2HS_231899	CCAAGCAGAGATTGGATCA	342125	TMC3
1	V2HS_104020	CTCTCACCTCAGTGTGTT	343406	OR10R2
1	V2HS_123598	CACTTTCTGCGAGCTTTAT	344332	LOC344332
1	V2HS_165618	GAAATGCAGTTCAACCGGT	345611	IRGM
1	V2HS_143861	CATGGAATCTCAAGGAAGT	347365	ITIH5L
2	V2HS_149689	CTTGGTGAGACATACTAGA	353164	TAS2R42
1	V2HS_286589	CATACTTCCTGTTTGACAT	387129	NPSR1
1	V2HS_274120	GTATGTATAGTAATATCTA	387921	NHLRC3
1	V2HS_180117	GTGAAGTTTGTGAAATATA	388272	C16orf87
1	V2HS_77701	CAAGGACGAGCATACCTCT	388403	YPEL2
1	V2HS_284594	GACCTTCTCAACATGGAAT	389152	FLJ46210
1	V2HS_122663	CTGATAGGAAATGCCATTA	390093	OR10A6
1	V2HS_255821	CGTTGTAAACCCATTTAT	390327	OR6C70
1	V2HS_83891	GCTTAACCCTGTGATATAT	390439	OR11G2
1	V2HS_17906	GATATATATACCTCTCTAC	391160	LOC391160
1	V2HS_207978	CTTGGACATTCAAAGAGAT	392382	LOC392382
1	V2HS_89876	CCTACAGGCTCATGCAAAT	399716	DKFZp667F0711
1	V2HS_16803	CTCGCTGTAGTTGTACATT	400500	BCAR4

1	V2HS_90609	GTGTCAATCTGTAGTAATT	400541	LOC400541
1	V2HS_183447	GAGATGACACTGGGCTTTA	400831	LOC400831
1	V2HS_119759	CAAACATCTTCCTTAAAGT	400891	LOC400891
1	V2HS_121076	GAAGGAAACACTTTATAGA	401242	LOC401242
1	V2HS_187186	GGCATAGGAACCAATCAGA	401504	LOC401504
1	V2HS_75780	ACCATTCTGAGTTTATTAA	431707	LHX8
1	V2HS_164049	GAAATATTGGTGTCATATA	440338	LOC440338
1	V2HS_141684	CATCTTCACCATTCAACTA	641977	40069
1	V2HS_12094	CAGAAAGTAGTGTGGGATA	643790	LOC643790
1	V2HS_122234	GAGACGTATACCCAGATGA	645312	GLTPP1
1	V2HS_86571	GACATAGATTTATAGATAT	645974	PABPC1L2B
1	V2HS_164919	GATATAACCAGGCCCTTTA	646603	LOC646603
1	V2HS_165267	CTGAGATTGCTCACAATGT	728572	LOC728572
1	V2HS_207434	CACTGATCTGTGAAGTAAA	728626	LOC728626
23	V2HS_29650	GTGCTTTATTCAAATCTAA	729324	hCG_1986447
1	V2HS_90173	GCCAAGTCAGCCAGTGAAA	729652	LOC729652
1	V2HS_25168	GTGTACACATGCTGACACA	730139	LOC730139
1	V2HS_50590	CTGACACTAGCCACACAGA	730461	LOC730461
1	V2HS_205677	GGAACAGAAGTAGAGGAAT	100130102	LOC100130102
1	V2HS_103839	CCAGCAATGAGCACAGAAT	100130269	LOC100130269
1	V2HS_178914	CCTTGAAGAAGTTATACCA	100130934	ZNF663
1	V2HS_268515	CTCACAGTTCCACAATAAT	100131539	LOC100131539

1	V2HS_20030	ATTATTATCTTGGCTAGTA	100132003	LOC100132003
1	V2HS_184330	CTCCTTATCGAAAGCGAGT	100132051	LOC100132051
1	V2HS_233813	CACCTACAGCCAAGTCCA	100132962	LOC100132962
1	V2HS_164471	CTCTGGAAGCCCTACATTT	100133405	LOC100133405
1	V2HS_209258	AGGCTAGTCCGAAGGTGCA	10408, 100133778, 100134408	LOC100133778, LOC100134408, MYCNOS
1	V2HS_75385	GTGAATTACATCTTCTTTA	12, 5104, 5265	SERPINA1, SERPINA3, SERPINA5
1	V2HS_234941	GAAACTCTAAGCAGTATGA	1278, 5406	COL1A2, PNLIP
1	V2HS_196358	CATTCAATCTCTAGGTGTA	143, 645771, 100130199	LOC100130199, LOC645771, PARP4
1	V2HS_19596	CATAGCGTCTTAATTGTTT	147341, 494470	C18orf23, RNF165
1	V2HS_164291	CCCTCCATCACTGCTGAGA	150622, 439921	FLJ30594, MXRA7
1	V2HS_113840	CACATATTCTTGCCAATTA	2252, 387628, 654466, 728832, 100132771	FGF7, KGFLP1, KGFLP2, LOC100132771, LOC728832
1	V2HS_19850	GCTGCTATGTGGAATGTTC	23433, 284988, 730041	LOC284988, LOC730041, RHOQ
1	V2HS_88200	CTCTCAGAGGTCCAGATAA	27287, 391518	VENTX, VENTXP7
1	V2HS_132064	CTATTAGTCAGATTGGAAT	2966, 653238, 728340, 730394	GTF2H2, GTF2H2B, LOC728340, LOC730394

1	V2HS_66140	GTGGAACATTGAGAATTTA	4301, 730031	LOC730031, MLLT4
1	V2HS_152288	CGCACCTTCATCGCCATCA	4831, 654364	NME1-NME2, NME2
1	V2HS_173673	CAATGGCCCTGTTTATTAA	55629, 100131261, 100132235	LOC100131261, LOC100132235, PNRC2
1	V2HS_37080	GAAAGCGTCTTAAAGCATA	5612, 644363	LOC644363, PRKRIR
1	V2HS_39928	GAGACAGCTGTAGATAACA	57019, 728599	CIAPIN1, CIAPIN1P
1	V2HS_72006	GAGAGTATCTTGCAGAAAT	57180, 653857	ACTR3B, ARP11
1	V2HS_228820	GTGGCCTATTCTGCATATA	57335, 254272, 353149	TBC1D26, TBC1D28, ZNF286A
1	V2HS_270169	CAAATCCATTATTTAATGA	6184, 285226	FLJ40473, RPN1
1	V2HS_61131	GAAAGAAGTGGTCAATAAA	6189, 146053, 400652, 439992, 643932, 643981, 646527, 652670, 727951, 727970, 729150, 730861, 731260, 731542, 100129742	LOC100129742, LOC146053, LOC400652, LOC439992, LOC643932, LOC643981, LOC646527, LOC652670, LOC727951, LOC727970, LOC729150, LOC730861, LOC731260, LOC731542, RPS3A
53	V2HS_93855	CTTAGTAAAGGACTTATCA	6230, 100131196	LOC100131196, RPS25
1	V2HS_112625	CAAACAATCTGTGGATGGA	689, 503543	BTF3, CTD- 2090I13.4
1	V2HS_202686	GGCGCCTAATGATGTCTAA	7335, 387522	TMEM189- UBE2V1, UBE2V1

1	V2HS_98101	GGCGCCTAATGATGTCTAA	7335, 387522	TMEM189- UBE2V1, UBE2V1
1	V2HS_188921	CTTATTAAACATCAGAGAA	7699, 7710, 84874, 388536	ZNF140, ZNF154, ZNF514, ZNF790
1	V2HS_89833	GGATTATGTTCAAGAAGTT	79031, 285359	LOC285359, PDCL3
755	V2HS_17314	GATATTCTGAGTGTCATAA	8741, 407977	TNFSF12- TNFSF13, TNFSF13
52	V2HS_17317	GAGACTCTATCCGATGTA	8741, 407977	TNFSF12- TNFSF13, TNFSF13
123	V2HS_17313	GCCGCCCTCTGCTAGGGAA	8741, 407977	TNFSF12- TNFSF13, TNFSF13
299	V2HS_17318	CTCCAGAGATGTAGCTATT	8741, 407977	TNFSF12- TNFSF13, TNFSF13
44	V2HS_17316	GGTGCCTTCGCAGTCAAAT	8741, 407977	TNFSF12- TNFSF13, TNFSF13
1	V2HS_23046	GGACCAGTAGCAAAGGAGT	9349, 646949	LOC646949, RPL23
1	V2HS_125487	CAAAGAGAGCAGAGACTTA	-	-
1	V2HS_187876	CACAGGAATGTTTAGAAAT	-	-
1	V2HS_167863	CACGTTCTGGCTACACATA	-	-
1	V2HS_126248	GAAGTTATCATTAGTCATA	-	-
1	V2HS_128780	GGAAGAGAACATGTGCTAA	-	-
1	V2HS_180486	GTCATGCCGGAACGATTCT	-	-
3	V2HS_167819	GTTCCCTGTTTCTGTTTCAGA	-	-
2	V2HS_17653	ATAAGCCATTGCCTCGATA	-	-

2	V2HS_226760	CAGATTCCTCATTAAATTAT	-	-
2	V2HS_187827	CAGCCACAAGGAATGCTGG	-	-
2	V2HS_126428	CAGTGTTGTGGTAACATTA	-	-
2	V2HS_77889	CATTCTAAGCTTGCACATA	-	-
2	V2HS_195878	CTCATTAGATGACAGTAAT	-	-
2	V2HS_226170	CTCTTCATTCTGAACAAT	-	-
2	V2HS_62017	GATATCCAGTGCATTAGAA	-	-
2	V2HS_86103	GTTTGAAAGTTAAGAGGAA	-	-
1	V2HS_71722	AACCCAACGAGAAATAGTA	-	-
1	V2HS_90995	AGAATATACTCTGTTCATA	-	-
1	V2HS_70405	AGCATCTGCCCAAACCTAA	-	-
1	V2HS_38009	AGGACCAATTACAACATAT	-	-
1	V2HS_31146	AGTACAAATTCCTATGCCA	-	-
1	V2HS_215437	AGTTGAATTTAGTAATGGA	-	-
1	V2HS_18226	CAAAGCTTCTGGATTTCAA	-	-
1	V2HS_123869	CAAGGTCACTGACACAGAA	-	-
1	V2HS_68703	CAAGTTTAGTTGTAACGAA	-	-
1	V2HS_229575	CAATACCAGCTAAATTAAT	-	-
1	V2HS_128328	CACAGGATCTGTCATTCAT	-	-
1	V2HS_129462	CACATACTCCCAGAAGAGA	-	-
1	V2HS_60788	CACATAGCCTTTCCCAAGC	-	-
1	V2HS_109586	CAGACACCACGATGCCAAA	-	-

1	V2HS_122479	CAGAGAGAGATGTCTAGGA	-	-
1	V2HS_190852	CAGAGTTACGAATTACGTT	-	-
1	V2HS_123450	CAGATCAAGACCCTTAACA	-	-
1	V2HS_127605	CAGATTAACCAAGATTGAT	-	-
1	V2HS_105348	CAGGAGCAAGTTTATCTTT	-	-
1	V2HS_108377	CAGTAAAGACAATAACGAA	-	-
1	V2HS_128185	CAGTATCTGGGAAGTGAAA	-	-
1	V2HS_167941	CATGCAAGGAGAGCCTCAT	-	-
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1	V2HS_214255	CATTGTTGATAGTAGAATA	-	-
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1	V2HS_123309	CCATACAGTTGTTGTGCAT	-	-
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1	V2HS_59571	CCCATTAGAGGAGATCCAT	-	-
1	V2HS_187558	CCCGTGATCTAATCCAGAA	-	-
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1	V2HS_127050	CCTTAATTCTCAGCTTAAT	-	-
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1	V2HS_142912	CGGAGACATTCTCCAGAAT	-	-
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1	V2HS_21591	CTCCTAAAGCCAAGGCCAA	-	-
1	V2HS_127321	CTCCTCAGCTCTCCAGTGA	-	-
1	V2HS_228360	CTCTTATGCAATTGATGGA	-	-
1	V2HS_147868	CTGCCATAAGTGGAACATA	-	-
1	V2HS_190045	CTGCCTGACACAGATGTAT	-	-
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1	V2HS_144118	CTGGCTCATCACTATAGTA	-	-
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1	V2HS_223954	CTTCACTTCTTAAGATAAT	-	-
1	V2HS_129996	CTTCTGGAAAGTGGCACTA	-	-
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1	V2HS_15504	GAGATTACAGGACCTAAGA	-	-
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1	V2HS_266928	GAGTGGATCTTGGAAATAA	-	-
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1	V2HS_149621	CTGTTTCACTTGTAGGTTA	-	-
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1	V2HS_147212	CTCATCTTGAAACACCAGA	-	-
1	V2HS_148382	CTGAGTTCCAGAGATAAGA	-	-
1	V2HS_186763	CTGTCCAGAATTAATGAGA	-	-
1	V2HS_45232	GAGTACAGACCGAATATTT	-	-

\* a hyphen (-) indicates unannotated genes; no gene symbol or gene ID is associated.

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