MICRORNA REGULATION AND HUMAN PROTEIN KINASE GENES REQUIRED FOR INFLUENZA VIRUS REPLICATION

by

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(Under the Direction of Ralph A. Tripp)

ABSTRACT

Human protein kinases (HPKs) have profound effects on cellular responses. To better understand the role of HPKs and the signaling networks that influence influenza replication, a siRNA screen of 720 HPKs was performed. From the screen, 17 “hit” HPKs (NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5, CALM2, PKN3, and HK2) were validated as important for A/WSN/33 influenza virus replication, and 6 HPKs (CDC2L5, HK2, NEK3, PANK4, PLK4 and SGK3) identified as important for A/New Caledonia/20/99 influenza virus replication. Meta-analysis of the hit HPK genes identified important for influenza virus replication showed a level of overlap, most notably with the p53/DNA damage pathway. In addition, microRNAs (miRNAs) predicted to target the validated HPK genes were determined based on miRNA seed site predictions from computational analysis and then validated using a panel of miRNA agonists and antagonists. The results identify miRNA regulation of hit HPK genes identified, specifically miR-148a by targeting CDC2L5 and miR-181b by targeting SGK3, and suggest these miRNAs also have a role in regulating influenza virus replication. Together these data advance our understanding of miRNA regulation of genes critical for virus replication and are important for development novel influenza intervention strategies.

INDEX WORDS: Influenza virus, host protein kinase, antiviral signaling, RNA interference, RNAi, short interfering RNA, siRNA, genome screen, microRNA, miRNA

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DEDICATION

To my sisters, Emily and Lindsey, for your unfailing love and encouragement. Thank you for making me smile is all your little ways. And to my Mom and Daddy, I love you.

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CHAPTER 1

INTRODUCTION

Influenza A virus is the causative agent of the common flu which affects 5 to 20% of the population during the fall and winter seasons and results in an estimated 226,000 hospitalizations and >36,000 deaths in the US every year [1]. Studies of influenza virus biology have revealed elaborate mechanisms by which the virus interacts with its host cell as it affects various cellular pathways involved in the synthesis of viral and cellular proteins to facilitate replication and to evade the innate antiviral response [2-5]. However, limited information is available concerning the role of host genes and influenza virus replication. Our long-term goal is to identify host kinase genes utilized by influenza A so that we may better understand the biology of virus replication and develop novel disease intervention strategies. The specific hypothesis of the research proposed is that host genes involved in protein kinase signaling pathways are critical for influenza virus replication and provide novel therapeutic targets that may provide a disease intervention approach to reduce or prevent influenza virus replication. This hypothesis is based on the known phosphorylation-regulated signaling events triggered when a host cell becomes infected with influenza [6]. These signals affect specific cellular actions, such as the interferon induced antiviral state, and are generally utilized to defend the host from infection and virus replication. Recent studies indicate that influenza viruses have acquired the capability to modify host cell antiviral responses to infection to support virus replication. For example, activation of the RAF-MEK-ERK host signaling pathway is required for influenza virus replication, and blocking this pathway causes the viral RNA to be retained in the nucleus resulting in a strong impairment of virus replication [7]. Additionally, nuclear factor κB (NF-κB), a hallmark antiviral transcription factor, has been identified as a cofactor contributing to influenza replication, and a recent study showed that acetylsalicylic acid (ASA) treatment blocked influenza virus replication by ameliorating influenza-mediated NF-kB-inhibiting activity [8]. Last, and most related to kinome pathways, PKCβII (a member of the protein kinase superfamily) was identified as an important regulator of the late endosomal sorting events used by the influenza virus, and protein kinase inhibitors have been shown to block influenza virus from entering the host cell [9, 10]. Taken together, these and related studies showing host gene regulation of influenza virus infection and replication provide rationale for the proposed studies as other host genes, and likely protein kinase genes, may be important for influenza virus replication. Identifying these genes will provide the framework for better understanding the biology of the virus-host interface, and may provide therapeutic targets for disease intervention approaches. Here we address the following specific aims:

Specific Aim 1. To identify host genes in the human kinome required for influenza replication. The working hypothesis is that influenza virus requires host protein kinase gene expression to support virus replication, and these genes can be identified using siRNAs to selectively silence individual host kinase genes.

Specific Aim 2. To determine cellular pathways associated with kinase genes identified as important for influenza virus replication. The working hypothesis is that influenza virus co-opts kinase gene pathways to facilitate replication.

Specific Aim 3. To understand other host protein kinase gene regulation related to microRNAs (miRNA) expression. The working hypothesis is that host cell miRNA regulates host protein kinase genes providing an additional mechanism that affects influenza virus replication.

References

1. Fiore, A.E., et al., *Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP), 2007.* MMWR Recomm Rep, 2007. **56**(RR-6): p. 1-54.
2. Basler, C.F., *Influenza viruses: basic biology and potential drug targets.* Infect Disord Drug Targets, 2007. **7**(4): p. 282-93.
3. Epstein, S.L., *Control of influenza virus infection by immunity to conserved viral features.* Expert Rev Anti Infect Ther, 2003. **1**(4): p. 627-38.
4. Katze, M.G., Y. He, and M. Gale, Jr., *Viruses and interferon: a fight for supremacy.* Nat Rev Immunol, 2002. **2**(9): p. 675-87.
5. Nagata, K., A. Kawaguchi, and T. Naito, *Host factors for replication and transcription of the influenza virus genome.* Rev Med Virol, 2008. **18**(4): p. 247-60.

6. Ludwig, S., et al., *Influenza-virus-induced signaling cascades: targets for antiviral therapy?* Trends Mol Med, 2003. **9**(2): p. 46-52.

7. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* Febs Letters, 2004. **561**(1-3): p. 37-43.

8. Mazur, I., et al., *Acetylsalicylic acid (ASA) blocks influenza virus propagation via its NF-kappaB-inhibiting activity.* Cell Microbiol, 2007. **9**(7): p. 1683-94.

9. Sieczkarski, S.B., H.A. Brown, and G.R. Whittaker, *Role of protein kinase C betaII in influenza virus entry via late endosomes.* J Virol, 2003. **77**(1): p. 460-9.

10. Root, C.N., et al., *Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor.* J Gen Virol, 2000. **81**(Pt 11): p. 2697-705.

CHAPTER 2

LITERATURE REVIEW

Overview of Influenza Virus

Influenza viruses have been causing disease for centuries as they were likely the disease described by Hippocampus back in 412 BC [1]. Influenza virus infection can cause disease in a variety of animals including birds, humans and a variety of others such as seals, whales, horses, and cats [2]. Annual influenza virus epidemics generally result in lost workforce productivity and strain health services. Worldwide, an estimated three to five million individuals develop severe disease, resulting in >500,000 deaths, most of who are either very young, elderly, or immunologically compromised [3]. The virus also has the propensity to cause pandemics which affect a larger proportion of the population, often with higher morbidity and mortality rates. The most notable pandemic, the 1918 “Spanish influenza”, was responsible for killing an estimated 40–50 million people worldwide [4, 5]. More recently, the world experienced a less severe swine-origin H1N1 2009 pandemic [6].

Influenza viruses are in the *Orthomyxoviridae* family and have a negative-sense RNA genome. The virus has an eight segmented genome that encodes for 10-11 viral proteins. On the surface of the mature virion are hemagglutinin (HA), neuraminidase (NA), and the membrane ion channel protein, M2. HA and NA are both surface glycoproteins and the antigenic determinants of the virus, with HA being the most abundant surface protein [7]. Inside the virion are the nucleoprotein (NP), the matrix protein (M1), as well as a polymerase complex (PB1, PB2, PA). In some viruses, the PB1 segment encodes a 90-amino acid protein, PB1- F2, which preferentially localizes to the mitochondria of infected cells [8]. The virus has two nonstructural proteins, NS1 and NS2, the latter being involved in nuclear export [9]. Due to its negative polarity, the influenza genome cannot be translated directly into protein upon entering the host cell, and thus encodes its own RNA-dependent RNA polymerase (RdRp). The polymerase forms a complex with the viral RNA, and NP, termed the ribonucleoprotein (RNP) complex. The NP composes most of the RNP as it forms the core to which the RNA wraps around. The exact interaction between the three in the complex has yet to be fully defined.

There are three influenza types, i.e. influenza A, B and C [10]. Influenza virus types B and C are primarily human pathogens and rarely infect other species [11]. Influenza B viruses periodically cause epidemics, but rarely cause pandemics, while influenza C viruses are endemic and sporadically cause mild respiratory disease in humans. Type A influenza viruses are ubiquitous, affecting many different avian and mammalian species with wild aquatic birds being the natural reservoir [12]. Only influenza virus A has established as subtypes and there are currently 16 HA and 9 NA subtypes. Although all RNA viruses mutate, mostly Influenza A viruses are associated with exchange of genetic material between strains, referred to antigenic shift [13]. Antigenic drift describes the accumulation of changes in the antigenic sites of the HA or NA and is responsible for the annual influenza epidemics. Due to antigenic drift, a new vaccine must be developed and produced each year to match the predicted circulating strain. Antigenic shift, with its pandemic potential, occurs when an entirely different HA or NA is obtained by an exchange of genes with another virus, termed reassortment. Influenza pandemics occur when a human population is introduced to an influenza virus with a HA to which they are immunologically naive. With the constant emergence of new influenza strains, a systematic nomenclature was developed. New influenza viruses are named based on their genera (A, B, or C), the host animal from with the virus was isolated, the geographic origin of the isolate, the unique laboratory or other reference number, the year of isolation, as well as the HA and NA subtype [14].

Influenza Virus Replication

Initial interaction of influenza virus with the host involves binding of the HA protein to a sialic acid receptor on the host cell surface [15-19]. It is thought that influenza virus requires N-linked glycoproteins for entry into a host cell, and that *in vivo*, sialic acid alone may not be a sufficient receptor [20]. Sialic acid refers to the terminal sugars found in N- and O-linked glycoproteins that can be made of many derivatives of neuraminic acid. Sialic acid molecules are classified as to how they are linked to the underlying sugars, i.e. according to the α2-carbon. Different α2-carbon linkages results in different conformations of the host receptor and thus has a role in determining host specificity [21]. It is likely that the HA of different influenza virus strains can have a preference for binding to different sialic acid linkages found in different species, an effect that may contribute to different tissue tropisms. Although not absolute, human influenza virus prefers an α2,6 linkage and avian influenza virus an α2,3 linkage [16, 22]. In addition, humans, ferrets [16, 17, 23-25], swine [26], and some avian species [27-31] can express both α2,6 and α2,3 linked sialic acids but with different tissue distribution. Also, some viruses can bind to both α2,6 and α2,3 linked sialic acids [16, 32].

Once attached, the influenza virus is internalized via one of four processes: clathrin-coated pits, caveolae, macropinocytosis, or a pathway not involving these [33]. Like other enveloped viruses, influenza requires a low pH to initiate fusion. As it travels from the early to late endosome, the pH is lowered and the viral HA is triggered to release its fusion domain and the viral and host endosomal membranes fuse. The influenza virus M2 allows an influx of H^+ ions into the virion and the viral RNA, in the form of an uncoated viral RNP complex, is released into the cytoplasm [34].

The influenza NP contains nuclear localization signals that are necessary for nuclear import, and once the RNP is free in the cytoplasm, it is actively transported to the nucleus [35]. This energy-dependent nuclear import, or active transport, is accomplished by NP binding to the host factor importin α, which in turn binds another host factor, importin β, allowing viral RNP nuclear import [36]. Influenza dependence on the host nucleus and nuclear functions for RNA synthesis is a hallmark of this virus, and these processes are tightly regulated.

Once in the nucleus, primary transcription can occur as the negative-sense viral RNA (vRNA) is transcribed into positive-sense messenger RNA (mRNA) using its own RNA-dependent RNA polymerase as well as a host RNA polymerase II [37]. Unlike the vRNA, the mRNA is capped and has a polyadenylated (poly-A) tail. Binding of the RdRp to both ends of the vRNA is necessary for the activation of endonuclease activity and transcription initiation [38]. In a secondary transcription reaction, the newly transcribed vRNA is used as a template for additional viral mRNA synthesis. This viral mRNA synthesis requires a 5’ capped primer that is hijacked from host mRNA by viral PB2, a process termed cap snatching. Therefore, influenza viral RNA synthesis involves vRNA→mRNA, vRNA→complementary RNA (cRNA), and cRNA→vRNA reactions. Termination of transcription occurs when the RdRp reached a stretch of U residues in the mRNA in which it copies into a poly-A tail [38].

The RdRp complex is very important as it is required for the synthesis of mRNA, cRNA, and vRNA. As noted, it is composed of the PB1, PB2, and PA, where the PB1 encompasses much of the actual polymerase activity as it drives the addition of nucleotides to the RNA transcript [38]. PB1 also contains the endonuclease domain and is probably responsible for endonucleolytic cleavage of cellular pre-mRNAs. The PB2 is the “cap snatcher”, and binds to the cap structures of the host cell pre-mRNA molecules [38]. PA is involved in the assembly of functional polymerase complex, cap binding and vRNA promoter binding [39]. The physical interactions in the RdRp have been characterized by extensive mutagenesis, showing that the N-terminal tip of PB1 binds to the C terminus of PA, and with the loss of PA comes the loss of RNA polymerase activity and viral replication [39].

The switch from transcriptase mode to replicase mode is dependent on viral protein synthesis. [40]. The first step in replication is when vRNA is copied into cRNA, a full length copy of the vRNA, and does not require a primer like mRNA synthesis. This requires a switch from the “capped” initiation to unprimed initiation with the help of viral NP and also the stretch of U’s at the 5’ end of the vRNA [38, 41]. The viral mRNA then leaves the nucleus and travels to the cytoplasm where it has access to host cellular machinery needed for viral protein translation.

The second step in replication is the copying of cRNA back to vRNA. While in the nucleus, the positive-sense RNA also serves as a template to make vRNA that will be packaged into the newly formed virions. Influenza M1 and NEP are crucial for trafficking of viral proteins to and from the nucleus as well as for assembly of the virion [42]. Viral assembly proceeds with HA, NA, and M2 viral proteins traveling through the endoplasmic reticulum, where they are folded and glycosylated [43]. Once the viral proteins are expressed and processed, they will assemble with the vRNAs at budding sites within the host cell membrane and M1 is believed to be an important regulator of this event. Once packaging is complete, the virus particle buds from the cell when influenza NA removes the sialic acid from the surface glycoprotein.

Pathogenesis of Influenza and the Host Response

Influenza virus replicates in epithelial cells lining the respiratory tract and both virus replication and the inflammatory response to infection contribute to lung pathology. The sialic acid receptor distribution in the host and the HA receptor specificity of influenza virus affects sites of virus replication and pathogenesis in the host [44-46]. The HA of most seasonal influenza viruses binds to α2-6 sialylated glycans expressed on the surface of the epithelial cells of the upper respiratory tract in humans [16]. In this case, infection is mostly limited to the upper respiratory tract causing milder disease and inflammation. Nasal discharges containing high titers of infectious virus facilitate spread among the human population. Highly pathogenic avian influenza (HPAI) viruses primarily bind to α2-3 sialylated glycans on type 2 pneumocytes in the lower respiratory tract of birds [16]. Targeting α2-6 sialic acids expressed on cells deeper in the respiratory tract makes it more difficult for HPAI to cause widespread infection among humans, however this cell tropism increases the severity of pneumonia in humans [47]. Several studies suggest that when influenza virus is passaged in a particular host, they virus adapts to that host by mutating the receptor binding site on the HA [48, 49]. It is thought that the HA of 1918 pandemic influenza virus originated in avian species and upon an amino acid change (E190D) the virus acquired the ability to bind to α2, 6-linked sialic acid [50-52]. Similarly, changes in the H5N1 HA have facilitated the ability to bind to α2-6 as well as α2-3 sialic acid [53]. Regarding the recent swine-origin H1N1 pandemic influenza A occurring in 2009, severe and fatal cases had a D222G substitution in the HA which changed the receptor binding specificity of the virus from α2-6 to α2-3 sialic acid and allowed it to infect ciliated bronchial cells [46, 54]. Therefore, amino acid mutations within the HAs of influenza viruses should be monitored to identify mutations that produce strains with potentially higher pathogenicity.

HA may also influence pathogenicity with respect to its susceptibility to host proteases. The HA (in a HA0 form) must be cleaved into two subunits, HA1 and HA2, for influenza viruses to be infectious [55]. The HA of seasonal influenza virus possesses a single arginine at the cleavage site and is cleaved by trypsin-like proteases that are produced by cells in the respiratory and gastrointestinal tract. For some notable HPAI, the HA possesses multiple basic amino acids at the cleavage site and can be cleaved by ubiquitous proteases, such as furin and PC6, and cause severe systemic infection leading to multiple organ failure and death [56].

The influenza virus RNA polymerase complex (PB1, PB2, PA) is able to mutate PA and PB2 to better support replication of avian viruses in mammalian cells [57-61]. Accordingly, A/Vietnam/1203/04 H5N1 virus isolated from a fatal human case was found to be highly lethal to ferrets and mice [62, 63]. When the viral RNA polymerase genes were replaced with those of a low pathogenic H5N1 virus, the pathogenicity of A/Vietnam/1203/04 H5N1 was significantly reduced in these animals [63]. It is also thought the RNA polymerase complex, along with NP, contributed to the pathogenicity of the 1918 influenza virus pandemic [64]. In some strains of influenza virus, the PB1 segment encodes a 90-amino acid protein, i.e. PB1-F2 that preferentially localizes to the mitochondria of infected cells and can induce apoptosis [8, 65]. For both the 1918 pandemic and H5N1 influenza viruses, a mutation (N665S) in PB1-F2 was shown to increase the secretion of proinflammatory cytokines and virus titers in the lungs [66]. Influenza NA and NS1 are also implicated in influenza virus virulence. NA is important for efficient viral replication [67] while NS1 antagonizes interferon production in virus-infected cells. Taken together, influenza HA, NA, NS1, its RNA polymerase complex and NP can all contribute to disease pathogenicity in mammals.

Pathogenesis of influenza virus is linked to virus infection and the host immune response to infection. Upon influenza virus infection of respiratory epithelial cells or alveolar macrophages, the single-stranded RNA of the virus is recognized by Toll-like receptors (TLR) including TLR7, as well as retinoic acid-inducible gene-I (RIG-I) [68, 69]. The signaling pathways of TLR7 and RIG-I induce the production of type I IFNs and activate antiviral host responses [70]. In turn, influenza virus avoids the innate immune response via NS1 which interferes with the RIG-I signaling pathway [71-75]. Specifically, NS1 interferes with an essential step in the type I IFN response by inhibiting the function of a tripartite motif (TRIM) 25 in the ubiquitination of RIG-I [76]. For example, it is known that NS1 of the 1918 pandemic influenza virus strain can suppress the expression of IFN-regulated genes and modify antiviral innate immune responses [77]. Influenza NS1 has been shown to also bind to the host antiviral protein kinase R (PKR) and inhibits its antiviral function by down-regulating the translation of the viral mRNA, which is mediated by phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) [78]. In mice, PKR was bound to NS1 at positions

123–127, and a mutation of these residues affected pathogenicity [73, 74]. It has also been shown that RIG-I and TLR7 induce the production of inflammatory proteins mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [79]. As a result, influenza virus infection may induce the upregulation of several inflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, TNFα, CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), and CXCL10 (IP-10) [80]. Of these, CCL2 recruits macrophages to the virus-infected lung [81]. CCR2-(a receptor of CCL2) positive macrophages express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induces alveolar epithelial cell apoptosis [82]. CCR2 deficient mice infected with influenza virus showed an inhibition of macrophage migration to the lung and increases survival rates [83]. This indicates that macrophages migrating to a influenza virus-infected lung may have a pathogenic role in pulmonary inflammation. Following infection with the 1918 virus, or HPAI H5N1 virus, substantial numbers of neutrophils have been shown to be recruited to the lung suggesting that neutrophils may also contribute to pathogenesis [80]. This is consistent with neutrophils functioning to limit virus replication and as a consequence lung inflammation [84].

The adaptive immune response has a role in protection and is sometimes linked with immune pathology. Seasonal influenza virus infection or influenza virus vaccination results in CD4 and CD8 T cells which provide cross-protection from other influenza virus strains by direct and indirect cytolytic activity and by antiviral cytokines during infection in the lung [85-87]. Interestingly, resident T cells may impair the innate immune response through inappropriate inflammatory responses. For example, poly(I:C) or LPS treatment in mice has been shown to result in pro-inflammatory response in both Rag-1 knockout mice lacking functional lymphocytes, and CD4/CD8 T cell depleted mice [88]. In addition, effector and memory CD4 T cells have been shown to inhibit macrophage inflammasome-mediated caspase-1 activation and subsequent IL-1b release, suppressing potentially damaging inflammation [89]. However, much of the inflammatory response is initiated via antigen presenting cell interaction and activation. For example, peripheral T cells require an initial interaction with dendritic cells (DCs) which migrate from the infected lung to draining lymph nodes for proper signaling and antigen activation a feature that promotes clonal expansion and increased T cell survival and accumulation [90, 91]. The interaction with pulmonary DCs can include pulmonary plasmacytoid DCs, CD8a+ DCs, or TNF-a inducible nitric oxide synthase (iNOS) producing DCs (tipDC). Although CD8 T cells have an important role in viral clearance, the release of the cytotoxic molecules such as granzyme, perforin and TNF-α and IFN-γ antiviral cytokines may also contribute to lung pathology. In transgenic mice expressing influenza HA antigen on the alveolar epithelial cells, it was shown that transfer of HA-specific CD8 T cell clones caused progressive lethal lung injury in the absence of active viral replication [92]. However, if these mice lacked Egr-1 (epithelial early growth response-1), no lung injury was reported, suggesting a role for ERK kinases induced Egr-1 in CD8 cell mediated immunopathology [93].

The subsets of CD4 T cells include Th1, Th2, and the more recently described Th17. Th17 cellular roles include anti-fungal defenses, inflammation regulation and promotes autoimmunity [94]. Th1 and Th17 hypercytokinemia has been correlated with severe pandemic H1N1 influenza [95]. Accordingly, expression of Th17-associated cytokines in the lungs of IL-10 knockout mice correlates with better survival upon lethal influenza virus challenge [96]. However, the role of IL-10 is controversial. In another study, IL-10 knockout mice showed better survival and increased viral clearance upon challenge with H1N1 (PR8) [96]; however, administration of monoclonal antibody to IL-10 receptor to block IL- 10 signaling resulted in increased and accelerated mortality and elevated inflammatory mediators [97]. Another factor that may contribute to immune-mediated pathology is the CD200 membrane glycoprotein that is highly expressed on resting airway epithelium. Interaction with its ligand CD200R on alveolar macrophages has been shown to provide inhibitory signals to maintain immune homeostasis [98]. CD200 knockout mice have been shown to lost more weight and have increased lung infiltration and lung endothelium damage following influenza virus infection; however, depletion of T cells prevented this increased pathology although with decreased viral clearance [98, 99]. These results suggest that both CD4 and CD8 T cells contribute to influenza clearance and immune pathology. It is becoming clearer that the balance between protection and pathological effects rely in part on a panel of inhibitory regulators, such as IL-10 and the CD200R-CD200 interaction.

Host innate and adaptive immune responses are complex, interconnected, and critical for defense against infectious diseases like influenza virus. Unfortunately, some of these immune responses lead to harmful consequences that are linked to the presence or abundance of viral antigen. Antiviral therapy offers an alternative, and for viruses lacking approved vaccines, is the main method of treatment for viruses such as H5N1. Specifically, oseltamivir treatment is quite effective for H5N1 as shown in a study where treatment commenced within 4 days of disease onset reduced mortality rates [100]. Although, there is a need for novel or adjunctive therapeutic options, recent studies suggest that selective down-regulation of harmful host responses may be possible. Interventions that affect host cellular pathways that are crucial for viral replication are of particular interest. For example, inhibition of the Raf/MEK/ ERK kinase cascade and activation of NF-kB lead to impaired virus replication and lessen the host pro-inflammatory cytokine responses suggesting potential therapeutic roles in influenza [101]. Unlike antiviral drugs, strategies that target the host will prevent the emergence of drug resistant viruses.

Disease Intervention

Vaccination is the most effective means in controlling influenza infection. However, there are currently several limitations related to vaccine production and use. For example, vaccine efficacy is reduced in those individuals generally most affected by influenza virus, i.e. the very young, elderly and immunocompromised. Additionally, the influenza virus is constantly changing (antigenic drift) and a new vaccine must be developed each year to match the predicted circulating strain. This is a laborious and time consuming process, taking minimally nine months from start to finish. This time constraint is problematic, and this issue is made more evident in the event of a pandemic. Thus, other options to control influenza virus are being investigated including the development of novel antiviral drugs approaches.

Antiviral drugs can be used to reduce symptoms and complications when a host becomes infected with influenza virus; however, current options for clinically-approved and effective antiviral drugs against influenza are limited. To date, two classes of antiviral drugs are available for influenza: the adamantanes which target the M2-ion channel, and the neuraminidase inhibitors [102, 103]. The two neuraminidase inhibitors available are oseltamivir (Tamiflu) and zanamivir. However, with any antiviral drug virus resistance develops, and in October of 2008, a Center for Disease Control survey found that greater than 99% of circulating H1N1 viruses were resistant to the neuraminidase inhibitor, oseltamivir [104May 25, 2009]. Aside from viral resistance, current antiviral drugs face issues such as sequence diversity among virus strains and toxic side effects from the drug. Thus, a consensus remains among many experts that alternative approaches for influenza virus therapy are crucial.

Various antiviral strategies have been explored. For example, several groups studying small molecule drugs showed that arbidol, a plant-derived agent, was able to block influenza virus attachment and fusion [105, 106]. Other novel approaches are being directed toward targeting non-essential host genes that are critically required for influenza virus replication. In principle, such an approach may pose an insurmountable barrier against the development of virus resistance related to the development of escape mutants. However, it is likely that viruses would adapt and use different but perhaps related cellular proteins for the same purpose.

RNA interference (*RNAi)*

RNAi was first described in *C. elegans* by Fire and Mello [107]. RNAi is an efficient mechanism for the sequence-specific inhibition of gene expression [108, 109], and is an evolutionary conserved process that is mediated by double-stranded small interfering RNA (siRNA) whose effect is to silence gene expression. There are several classes of RNAi molecules, e.g. siRNAs, microRNAs (miRNAs), and piwiRNA (piRNAs), which differ in size, targeting parameters, and biogenesis pathways [110]. In a cell, elements of the RNAi pathway are used to regulate endogenous genes, where endogenous non-coding RNAs are processed into siRNAs by RNase III ribonucleases [111]. RNAi can suppress gene expression via post transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS) [112]. There are three core components that are important for PTGS, i.e. Dicer, DROSHA, and Argonaute (Ago). Drosha and Dicer are both RNase III ribonucleases and convert the double-stranded RNA (dsRNA) into smaller guide dsRNAs. Dicer is in a complex with the human immunodeficiency virus type 1 (HIV-1) transactivating response (TAR) RNA binding protein (TRBP), Ago-2, and PACT [113]. Ago, specifically Ago-2, is a component of the RNA-Induced Silencing Complex (RISC) and uses the siRNAs as a guide to identify homologous sequences on mRNA. Ago-2 interacts with the 3' end of the siRNA strand via its PAZ domain [114]. Once the mRNA is identified, it is destroyed, inhibited, or transcriptionally silenced. In human cells, this mode of silencing can occur in either the cytoplasm or the nucleus [111, 115].

The siRNA is incorporated in RISC where the guide strand of the duplex can suppress protein expression or direct degradation of mRNAs that contain homologous sequences [116-118]. The siRNAs represent an extensive class of short, non-coding, regulatory RNA molecules that can control gene expression at both the transcriptional and translational levels [110, 119]. siRNAs in particular have become a valuable research tool for gene knockdown and silencing studies [120], and cell-based siRNA genome screens have been shown to have the ability to study of genes involved in an entire viral infection cycle [121-126].

microRNA (miRNA) Regulation in the Host

Following the discovery of the RNAi pathway, a novel class of gene regulators was discovered, i.e. miRNAs. miRNAs are noncoding RNA molecules that have been proposed as master regulators of gene expression [127]. miRNAs are endogenous and transcribed by the class II RNA polymerase into pre-miRNAs which are subsequently recognized by Drosha, which cleaves the pre-miRNA into a hairpin structure that is able to travel out of the nucleus and into the cytoplasm [128]. Once in the cytoplasm, the hairpin structure is processed by

Dicer to yield its fully active form. At the point of cleavage by Dicer, the processing of siRNA duplexes and miRNA follow a similar pathway with RISC and Ago, although the way they alter gene expression is different. siRNAs are phosphorylated on their 5’ end and are able to assemble into RISC with Ago-2 which in turn targets mRNA with exact complementarity to be cleaved by Ago-2 and silenced [129]. miRNA is loaded into RISC, and how it is loaded determines its function and due to the inherent base mismatching, when it is guided to the target mRNA, it represses protein expression [130].

In TGS, dsRNA silences regions complementary to the siRNA. This has effect has been shown in plants, yeast, and in mammalian cells [131]. TGS was first demonstrated in tobacco plants when they were doubly transformed they showed a suppressed phenotype of that transgene that was a result of RNA dependent DNA methylation (RdDM) at that transgene locus. Therefore, the RdDM with a dsRNA yields the short dsRNAs, which bind to and methlylate the homologous promoter region and reduce transgene expression [132]. It has also been shown in yeast that an Ago, specifically Ago-4, helps to direct the short dsRNA mediated silencing [133]. In mammalian cells, both an RNA/RNA and RNA/DNA siRNA mediated mode of silencing have been proposed, but the ability of siRNAs targeting promoter regions to induce TGS in mammalian cells has been controversial, and the mechanisms underlying this process are still being defined [134]. It is understood that this process involves changes to the histone code, with the main role of histones being its association with DNA in the nucleosome, and altering the methylation status of histone tails. It has been shown that the antisense strand of siRNA interacts with an RNA polymerase II-transcribed promoter-associated RNA species, which can direct sequence-specific transcriptional gene silencing [135]. It is also becoming clear that miRNAs can also induce changes in the histone code and this process is initiated by the ability of miRNA to suppress specific genes at the translational level [136]. There are therefore several mechanisms by which siRNA and miRNA can induce TGS [137].

RNAi and the Genome

Recently, siRNA libraries targeting each gene in the human genome have become available as it has become easier to synthesize siRNA [138]. RNAi libraries are available from multiple companies, and because of their different manufacture, they have varying silencing efficiency. Most libraries consist of pools of 2-4 siRNAs to ensure efficient silencing of the target genes. RNAi libraries to date have been generally applied to understanding virus-host interactions, particularly the host genes required for efficient HIV [139-141], dengue virus [122, 142], West Nile virus [122, 143], and influenza virus replication [122-126], and is discussed in detail in the next section.

Validation of primary RNAi screen results is crucial. While the design of the screen methodology is important, the validation process is essential to confirming positive hits. A siRNA will target its mRNA with perfect complementary [144, 145]. However, since siRNAs enter the same biological pathway as an endogenously derived miRNA, there is the possibility of the short siRNA sequence acting similar to a miRNA and targeting multiple mRNAs with imperfect complementarity instead of binding only to the matched target mRNA [144, 145]. Therefore the validation process is important to eliminate false positives that can be generated by the primary screen.

False negatives are also an issue with RNAi screens. The statistical method, i.e. the Z statistic employed to set thresholds of what is considered a hit, may result in too strict a cutoff resulting in missed positives. This is a common feature when performing HTS RNAi studies due to the fact that an RNAi screen cannot be optimized for every siRNA/gene pair included in the study. Proteins with a long half-life may not be completely degraded by the time of assay even though the siRNA has effectively silenced the message, and some genes may have functional redundancy meaning other genes can serve its purpose even after the target gene has been silenced [144].

In addition to screening with siRNA, it is possible to perform HTS based on miRNAs which can subtly modulate both host and viral gene expression. miRNA mimics increase the cellular concentration of miRNAs that can be loaded onto the RISC, while the miRNA inhibitors are designed to bind to mature miRNAs and prohibit cleavage [146]. By evaluating the effect on phenotype from both the miRNA mimic and corresponding inhibitor, it is possible to identify miRNAs that affect host genes critical for virus replication. However, there are limitations to miRNA screens. Careful consideration must be used when choosing the cell model to be studies as all miRNAs are not expressed in similar amounts, if at all, across multiple cell types due to the tight regulatory control over cellular processes that they exert [147]. Additionally, miRNA function in the host cell is subtle and multi-targeted; therefore, results must be thoroughly validated to prohibit confusion from off-target effects [148]. Finally, since one miRNA may potentially have hundreds of targets [149], one particular miRNA-mRNA interaction may not be sufficient to produce or explain the intended effect. Effective miRNA antivirals may require targeting multiple genes using different miRNAs [150].

siRNA Genome High-Throughput Screens (HTS)

RNAi screens have been used as a tool for studying many pathogens including Retroviridae, Flaviviridae, and influenza viruses. As HIV is a substantial public health burden, and the need to understand HIV virus-host interactions is crucial for the development of new therapies, several RNAi screens to identify host factors exploited by HIV have been examined. For example, a HIV LTR-driven β-galactosidase reporter assay as readout for HIV replication has been investigated to determine critical host genes required for replication at 48 and 96 hpi [141]. This study used a HeLa cell line altered to express CD4 to render the cells susceptible to HIV infection, and replication was determined by expressing the β-gal reporter [141]. Although artificial in many ways, this screen identified 311 hit genes that were expressed in human CD4$^+$ cells and macrophages of which 232 genes were validated by repeating the assay with a novel pool of siRNAs [141]. In contrast, an independent HIV RNAi screen examined host genes involved in early events of virus replication using an HIV reporter virus expressing luciferase that underwent a single round of replication [140]. This screen identified 213 host factors which were then used to conduct a meta-analysis to determine overall cellular factors and processes connected with HIV replication, some which had not previously been identified [140]. Together, these HTS studies identified several overlapping gene determined as critical for HIV replication using very different assay systems suggesting the utility of RNAi genome screening assays.

Similar RNAi genome screens have been performed for dengue virus and West Nile virus. Dengue fever is of particular concern as there is no vaccine available and previous exposure to one serotype can negatively impact a subsequent infection of another serotype [142]. A HTS RNAi genome screen for dengue virus identified 116 host cellular factors required for replication with some that had previously been associated with dengue virus pathogenesis such as V-ATPases and alpha-glucosidases, and others that were entirely novel. The screen was carried out in *Drosophila* cells, but 82 candidates had human homologs, and a secondary screen validated 42 of those genes in a human cell line [142]. West Nile virus, a causative agent of the neuroinvasive West Nile encephalitis, represents another serious global public health problem where RNAi screens for host factors proved useful. To evaluate host genes important for West Nile virus replication, an immunofluorescence assay based on the West Nile virus envelope protein was employed using HeLa cells transfected with siRNA pools targeting host genes. A rescreen with a second single siRNA was used to confirm phenotype and served as validation to identify 305 associated host factors [143].

To date, six RNAi HTS assays have been published for influenza virus, and our lab has performed RNAi sub-library screens targeting human kinase and protease genes (reviewed in Table 5.2). All RNAi HTS screens in the influenza virus studies used varying methodologies and endpoint assays. The first RNAi screen was examined in *Drosophila* cells using a recombinant A/WSN/33 virus where the hemagglutinin and neuraminidase segments had been replaced with Vesicular stomatitis virus (VSV) G protein and a luciferase reporter, respectively [123]. After transfection, cells were infected with the recombinant virus and infection was quantified by luciferase expression. However, this screen only allowed identification of host genes that affected viral entry, as the recombinant virus was unable to replicate. Furthermore, *Drosophila* cells are not permissive for influenza infection, so host genes identified may not translate to a real-life situation. The screen revealed 121 genes as being involved with early infection events, but no validations steps were done [123]. In a second RNAi screen, human U2OS cells were used, but these cells were also not permissive for natural influenza infection and the methodology again only allowed for single round viral replication. Despite this, 260 genes identified following infection of U2OS cells with A/PR8 whose replication was indirectly measured by staining for HA protein. The screen was not validated [122]. In a related screen, performed using a HBEC cell line that more closely mimics natural influenza virus infection, a yeast two-hybrid system identified 616 genes important for influenza virus replication [126]. However, the study had major caveats: 1) the RNAi library contained only 1200 genes (human ORFeome v3.1) so the selection of available host genes was limited, and 2) the A/PR8 virus used lacked the NS1 protein which is vital in modulation of antiviral responses as apoptosis-related genes [151]. Another RNAi genome screen used A549 cells and both recombinant A/WSN/33 and swine-origin influenza virus with luciferase readout [125]. Of the 295 gene hits identified, 219 were confirmed in wild-type WSN virus while 76 were not [125]. Finally, a related study using A549 cells was done where 168 genes identified following A/WSN/33 infection whose replication was measured by a 293T luciferase reporter cell line [124].

Each of the primary screens resulted in a large number of hit genes but these were not similarly validated. The validation process is important given the potential for the generation of both false negative and false positive hits. Elimination of false positives from the screen is accomplished by validating where the most common method is to deconvolute the pools of siRNA used in the RNAi HTS screen. For example, in one RNAi screen that utilized pools of four individual siRNAs for each host gene, a repeat of the assay using the four individual siRNAs to confirm that the same phenotype was served as a validation step [122]. Others used statistical parameters that did not specifically validate the screen hits but increased the likelihood of positives. For example, two studies used more than 2 unique siRNAs targeting the candidate gene to show a reduction in virus [124, 125]. Another screen validated phenotype by using the same phenotype but looking at multiple endpoint assays [126]. The screens performed by our laboratory combined multiple methods - not only utilizing a novel siRNA targeting a different seed region of the gene of interest, but also looking at endpoints denoting viral replication, infectious virus production, and viral assembly.

For our primary RNAi genome screen, our laboratory utilized a A549 (human lung epithelial) cell line that is permissive for influenza infection which allows WSN/33 influenza virus replication at low levels in the absence of exogenous trypsin [152]. After siRNA reverse transfection, we incubated the cells for 48 hours to allow for gene silencing, and then infected with WSN/33 allowing for multicycle replication so we could determine the host genes that affected virus replication at all points in the replication cycle. Virus titers were measured by $TCID_{50}$ to quantify the level of infectious virus. After performing two independent siRNA genome screens, we began validation of the positive hits by first adding other assay endpoints that included high-content analysis, i.e. NP localization in the cell, and M gene detection by qRT-PCR to quantify viral replication. The primary positive hits were then rescreened using all three endpoints for silencing of influenza replication using a novel siRNA that targeted a new seed site on the target gene. If the phenotype was confirmed, the genes were considered validated. Furthermore, to show that the genes discovered were not unique to WSN/33, we tested the validated genes with a clinical influenza isolate to ensure that the phenotype (increased or decreased virus) could be emulated.

When considering all of the RNAi genome screens for influenza virus, the screens generated a list of positive host genes that represented approximately

2% of the genes screened. However, these screens had very little overlap of hit genes among each other. Only three genes overall were independently validated in four of the six screens, nine genes were validated in 2 of 6 screens, and 86 genes were validated across two of the screens. The lack of consistency is troubling at first glance, but the relationships of the hit genes to each other may be more helpful to interpret the data than the individual hit genes themselves. For example, a global pathway analysis of our hits alone revealed grouping of the hit genes from the primary screen into six global cellular pathways (proteases: NF-κB, CREB signaling, apoptosis; kinases: NFAT, FOXO, p53 signaling). A meta-analysis of the hits identified in other influenza screens shows that while the individual hit genes differ, the cellular pathway nodes connected to the hits show repeatability from screen to screen.

Host Signaling Pathways Important for Influenza Virus Replication

Intracellular signaling events reflect the balance of virus replication and antiviral host responses. It is well established that the host cell will initiate signaling processes in response to an invading pathogen such as influenza, often in a phosphorylation-regulated manner. The signals are transduced into specific cellular actions, such as changes in gene expression, which aim to defend the host from the pathogen. However, it has also been shown that the virus can hijack these signaling processes to facilitate replication.

Cellular signaling pathways are affected early during the infection process and throughout virus replication as viral genes are expressed. As influenza HA binds to the host cell sialic acid receptor, protein kinase C (PKC) signaling is induced [153], and Toll-like receptors (TLR) can be activated. Inside the cell, expression of the influenza proteins HA, NP, and M1 activate NF-κB [154, 155]. Moreover, dsRNA is a pathogen associated molecular pattern (PAMP) associated with RNA viruses. When dsRNA is released from infected and dying cells, it is detected by TLR-3 and signals an immediate antiviral response [156]. When inside the cell, dsRNA is may be detected by a variety of proteins including RIG-1, melanoma differentiation-associated gene-5 (MDA-5), and IFN-inducible RNA-dependent PKR, initiating a signaling cascade that results in the production of interferons (IFN) [157-159]. The presence of viral dsRNA has been shown to activate signaling cascades involving IKK–NF-κB, c-Jun N-terminal kinase (JNK), and P38 mitogen-activated protein kinase (MAPK) cascades; all which regulate the expression of antiviral cytokines [160-162]. However, recent data suggests influenza virus has capitalized on these host cell processes, reprogramming these defense mechanisms to support of virus replication [163]. Two pathways of note are the Raf/MEK/ERK mitogenic kinase cascade and the IKK/NF-κB [164, 165].

The Raf/MEK/ERK signaling pathway is a member of the MAPK cascades. This pathway is initiated by either receptor tyrosine kinases or G-protein-coupled receptors and phosphorylates the serine threonine kinase Raf, activating MAPK kinase/ERK kinase (MEK) which activates MAPK extracellular signal-regulated kinase (ERK). ERK can regulate various cellular functions by phosphorylating a variety of substrates [166]. During an influenza infection, this pathway is activated in a protein kinase C α-dependent manner with membrane accumulation of the viral HA protein [167]. Data show Inhibiting this pathway decreases both influenza A and B virus propagation as the viral RNP complexes are retained in the nucleus [168, 169]. Inhibitors directed to the signaling mediator MEK provide broad antiviral activity and have shown little cytotoxicity in cell culture as well as *in vivo* [165, 169-172]. Using a specific inhibitor for MEK, U0126, virus titers were reduced in the lungs of infected mice after local aerosol administration [173]. Furthermore, targeting this host gene showed no tendency to induce viral resistance, a major downfall with current antiviral therapies [165]. Because the Raf/MEK/ERK cascade regulates many inflammatory cytokines, inhibiting this pathway may also provide a means to prevent excessive inflammation. Inhibitors of the Raf/MEK/ERK cascade are currently under clinical investigation and thus far show selective inhibition of this pathway is well tolerated in humans [174].

Another signaling pathway important for influenza virus replication is IKK/NF-κB. NF-κB is a transcription factor which regulates genes involved in many immunological responses. In a resting cell, NF-κB is bound to one or more inhibitory proteins called inhibitors of κB (IκB). However, upstream signals generated by a T cell receptor or a cytokine receptor will activate the IκB kinase complex (IKK). One of the three isoenzymes in this complex, IKK2, can then phosphorylate IκB causing IκB degradation and release from NF-κB [175]. Once NF-κB is freed from IκB, it is in its active form and can travel to the nucleus and in a sequence specific manner, bind the target gene DNA and initiate transcription [176].

NF-κB has a central role in the innate immune response as the regulator of the expression of IFNβ and other inflammatory cytokines and chemokines [161, 177]. Therefore, activation of this transcription factor is a hallmark of most viral infections, including influenza infection [178]. Influenza virus induces IFN expression through NF-κB since blocking NF-κB expression using a IKK2 or IκBα dominant negative mutant cell line impairs IFNβ promoter activity upon influenza infection [179, 180]. However, blocking the NF-κB pathway in cells does not enhance influenza virus replication as expected; rather it impairs replication. In cells expressing a constitutively active form of IKK2, influenza virus propagation was shown to be enhanced [180]. In a separate study, influenza replication was more efficient in an Epstein-Barr virus-immortalized cell line with high NF-κB activity cell line than in a low NF-κB activity Burkitt's Lymphoma cell line [181]. When the Burkitt's Lymphoma cells were modified to have increased NF-κB activity, they became more susceptible to influenza propagation [181]. Taken together, these data suggest a virus-supportive function of NF-κB in influenza virus replication.

Several observations have been made in understanding how NF-κB supports influenza virus replication. First, activation of NF-κB correlates with nuclear export of influenza vRNPs. This is because NF-κB activity induces TNF-related apoptosis-inducing ligand (TRAIL), FasL, and other pro-apoptotic factors which subsequently activate caspases, triggering apoptosis and leading to degradation of nuclear pore proteins and dissociation of the nuclear lamina [180, 182, 183]. The compromised integrity of the nuclear membrane allows the vRNP to diffuse more easily across the membrane. Second, Influenza virus inhibits type I IFN signaling via NF-κB. Data have shown that NF-κB activity negatively regulates type I IFN-induced gene (ISG) expression and anti-influenza activity by either directly suppressing ISG promoter regions or NF-κB induced expression of the suppressor of cytokine signaling 3 (SOCS-3) [184, 185]. Last, NF-κB signaling differentially regulates influenza RNA synthesis. When influenza infected cells were treated with NF-κB inhibitors, influenza genomic RNA levels were reduced while cRNA and mRNA levels were not [186].

As mentioned above, activation of the Raf/MEK/ERK pathway is required for efficient influenza virus growth. Inhibitors directed to the signaling mediator, MEK, provide broad antiviral activity and have shown little cytotoxicity in cell culture as well as *in vivo* [165, 169-172]. Using a specific inhibitor for MEK, e.g. U0126, influenza virus titers were reduced in the lungs of infected mice after local aerosol administration into the trachea [173]. Also, targeting this host gene showed no tendency to induce viral resistance, a major pitfall of current antiviral therapies, e.g. amantadine and oseltamivir [165]. Furthermore, inhibiting MEK also impaired virus replication for Borna-disease virus, Visna virus, and Coxsackie B3 virus [170, 187, 188]. Because the Raf/MEK/ERK cascade regulates many inflammatory cytokines, inhibiting this pathway may also prevent excessive inflammation. Inhibitors of the Raf/MEK/ERK cascade are currently under clinical investigation and thus far show selective inhibition of this pathway is well tolerated in humans [174]. Thus, targeting host intracellular signaling mediators in the kinase family that is modulated by influenza virus for replication appears to be a promising strategy for the development of novel antiviral drugs. Data indicate inhibitors of MEK are broadly active and may have additional beneficial effects, such as suppressing an overabundant cytokine response.

miRNA Regulation of Host Cell Signaling

Since their discovery [127, 189-191], miRNAs have emerged as important regulators of eukaryotic gene expression. These highly conserved small non-coding RNAs (21-25 nt) regulate gene expression post-transcriptionally by targeting one or more mRNAs for translational repression [192-194]. While siRNAs are exogenously introduced and are by design perfectly complementary to their target mRNA to achieve mRNA degradation, miRNAs are transcribed from an endogenous gene, or group of genes, and bind their target mRNAs through partially complementing sites in their 3′-untranslated region (UTR) leading to repression or cleavage [192, 195]. miRNAs have also been shown to bind within the mRNA coding sequence [192] and sometimes within the mRNA 5’-UTR [195].

Largely, the loci for human miRNA are located within non-coding or intronic regions and are transcribed in parallel with other transcripts [196-198]. Mature miRNAs sequences have been found in over 150 species. To date, 1424 miRNAs have been identified in the human genome (miRBase database, release 17; http://www.mirbase.org/), each of which can regulate about 200 target genes

[127, 189, 199, 200]. In lower metazoans and plants, only about 200 miRNAs identified [127, 189, 199, 200]. By identifying new miRNA-mRNA target relationships through functional studies, it is increasingly clear that miRNAs are involved in the regulation of virtually every cellular process [193]. Bioinformatics analysis indicates that mammalian miRNAs can regulate ~60% of all protein-coding genes further validating the global importance of miRNA regulation to the cell [149]. Changes in miRNA expression are critical for many cell signaling processes including development and cell differentiation, proliferation, apoptosis, and importantly, immunity. Therefore, it is important to understand the convergence of miRNAs and their target genes within a specific signaling cascade. Because a single miRNA usually targets many genes, the influence of miRNAs on the components of different signaling pathways could be complex. Studies in other model organisms, such as Drosophila and *C.elegans*, support this scenario.

The highly conserved MAPK signaling cascade, involved in cell proliferation, differentiation and migration [201], has been associated with miR-21, miR-155 and miR-34c. The expression of miR-2, expressed specifically in cardiac fibroblasts [202], acts as a positive feedback regulator of the ERK/MAPK signaling cascade. It is induced upon ERK1/2 activation and enhances ERK1/2 activity by repressing negative regulators of ERK⁄MAPK signaling. Within fibroblast of a failing heart, miR-21 is upregulated and functions to repress sprout homolog 1, a negative regulator of MAPK [203]. Also, miR-21 is upregulated during cardiac hypertrophic growth and inhibits the expression of Sprouty 2

(Spry2), which negatively regulates ERK1/2 [204]. Therefore, miR-21 increases the activity of ERK/MAPK signaling by inhibiting negative regulators of ERK/MAPK. Interestingly, the expression of miR-21 is also upregulated by over-expression of ERK1/2 activators. Specifically, in breast cancer cells the expression of miR-21 is upregulated via the ERK1/2 pathway upon stimulation of HER2/neu signaling, resulting in miR-21 suppression of the metastasis suppressor protein PDCD4 (programmed cell death 4) [205]. The expression of miR-21 is also upregulated by over-expression of other ERK1/2 activators, such as RASV12 and ID-1, in HER2/neu-negative breast cancer cells [205]. Others have reported the transcription factor activation protein 1 (AP-1) triggers the expression of miR-21 through binding to several AP-1 binding sites that are found in the promoter of the gene for miR-21 [206]. The induction of miR-155 also depends on a conserved AP-1 site and this site is approximately 40 bp upstream from the site of initiation of miR-155 transcription [207]. In addition, miR-34c is induced upon the activation of both p53 and p38/MAPK, and prevents Myc-dependent DNA replication by targeting c-Myc [208]. It has also been that the brain-derived neurotrophic factor upregulates miR-132 expression via the ERK/MAPK pathway, which results in the upregulation of glutamate receptors in cultured cortical neurons [209]. Expressions of miRNAs have important roles in various cellular functions and these data indicate that many miRNAs are involved in the MAPK signaling pathway.

Other miRNAs have been reported to be induced during cell signaling. Two miRNAs implicated as oncogenes in tumors of testicular germ cells, miR-

372 and miR-373, have been reported to target and regulate LATS2, which is a homolog of a component of the Hippo signaling pathway involved in the control of tissue growth [210]. Members of the transforming growth factor β (TGFβ) family have been associated with biogenesis of miRNAs [211]. For example, stimulation by an appropriate ligand causes the recruitment of receptor-regulated SMADs (R-smads) to specific pri-miRNAs that are bound to the Drosha–DiGeorge syndrome critical region gene 8 complex and RNA helicase p68. Recruitment of the R-smads leads to the production of miRNAs, repressing the expression of their target genes. Downstream mediators of TGFβ signaling, ZEB1 and ZEB2, negatively regulate the expression of E-cadherin. The miR-200 family has been shown to target these two transcription factors causing the inhibition of epithelial–mesenchymal transition (EMT) in vertebrate cell lines [212-214]. Accordingly, in TGFβ stimulated cells that have undergone EMT, the miR-200 family is markedly decreased [213]. Interestingly, ZEB1 has also been shown to repress the expression of the miR-200 cluster, promoting EMT in a feed forward manner [215].

Taken together, these data show the transcriptional events downstream of key signal cascades appears to include multiple miRNAs. It is likely that these miRNAs mediate cross-talk between the different signaling pathways via the repression of their target genes. Therefore, the expressions of miRNAs have important roles in various cellular functions and these data indicate that many miRNAs are involved in signaling pathways such as ERK/MAPK, Hippo, and the TGFβ signaling pathway.

miRNA Expression During Viral Infection

Changes in miRNA expression are critical for many cell signaling processes including development and cell differentiation, proliferation, apoptosis, and importantly, immunity. Dysregulation of miRNA expression during an immune response may promote human diseases as well as virus infection [216-219]. miRNAs are global regulators of gene expression within a cell and therefore ideal targets for viruses to facilitate replication. Accordingly, there is a growing range of viruses known to affect miRNA regulation of host responses. The discovery of miRNAs encoded by a number of viruses, including several human oncogenic viruses, has attracted renewed interest in the molecular mechanism of viral oncogenesis. A few examples are seen in Herpes Simplex Virus where virus encoded miRNAs target transcripts involved in apoptosis [220]. Also, Marek's Disease virus (MDV), a disease in avian species, has shown to encode a miRNA targeting the latency associated transcript and its expression in MDV induced tumors [221]. Although it has not been completely defined, there are clearly genetic interactions between the host miRNA and the virus. These interactions should be considered when looking at host genes involved in virus replication.

Cellular miRNA expression is intensely influenced by viral infection due to both host antiviral defenses and viral factors altering the cellular environment. Herpes simplex virus-1 (HSV-1) replication has been shown to be suppressed when miR-101 targets a subunit of mitochondrial ATP synthase (ATP5B) [222]. Alternatively, miR-132 has been shown to be highly upregulated after HSV-1 and human cytomegalovirus (HCMV) infection and to have a negative effect on the expression of interferon-stimulated genes, thereby facilitating viral replication [223]. HIV-1 down-regulates the expression of many cellular miRNAs [224] and suppression of miR-17-92 is required for efficient viral replication [136]. HIV-1 also suppresses host miR-17-5p and miR-20a which are known to target p300/CBP-associated factor, a cellular histone acetylase thought to be a cofactor of the HIV-1 Tat transactivator [225]. The Epstein-Barr Virus (EBV) latent membrane protein LMP1 induces miR-29b to repress the cell survival gene, TCL1 [226], and in B cells, EBV infection induces miR-146a expression [227]. Retrovirus primate foamy virus type 1 replication is suppressed by miR-32 by down-regulation of the replication-essential viral proteins [228]. Interestingly, IFN-β stimulation of hepatic cells results in the production of at least eight miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448) with perfect seed sequence complementarity to Hepatitis C Virus (HCV) mRNAs [229]. One miRNA, miR-122, is required for HCV replication and significantly reduced in response to IFN-β treatment [229]. Therefore, the host response to HCV likely utilizes miRNAs to suppress viral mRNAs as well as down-regulating miR-122 to inhibit viral replication. In mouse macrophages, VSV induces overexpression of miR-146a in a TLR–MyD88-independent but a RIG-I–NF-κB-dependent manner, suppressing type 1 IFN production and promoting virus replication [230]. Similarly, miR-132 is upregulated during Kaposi's sarcoma-associated herpesvirus (KSHV) infection [231]. In regards to influenza virus, cellular miRNAs miR-323, miR-491, and miR-654 have been shown to inhibit influenza replication by binding to the viral PB1 gene [232], while miR-507 and miR-136 have potential binding sites within the viral PB2 and HA genes [233]. In Madin-Darby Canine kidney (MDCK) cells, miR-26a and miR-939 have also been shown to regulate the replication of influenza virus [234].

While some host miRNAs are in place to protect against viral infection, viruses encode miRNAs of their own to regulate both viral and host genes [235]. Over 200 miRNAs have been identified in DNA viruses, namely herpesviruses, polyomaviruses, and adenoviruses [235, 236]. Biogenesis of these viral miRNAs is likely dependent on cellular factors since there is no known viral proteins found in miRNA processing [236]. At present, there are no known RNA viruses that encode miRNAs. Viral miRNAs hijack host miRNAs to regulate the latent-lytic switch, support viral replication by promoting cell survival, proliferation and/or differentiation and modulate immune responses [235, 236]. KSHV is known to encode 12 miRNA genes, including miR-K12-11 which is homologous to host miR-155 and likely cross-regulating the same targets [237, 238]. The exploitation of host miR-155-regulated pathways by viruses may contribute to viral oncogenesis as miR-155 is known to be involved in many malignancies [239]. EBV produces miRNAs including miR-BART2, which targets the EBV-DNA polymerase BALF5 during infection and contributes to viral maintenance and latency [240, 241]. HCMV-encoded miR-UL112 represses the expression of MHC-class I polypeptide-related sequence B, which is required for natural killer cell-mediated killing of virus-infected cells [242]. Therefore, for many viruses, miRNAs are essential for governing the host response to viral replication, and targeting miRNAs could provide a promising therapeutic option particularly since miRNAs are endogenously expressed within the cell and may trigger lower immunogenic responses having fewer off-target or side effects [243, 244].

Next Generation Antivirals

The identification of pro- and antiviral miRNAs opens the possibility of using miRNAs as potential therapeutics. However, clinical application of miRNA is dependent on our understanding of the consequences of perturbations in miRNA expression. [245] As miRNAs are known to be involved in basic cellular pathways such as proliferation and apoptosis [147], which can be dysregulated during cancer or viral infection, miRNAs can be harnessed as a means to control gene expression during illness.

Simple characterization of host miRNA expression can lead to understanding of disease pathogenesis. Aberrant miRNA expression profiles are known to be indicative of disease, and the miRNAs let-7 and miR-155 have been shown to be dysregulated in non-small cell lung cancer. [246] miRNA expression profiles can also be correlated with cancer relapse, survival rates, and treatment success. [247] miRNAs have also been used as therapeutics themselves. Delivery of miR-26a suppressed tumorigenesis in a murine hepatocarcinoma model [248], and over-expression of miR-155 was shown to enhance innate antiviral immunity and aid in hepatitis B virus clearance. [249] Similarly, miR-122 was found to facilitate hepatitis C virus replication of the viral genome, implying miR-122 is a potential therapeutic target for HCV [250].

References

1. Hoehling, A.A., *The great Epidemic*1961, Boston: Little, Brown & Co.
2. Sellwood, C., *Avian and Animal Influenza: Manifestation in Man*, in *Introduction to Pandemic Influenza*, J.V.-T.a.C. Sellwood, Editor 2010, CAB International: London, UK.
3. WHO. *Media centre: Influenza (Seasonal)*. http://www.who.int/mediacentre/factsheets/fs211/en/ April 2009.
4. Crosby, A.W., *America's forgotten pandemic : the influenza of 1918*1989, Cambridge [England] ; New York: Cambridge University Press. xiv, 337 p.
5. Johnson, N.P. and J. Mueller, *Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic.* Bull Hist Med, 2002. **76**(1): p. 105-15.
6. Wang, T.T. and P. Palese, *Unraveling the mystery of swine influenza virus.* Cell, 2009. **137**(6): p. 983-5.
7. Compans, R.W., et al., *Influenza virus proteins. I. Analysis of polypeptides of the virion and identification of spike glycoproteins.* Virology, 1970. **42**(4): p. 880-&.
8. Chen, W., et al., *A novel influenza A virus mitochondrial protein that induces cell death.* Nat Med, 2001. **7**(12): p. 1306-12.
9. O'Neill, R.E., J. Talon, and P. Palese, *The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins.* Embo Journal, 1998. **17**(1): p. 288-296.

10. Kawaoka, Y., Cox, N., Haller, O., et al., *Orthomyxoviridae*, in *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*, C.M. Fauquet, Mayo, M. A., Maniloff, J., et al., Editor 2005, Elsevier Academic Press: London. p. 681-693.
11. Palese P, S.M., *Fields Virology*. 5th ed, ed. H.P. Knipe DM2007, Philadelphia: Raven.
12. Shinya, K., A. Makino, and Y. Kawaoka, *Emerging and reemerging influenza virus infections.* Vet Pathol, 2010. **47**(1): p. 53-7.
13. Wright P, N.G., Kawaoka Y, *Orthomyxoviruses*, in *Fields Virology*, D.K.a.p. Howley, Editor 2007, Lippincott Williams & Wilkins: Philadelphia, PA.
14. Suarez, D., *Influenza A Viruses*, in *Avian Influenza*, D. Swayne, Editor 2008, Blackwell Publishing: Ames, Iowa.
15. Kuiken, T., et al., *Host species barriers to influenza virus infections.* Science, 2006. **312**(5772): p. 394-7.
16. Shinya, K., et al., *Avian flu: influenza virus receptors in the human airway.* Nature, 2006. **440**(7083): p. 435-6.
17. van Riel, D., et al., *H5N1 Virus Attachment to Lower Respiratory Tract.* Science, 2006. **312**(5772): p. 399.
18. Srinivasan, A., et al., *Quantitative biochemical rationale for differences in transmissibility of 1918 pandemic influenza A viruses.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 2800-5.

19. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**: p. 531-69.

20. Chu, V.C. and G.R. Whittaker, *Influenza virus entry and infection require host cell N-linked glycoprotein.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 18153-8.

21. Swayne, D.E., *Avian Influenza*. 1st ed2008: Blackwell Professional Publishing. 628.

22. Connor, R.J., et al., *Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates.* Virology, 1994. **205**(1): p. 17-23.

23. Baum, L.G. and J.C. Paulson, *Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity.* Acta Histochem Suppl, 1990. **40**: p. 35-8.

24. Leigh, M.W., et al., *Receptor specificity of influenza virus influences severity of illness in ferrets.* Vaccine, 1995. **13**(15): p. 1468-73.

25. Belser, J.A., et al., *Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility.* Proc Natl Acad Sci U S A, 2008. **105**(21): p. 7558-63.

26. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.

27. Gambaryan, A.S., et al., *Differences between influenza virus receptors on target cells of duck and chicken and receptor specificity of the 1997 H5N1*

chicken and human influenza viruses from Hong Kong. Avian Dis, 2003. **47**(3 Suppl): p. 1154-60.

28. Gambaryan, A., R. Webster, and M. Matrosovich, *Differences between influenza virus receptors on target cells of duck and chicken.* Arch Virol, 2002. **147**(6): p. 1197-208.

29. Kim, J.A., S.Y. Ryu, and S.H. Seo, *Cells in the respiratory and intestinal tracts of chickens have different proportions of both human and avian influenza virus receptors.* J Microbiol, 2005. **43**(4): p. 366-9.

30. Wan, H. and D.R. Perez, *Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses.* Virology, 2006. **346**(2): p. 278-86.

31. Guo, C.T., et al., *The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors.* Glycobiology, 2007. **17**(7): p. 713-24.

32. Oshansky, C.M., et al., *Avian influenza viruses infect primary human bronchial epithelial cells unconstrained by sialic acid alpha2,3 residues.* PLoS One, 2011. **6**(6): p. e21183.

33. Knipe, D.M., Roizman, B., Howley, P. M., Straus, S. E., Griffin, D. E., *Fields Virology*. 5th ed, ed. D.M. Knipe2006: Lippincott Williams & Wilkins. 3177.

34. Pinto L.H., a.R.A.L., *Controlling influenza virus replication by inhibiting its proton channel.* Molecular Biosystems, 2007. **3**: p. 18-23.

35. Cros, J.F., A. Garcia-Sastre, and P. Palese, *An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein.* Traffic, 2005. **6**(3): p. 205-13.
36. Cros, J.F. and P. Palese, *Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses.* Virus Res, 2003. **95**(1-2): p. 3-12.
37. Engelhardt, O.G., M. Smith, and E. Fodor, *Association of the influenza a virus RNA-dependent RNA polymerase with cellular RNA polymerase II.* J Virol, 2005. **79**(9): p. 5812-5818.
38. Mikulasova, A., E. Vareckova, and E. Fodor, *Transcription and replication of the influenza a virus genome.* Acta Virol, 2000. **44**(5): p. 273-82.
39. Obayashi, E., et al., *The structural basis for an essential subunit interaction in influenza virus RNA polymerase.* Nature, 2008. **454**(7208): p. 1127-31.
40. Vreede, F.T. and G.G. Brownlee, *Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA in vitro.* J Virol, 2007. **81**(5): p. 2196-204.
41. Newcomb, L.L., et al., *Interaction of the influenza A virus nucleocapsid protein with the viral RNA polymerase potentiates unprimed viral RNA replication.* J Virol, 2008.
42. Bourmakina, S.V., and A. Garcia-Sastre, *Reverse genetics studies on the filamentous morphology of influenza A virus.* Journal of General Virology, 2003. **84**: p. 517-527.

43. Barman, S., et al., *Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses.* Virus Research, 2001. **77**(1): p. 61-69.

44. de Wit, E., et al., *Molecular determinants of adaptation of highly pathogenic avian influenza H7N7 viruses to efficient replication in the human host.* J Virol. **84**(3): p. 1597-606.

45. Tumpey, T.M., et al., *A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission.* Science, 2007. **315**(5812): p. 655-9.

46. Liu, Y., et al., *Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus.* J Virol. **84**(22): p. 12069-74.

47. Korteweg, C. and J. Gu, *Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans.* Am J Pathol, 2008. **172**(5): p. 1155-70.

48. Mochalova, L., et al., *Receptor-binding properties of modern human influenza viruses primarily isolated in Vero and MDCK cells and chicken embryonated eggs.* Virology, 2003. **313**(2): p. 473-80.

49. Gambaryan, A.S., J.S. Robertson, and M.N. Matrosovich, *Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses.* Virology, 1999. **258**(2): p. 232-9.

50. Vines, A., K. Wells, M. Matrosovich, M.R. Castrucci, T. Ito, and Y. Kowaoka, *The role of influenza A virus hemagglutinin residues 226 and*

228 in receptor specificity and host range restriction. J Virol, 1998. **72**: p. 7626-7631.

51. Glaser, L., et al., *A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity.* J Virol, 2005. **79**(17): p. 11533-6.
52. Stevens, J., et al., *Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities.* J Mol Biol, 2006. **355**(5): p. 1143-55.
53. Yamada, S., et al., *Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors.* Nature, 2006. **444**(7117): p. 378-82.
54. Mak, G.C., et al., *Association of D222G substitution in haemagglutinin of 2009 pandemic influenza A (H1N1) with severe disease.* Euro Surveill. **15**(14).
55. Klenk, H.D. and W. Garten, *Host cell proteases controlling virus pathogenicity.* Trends Microbiol, 1994. **2**(2): p. 39-43.
56. Horimoto, T., et al., *Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses.* J Virol, 1994. **68**(9): p. 6074-8.
57. Bussey, K.A., et al., *PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells.* J Virol. **84**(9): p. 4395-406.

58. Hatta, M., et al., *Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice.* PLoS Pathog, 2007. **3**(10): p. 1374-9.

59. Munster, V.J., et al., *The molecular basis of the pathogenicity of the Dutch highly pathogenic human influenza A H7N7 viruses.* J Infect Dis, 2007. **196**(2): p. 258-65.

60. Song, M.S., et al., *The polymerase acidic protein gene of influenza a virus contributes to pathogenicity in a mouse model.* J Virol, 2009. **83**(23): p. 12325-35.

61. Li, Z., et al., *Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model.* J Virol, 2005. **79**(18): p. 12058-64.

62. Govorkova, E.A., et al., *Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004.* J Virol, 2005. **79**(4): p. 2191-8.

63. Salomon, R., et al., *The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04.* J Exp Med, 2006. **203**(3): p. 689-97.

64. Watanabe, T., et al., *Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets.* Proc Natl Acad Sci U S A, 2009. **106**(2): p. 588-92.

65. Zamarin, D., et al., *Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1.* PLoS Pathog, 2005. **1**(1): p. e4.

66. Conenello, G.M., et al., *A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence.* PLoS Pathog, 2007. **3**(10): p. 1414-21.

67. Pappas, C., et al., *Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3064-9.

68. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.* Science, 2004. **303**(5663): p. 1529-31.

69. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates.* Science, 2006. **314**(5801): p. 997-1001.

70. Kumagai, Y., O. Takeuchi, and S. Akira, *Pathogen recognition by innate receptors.* J Infect Chemother, 2008. **14**(2): p. 86-92.

71. Jiao, P., et al., *A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice.* J Virol, 2008. **82**(3): p. 1146-54.

72. Zhou, H., et al., *Effect on virulence and pathogenicity of H5N1 influenza A virus through truncations of NS1 eIF4GI binding domain.* J Infect Dis. **202**(9): p. 1338-46.

73. Pu, J., et al., *Synergism of co-mutation of two amino acid residues in NS1 protein increases the pathogenicity of influenza virus in mice.* Virus Res. **151**(2): p. 200-4.

74. Min, J.Y., et al., *A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis.* Virology, 2007. **363**(1): p. 236-43.

75. Seo, S.H., E. Hoffmann, and R.G. Webster, *Lethal H5N1 influenza viruses escape host anti-viral cytokine responses.* Nat Med, 2002. **8**(9): p. 950-4.

76. Gack, M.U., et al., *Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I.* Cell Host Microbe, 2009. **5**(5): p. 439-49.

77. Geiss, G.K., et al., *Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10736-41.

78. Li, S., et al., *Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA.* Virology, 2006. **349**(1): p. 13-21.

79. Kawai, T. and S. Akira, *Toll-like receptor and RIG-I-like receptor signaling.* Ann N Y Acad Sci, 2008. **1143**: p. 1-20.

80. Perrone, L.A., et al., *H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice.* PLoS Pathog, 2008. **4**(8): p. e1000115.

81. Lin, K.L., et al., *CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality.* J Immunol, 2008. **180**(4): p. 2562-72.

82. Herold, S., et al., *Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand.* J Exp Med, 2008. **205**(13): p. 3065-77.

83. Dawson, T.C., et al., *Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus.* Am J Pathol, 2000. **156**(6): p. 1951-9.

84. Tate, M.D., et al., *The role of neutrophils during mild and severe influenza virus infections of mice.* PLoS One, 2011. **6**(3): p. e17618.

85. Ge, X., et al., *Assessment of seasonal influenza A virus-specific CD4 T-cell responses to 2009 pandemic H1N1 swine-origin influenza A virus.* J Virol, 2010. **84**(20071564): p. 3312-3319.

86. Kreijtz, J.H.C.M., et al., *Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus.* J Virol, 2008. **82**(18353950): p. 5161-5166.

87. Tu, W., et al., *Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus.* J Virol, 2010. **84**(20410263): p. 6527-6535.

88. Kim, K.D., et al., *Adaptive immune cells temper initial innate responses.* Nat Med, 2007. **13**(17891146): p. 1248-1252.

89. Guarda, G., et al., *T cells dampen innate immune responses through inhibition of NLRP1 and NLRP3 inflammasomes.* Nature, 2009. **460**(19494813): p. 269-273.

90. Aldridge, J.R., et al., *TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection.* Proc Natl Acad Sci U S A, 2009. **106**(19279209): p. 5306-5311.

91. McGill, J., N. Van Rooijen, and K.L. Legge, *Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs.* J Exp Med, 2008. **205**(18591411): p. 1635-1646.

92. La Gruta, N.L., et al., *A question of self-preservation: immunopathology in influenza virus infection.* Immunol Cell Biol, 2007. **85**(2): p. 85-92.

93. Ramana, C.V., et al., *Role of alveolar epithelial early growth response-1 (Egr-1) in CD8+ T cell-mediated lung injury.* Mol Immunol, 2009. **47**(19786304): p. 623-631.

94. Weaver, C.T., et al., *Th17: an effector CD4 T cell lineage with regulatory T cell ties.* Immunity, 2006. **24**(16782025): p. 677-688.

95. Berkhoff, E.G., et al., *A mutation in the HLA-B*2705-restricted NP383-391 epitope affects the human influenza A virus-specific cytotoxic T-lymphocyte response in vitro.* J Virol, 2004. **78**(10): p. 5216-22.

96. McKinstry, K.K., et al., *IL-10 deficiency unleashes an influenza-specific Th17 response and enhances survival against high-dose challenge.* J Immunol, 2009. **182**(19494257): p. 7353-7363.

97. Sun, J., et al., *Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10.* Nat Med, 2009. **15**(3): p. 277-84.

98. Snelgrove, R.J., et al., *A critical function for CD200 in lung immune homeostasis and the severity of influenza infection.* Nat Immunol, 2008. **9**(18660812): p. 1074-1083.

99. Rygiel, T.P., et al., *Lack of CD200 enhances pathological T cell responses during influenza infection.* J Immunol, 2009. **183**(19587022): p. 1990-1996.

100. Grebe, K.M., et al., *Cutting edge: Sympathetic nervous system increases proinflammatory cytokines and exacerbates influenza A virus pathogenesis.* J Immunol, 2010. **184**(2): p. 540-4.

101. Ludwig, S., *Targeting cell signalling pathways to fight the flu: towards a paradigm change in anti-influenza therapy.* J Antimicrob Chemother, 2009. **64**(1): p. 1-4.

102. Hay, A.J., et al., *THE MOLECULAR-BASIS OF THE SPECIFIC ANTI-INFLUENZA ACTION OF AMANTADINE.* Embo Journal, 1985. **4**(11): p. 3021-3024.

103. Moscona, A., *Drug therapy - Neuraminidase inhibitors for influenza.* New England Journal of Medicine, 2005. **353**(13): p. 1363-1373.

104. CDC. *Fluview: Antiviral Resistance*. 2009 May 25 2009].

105. Droebner, K., et al., *CYSTUS052, a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice.* Antiviral Res, 2007. **76**(1): p. 1-10.

106. Ehrhardt, C., et al., *A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance.* Antiviral Res, 2007. **76**(1): p. 38-47.

107. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(9486653): p. 806-811.

108. Leung, R.K. and P.A. Whittaker, *RNA interference: from gene silencing to gene-specific therapeutics.* Pharmacol Ther, 2005. **107**(2): p. 222-39.

109. Tripp, R.A. and S.M. Tompkins, *Therapeutic applications of RNAi for silencing virus replication.* Methods Mol Biol, 2009. **555**: p. 43-61.

110. Carthew, R.W. and E.J. Sontheimer, *Origins and Mechanisms of miRNAs and siRNAs.* Cell, 2009. **136**(4): p. 642-55.

111. Paddison, P.J., *RNA interference in mammalian cell systems.* Curr Top Microbiol Immunol, 2008. **320**: p. 1-19.

112. Pal-Bhadra, M., U. Bhadra, and J.A. Birchler, *RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila.* Mol Cell, 2002. **9**(2): p. 315-27.

113. Lee, Y., et al., *The role of PACT in the RNA silencing pathway.* EMBO J, 2006. **25**(3): p. 522-32.

114. Song, J.J., et al., *Crystal structure of Argonaute and its implications for RISC slicer activity.* Science, 2004. **305**(5689): p. 1434-7.

115. Langlois, M.A., et al., *Cytoplasmic and nuclear retained DMPK mRNAs are targets for RNA interference in myotonic dystrophy cells.* J Biol Chem, 2005. **280**(17): p. 16949-54.

116. Haasnoot, J., E.M. Westerhout, and B. Berkhout, *RNA interference against viruses: strike and counterstrike.* Nat Biotechnol, 2007. **25**(12): p. 1435-43.

117. Kim, D.H. and J.J. Rossi, *Strategies for silencing human disease using RNA interference.* Nat Rev Genet, 2007. **8**(3): p. 173-84.

118. Wiznerowicz, M., J. Szulc, and D. Trono, *Tuning silence: conditional systems for RNA interference.* Nat Methods, 2006. **3**(9): p. 682-8.

119. He, S., et al., *The properties and functions of virus encoded microRNA, siRNA, and other small noncoding RNAs.* Crit Rev Microbiol, 2008. **34**(3-4): p. 175-88.

120. Mocellin, S. and M. Provenzano, *RNA interference: learning gene knock-down from cell physiology.* Journal of translational medicine, 2004. **2**(1): p. 39.

121. Cherry, S., *What have RNAi screens taught us about viral-host interactions?* Current opinion in microbiology, 2009. **12**(4): p. 446-52.

122. Brass, A.L., et al., *The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus.* Cell, 2009. **139**(7): p. 1243-54.

123. Hao, L., et al., *Drosophila RNAi screen identifies host genes important for influenza virus replication.* Nature, 2008. **454**(7206): p. 890-3.

124. Karlas, A., et al., *Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication.* Nature, 2010.

125. Konig, R., et al., *Human host factors required for influenza virus replication.* Nature, 2009. **463**(7282): p. 813-7.

126. Shapira, S.D., et al., *A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection.* Cell, 2009. **139**(7): p. 1255-67.

127. Lau, N.C., et al., *An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 858-62.

128. Wolters, N.M. and J.P. MacKeigan, *From sequence to function: using RNAi to elucidate mechanisms of human disease.* Cell Death Differ, 2008. **15**(5): p. 809-19.

129. Rana, T.M., *Illuminating the silence: understanding the structure and function of small RNAs.* Nat Rev Mol Cell Biol, 2007. **8**(1): p. 23-36.

130. Petersen, C.P., et al., *Short RNAs repress translation after initiation in mammalian cells.* Mol Cell, 2006. **21**(4): p. 533-42.

131. Matzke, M.A. and J.A. Birchler, *RNAi-mediated pathways in the nucleus.* Nat Rev Genet, 2005. **6**(1): p. 24-35.

132. Matzke, M.A., et al., *Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants.* EMBO J, 1989. **8**(3): p. 643-649.

133. Zilberman, D., X. Cao, and S.E. Jacobsen, *ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation.* Science, 2003. **299**(5607): p. 716-9.

134. Morris, K.V., *RNA-mediated transcriptional gene silencing in human cells.* Curr Top Microbiol Immunol, 2008. **320**: p. 211-24.

135. Weinberg, M.S., et al., *An RNA targeted to the HIV-1 LTR promoter modulates indiscriminate off-target gene activation.* Nucleic Acids Res, 2007. **35**(21): p. 7303-12.

136. Triboulet, R., et al., *Suppression of microRNA-silencing pathway by HIV-1 during virus replication.* Science, 2007. **315**(5818): p. 1579-82.

137. Suzuki, K., et al., *Closed chromatin architecture is induced by an RNA duplex targeting the HIV-1 promoter region.* J Biol Chem, 2008. **283**(34): p. 23353-63.

138. Hughes, R.A., A.E. Miklos, and A.D. Ellington, *Gene synthesis methods and applications.* Methods in enzymology, 2011. **498**: p. 277-309.

139. Brass, A.L., et al., *Identification of host proteins required for HIV infection through a functional genomic screen.* Science, 2008. **319**(5865): p. 921-6.

140. Konig, R., et al., *Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication.* Cell, 2008. **135**(1): p. 49-60.

141. Zhou, H., et al., *Genome-scale RNAi screen for host factors required for HIV replication.* Cell Host & Microbe, 2008. **4**(5): p. 495-504.

142. Sessions, O.M., et al., *Discovery of insect and human dengue virus host factors.* Nature, 2009. **458**(7241): p. 1047-50.

143. Krishnan, M.N., et al., *RNA interference screen for human genes associated with West Nile virus infection.* Nature, 2008. **455**(7210): p. 242-5.

144. Sigoillot, F.D. and R.W. King, *Vigilance and validation: Keys to success in RNAi screening.* ACS chemical biology, 2011. **6**(1): p. 47-60.

145. Hirsch, A.J., *The use of RNAi-based screens to identify host proteins involved in viral replication.* Future Microbiol. **5**(2): p. 303-11.

146. Vermeulen, A., et al., *Double-stranded regions are essential design components of potent inhibitors of RISC function.* RNA, 2007. **13**(5): p. 723-30.

147. Winter, J., et al., *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nature cell biology, 2009. **11**(3): p. 228-34.

148. Birmingham, A., et al., *Statistical methods for analysis of high-throughput RNA interference screens.* Nat Methods, 2009. **6**(8): p. 569-75.

149. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome research, 2009. **19**(1): p. 92-105.

150. Santhakumar, D., et al., *Combined agonist-antagonist genome-wide functional screening identifies broadly active antiviral microRNAs.* Proc Natl Acad Sci U S A, 2010. **107**(31): p. 13830-5.

151. Hale, B.G., et al., *The multifunctional NS1 protein of influenza A viruses.* J Gen Virol, 2008. **89**(Pt 10): p. 2359-76.

152. Lazarowitz, S.G., A.R. Goldberg, and P.W. Choppin, *Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: host cell activation of serum plasminogen.* Virology, 1973. **56**(1): p. 172-80.

153. Arora, D.J. and N. Gasse, *Influenza virus hemagglutinin stimulates the protein kinase C activity of human polymorphonuclear leucocytes.* Arch Virol, 1998. **143**(10): p. 2029-37.

154. Pahl, H.L. and P.A. Baeuerle, *Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B.* J Virol, 1995. **69**(3): p. 1480-4.

155. Flory, E., et al., *Influenza virus-induced NF-kappaB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IkappaB kinase.* J Biol Chem, 2000. **275**(12): p. 8307-14.

156. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.

157. Stark, G.R., et al., *How cells respond to interferons.* Annu Rev Biochem, 1998. **67**: p. 227-64.

158. Kumar, A., et al., *Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B.* Proc Natl Acad Sci U S A, 1994. **91**(14): p. 6288-92.

159. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.* Nature, 2006. **441**(7089): p. 101-5.

160. Majde, J.A., *Viral double-stranded RNA, cytokines, and the flu.* J Interferon Cytokine Res, 2000. **20**(3): p. 259-72.

161. Chu, W.M., et al., *JNK2 and IKKbeta are required for activating the innate response to viral infection.* Immunity, 1999. **11**(6): p. 721-31.

162. Ludwig, S., et al., *Ringing the alarm bells: signalling and apoptosis in influenza virus infected cells.* Cell Microbiol, 2006. **8**(3): p. 375-86.

163. Ludwig, S., *Exploited defense: how influenza viruses take advantage of antiviral signaling responses.* Future Virology, 2007. **2**(1): p. 91-100.

164. Ludwig, S. and O. Planz, *Influenza viruses and the NF-kappaB signaling pathway - towards a novel concept of antiviral therapy.* Biol Chem, 2008. **389**(10): p. 1307-12.

165. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* FEBS Lett, 2004. **561**(1-3): p. 37-43.

166. Chang, L. and M. Karin, *Mammalian MAP kinase signalling cascades.* Nature, 2001. **410**(6824): p. 37-40.

167. Marjuki, H., et al., *Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Calpha-mediated activation of ERK signaling.* J Biol Chem, 2006. **281**(24): p. 16707-15.

168. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* Febs Letters, 2004. **561**(1-3): p. 37-43.

169. Pleschka, S., et al., *Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade.* Nature cell biology, 2001. **3**(3): p. 301-5.

170. Planz, O., S. Pleschka, and S. Ludwig, *MEK-specific inhibitor U0126 blocks spread of Borna disease virus in cultured cells.* J Virol, 2001. **75**(10): p. 4871-7.

171. Sebolt-Leopold, J.S., et al., *Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo.* Nat Med, 1999. **5**(7): p. 810-6.

172. Cohen, P., *Protein kinases--the major drug targets of the twenty-first century?* Nat Rev Drug Discov, 2002. **1**(4): p. 309-15.

173. Ludwig, S., et al., *Influenza-virus-induced signaling cascades: targets for antiviral therapy?* Trends Mol Med, 2003. **9**(2): p. 46-52.

174. Montagut, C. and J. Settleman, *Targeting the RAF-MEK-ERK pathway in cancer therapy.* Cancer Lett, 2009. **283**(2): p. 125-34.

175. Karin, M., *How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex.* Oncogene, 1999. **18**(49): p. 6867-74.

176. Karin, M., *The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation.* J Biol Chem, 1999. **274**(39): p. 27339-42.

177. Pahl, H.L., *Activators and target genes of Rel/NF-kappaB transcription factors.* Oncogene, 1999. **18**(49): p. 6853-66.

178. Hiscott, J., H. Kwon, and P. Genin, *Hostile takeovers: viral appropriation of the NF-kappaB pathway.* J Clin Invest, 2001. **107**(2): p. 143-51.

179. Wang, X., et al., *Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon.* J Virol, 2000. **74**(24): p. 11566-73.

180. Wurzer, W.J., et al., *NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation.* J Biol Chem, 2004. **279**(30): p. 30931-7.

181. Nimmerjahn, F., et al., *Active NF-kappaB signalling is a prerequisite for influenza virus infection.* J Gen Virol, 2004. **85**(Pt 8): p. 2347-56.

182. Wurzer, W.J., et al., *Caspase 3 activation is essential for efficient influenza virus propagation.* EMBO J, 2003. **22**(11): p. 2717-28.

183. Kramer, A., et al., *Apoptosis leads to a degradation of vital components of active nuclear transport and a dissociation of the nuclear lamina.* Proc Natl Acad Sci U S A, 2008. **105**(32): p. 11236-41.

184. Pauli, E.K., et al., *Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression.* PLoS Pathog, 2008. **4**(11): p. e1000196.

185. Wei, L., et al., *NFkappaB negatively regulates interferon-induced gene expression and anti-influenza activity.* J Biol Chem, 2006. **281**(17): p. 11678-84.

186. Kumar, N., et al., *NF-kappaB signaling differentially regulates influenza virus RNA synthesis.* J Virol, 2008. **82**(20): p. 9880-9.

187. Barber, S.A., et al., *Visna virus-induced activation of MAPK is required for virus replication and correlates with virus-induced neuropathology.* J Virol, 2002. **76**(2): p. 817-28.

188. Luo, H., et al., *Coxsackievirus B3 replication is reduced by inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway.* J Virol, 2002. **76**(7): p. 3365-73.

189. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**: p. 843-854.

190. Lee, R.C. and V. Ambros, *An extensive class of small RNAs in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 862-4.

191. Lagos-Quintana, M., et al., *Identification of novel genes coding for small expressed RNAs.* Science, 2001. **294**(5543): p. 853-8.

192. Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg, *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nat Rev Genet, 2008. **9**(2): p. 102-14.

193. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.

194. Ambros, V., *The functions of animal microRNAs.* Nature, 2004. **431**(7006): p. 350-5.

195. Grey, F., et al., *A viral microRNA down-regulates multiple cell cycle genes through mRNA 5'UTRs.* PLoS Pathog. **6**: p. e1000967.

196. Kim, V.N. and J.W. Nam, *Genomics of microRNA.* Trends in Genet, 2006. **22**: p. 165-173.

197. Kim, Y.K. and V.N. Kim, *Processing of intronic microRNAs.* EMBO J, 2007. **26**: p. 775-783.

198. Rodriguez, A., et al., *Identification of mammalian microRNA host genes and transcription units.* Genome research, 2004. **14**: p. 1902-1910.

199. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**(Database issue): p. D140-4.

200. Griffiths-Jones, S., *The microRNA Registry.* Nucleic Acids Res, 2004. **32**(Database issue): p. D109-11.

201. Nishida, E. and Y. Gotoh, *The MAP kinase cascade is essential for diverse signal transduction pathways.* Trends Biochem Sci, 1993. **18**(8388132): p. 128-131.

202. Thum, T., et al., *MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts.* Nature, 2008. **456**(19043405): p. 980-984.

203. Hanafusa, H., et al., *Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway.* Nature cell biology, 2002. **4**(12402043): p. 850-858.

204. Sayed, D., et al., *MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths.* Mol Biol Cell, 2008. **19**(18508928): p. 3272-3282.

205. Huang, T.-H., et al., *Up-regulation of miR-21 by HER2/neu signaling promotes cell invasion.* J Biol Chem, 2009. **284**(19419954): p. 18515-18524.

206. Fujita, S., et al., *miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism.* J Mol Biol, 2008. **378**(18384814): p. 492-504.

207. Yin, Q., et al., *MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation.* J Virol. **84**(13): p. 6318-27.

208. Cannell, I.G., et al., *p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication.* Proc Natl Acad Sci U S A, 2010. **107**(20212154): p. 5375-5380.

209. Kawashima, H., et al., *Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression.* Neuroscience, 2010. **165**(19958814): p. 1301-1311.

210. Voorhoeve, P.M., et al., *A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors.* Cell, 2006. **124**(16564011): p. 1169-1181.

211. Davis, B.N., et al., *SMAD proteins control DROSHA-mediated microRNA maturation.* Nature, 2008. **454**(18548003): p. 56-61.

212. Christoffersen, N.R., et al., *miR-200b mediates post-transcriptional repression of ZFHX1B.* RNA, 2007. **13**(17585049): p. 1172-1178.

213. Gregory, P.A., et al., *The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.* Nature cell biology, 2008. **10**(18376396): p. 593-601.

214. Hurteau, G.J., et al., *Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin.* Cancer Res, 2007. **67**(17804704): p. 7972-7976.

215. Burk, U., et al., *A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells.* EMBO Rep, 2008. **9**(18483486): p. 582-589.

216. Baltimore, D., et al., *MicroRNAs: new regulators of immune cell development and function.* Nat Immunol, 2008. **9**: p. 839-845.

217. Lodish, H.F., et al., *Micromanagement of the immune system by microRNAs.* Nat Rev Immunol, 2008. **8**: p. 120-130.

218. Kingwell, K., *Cardiovascular disease: microRNA protects the heart.* Nat Rev Drug Discov. **10**(2): p. 98.

219. Ceribelli, A., et al., *MicroRNAs in rheumatoid arthritis*.

220. Gupta, A., et al., *Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript.* Nature, 2006. **442**(7098): p. 82-5.

221. Burnside, J., et al., *Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript.* J Virol, 2006. **80**(17): p. 8778-86.

222. Zheng, S.Q., et al., *MiR-101 regulates HSV-1 replication by targeting ATP5B.* Antiviral Res. **89**(3): p. 219-26.

223. Lagos, D., et al., *miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator.* Nature cell biology. **12**(5): p. 513-9.

224. Yeung, M.L., et al., *Changes in microRNA expression profiles in HIV-1-transfected human cells.* Retrovirology, 2005. **2**: p. 81.

225. Triboulet, R., et al., *Suppression of microRNA-silencing pathway by HIV-1 during virus replication.* Science, 2007. **315**: p. 1579-1582.

226. Anastasiadou, E., et al., *Epstein-Barr virus encoded LMP1 downregulates TCL1 oncogene through miR-29b.* Oncogene. **29**: p. 1316-1328.

227. Cameron, J.E., et al., *Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways.* J Virol, 2008. **82**: p. 1946-1958.

228. Lecellier, C.H., et al., *A cellular microRNA mediates antiviral defense in human cells.* Science, 2005. **308**: p. 557-560.

229. Pedersen, I.M., et al., *Interferon modulation of cellular microRNAs as an antiviral mechanism.* Nature, 2007. **449**: p. 919-922.

230. Hou, J., et al., *MicroRNA-146a feedback inhibits RIG-I-dependent type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2.* J Immunol, 2009. **183**: p. 2150-2158.

231. Lagos, D., et al., *miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator.* Nature cell biology. **12**: p. 513-519.

232. Song, L., et al., *Cellular microRNAs inhibit replication of the H1N1 influenza A virus in infected cells.* J Virol. **84**(17): p. 8849-60.

233. Scaria, V., et al., *Host-virus interaction: a new role for microRNAs.* Retrovirology, 2006. **3**: p. 68.

234. Liu, H., L. Song, and W. Huang, *[MiR26a and miR939 regulate the replication of H1N1 influenza virus in MDCK cell].* Wei Sheng Wu Xue Bao. **50**(10): p. 1399-405.

235. Plaisance-Bonstaff, K. and R. Renne, *Viral miRNAs.* Methods Mol Biol. **721**: p. 43-66.

236. Skalsky, R.L. and B.R. Cullen, *Viruses, microRNAs, and host interactions.* Annu Rev Microbiol. **64**: p. 123-141.

237. Gottwein, E., et al., *A viral microRNA functions as an orthologue of cellular miR-155.* Nature, 2007. **450**: p. 1096-1099.

238. Skalsky, R.L., et al., *Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155.* J Virol, 2007. **81**: p. 12836-12845.

239. Xiao, C. and K. Rajewsky, *MicroRNA control in the immune system: basic principles.* Cell, 2009. **136**: p. 26-36.

240. Pfeffer, S., et al., *Identification of microRNAs of the herpesvirus family.* Nat Methods, 2005. **2**: p. 269-276.

241. Pfeffer, S., et al., *Identification of virus-encoded microRNAs.* Science, 2004. **304**: p. 734-736.

242. Stern-Ginossar, N., et al., *Host immune system gene targeting by a viral miRNA.* Science, 2007. **317**: p. 376-381.

243. Bell, J.C. and D. Kirn, *MicroRNAs fine-tune oncolytic viruses.* Nat Biotechnol, 2008. **26**(12): p. 1346-8.

244. Cullen, B.R., *Viruses and microRNAs.* Nat Genet, 2006. **38 Suppl**: p. S25-30.

245. Shahab, S.W., et al., *Evidence for the Complexity of MicroRNA-Mediated Regulation in Ovarian Cancer: A Systems Approach.* PLoS One, 2011. **6**(7): p. e22508.

246. Jay, C., et al., *miRNA profiling for diagnosis and prognosis of human cancer.* DNA Cell Biol, 2007. **26**(5): p. 293-300.

247. Megiorni, F., A. Pizzuti, and L. Frati, *Clinical Significance of MicroRNA Expression Profiles and Polymorphisms in Lung Cancer Development and Management.* Pathology research international, 2011. **2011**: p. 780652.

248. Kota, J., et al., *Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model.* Cell, 2009. **137**(6): p. 1005-17.

249. Su, C., et al., *Ectopic expression of microRNA-155 enhances innate antiviral immunity against HBV infection in human hepatoma cells.* Virology Journal, 2011. **8**(1): p. 354.

250. Jopling, C.L., et al., *Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA.* Science, 2005. **309**(5740): p. 1577-81.

CHAPTER 3

USING A RNAI GENOME SCREEN TO IDENTIFY HUMAN PROTEIN KINASE GENES REQUIRED FOR INFLUENZA A REPLICATION IN HUMAN ALVEOLAR EPITHELIAL CELLS[1]

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Abstract

Influenza virus is a global threat to public health causing seasonal epidemics and recurring pandemics. High mutation rates lead to the generation of viral escape mutants rendering vaccines and antiviral drugs ineffective. An alternative disease intervention approach that is refractory to viral escape is targeting of host cell factors that are critical for viral replication, but not essential for host cell survival. To this end, we utilized a kinome-wide RNA interference (RNAi) screening approach in a mammalian cell culture system to identify host kinase genes influencing influenza virus replication. Here we identify 22 human host kinase genes modulated during A/WSN/33 influenza virus (WSN) replication and provide new insight into how influenza virus interacts with its host at the molecular level.

Introduction

Seasonal influenza virus infection is a leading cause of morbidity and mortality worldwide [1]. Each year, seasonal influenza virus affects up to 15% of the human population, causing severe illness in an estimated 3-5 million people, with approximately 500,000 deaths [1]. Moreover, the economic burden associated with influenza virus infection is considerable. In the US alone, it is estimated to total $87.1 billion annually [2]. The emergence of H5N1 avian influenza virus and the swine-origin H1N1 influenza virus highlight the ever-increasing risk influenza viruses pose to human health. In 2009, the swine-origin H1N1 pandemic virus spread to 214 countries and superseded pre-existing circulating H1N1 and H3N2 viruses in the human population [3].

Influenza viruses are members of the *Orthomyxoviridae* family of viruses. These are enveloped viruses having an eight segmented, negative-sense, single-stranded RNA genome that encodes up to 11 proteins [4]. The viral envelope contains hemagglutinin (HA) and neuraminidase (NA) glycoproteins, and the membrane ion channel protein, M2. Within the virion, the matrix protein (M1) provides structure and secures the viral ribonucleoprotein (RNP) complexes consisting of viral RNA coupled to nucleoprotein (NP) and the three polymerase proteins (PB1, PB2 and PA). The virus also encodes two nonstructural proteins, NS1 and NS2, and the recently identified PB1-F2 protein found in some virus species.

Antiviral drugs can be used to reduce symptoms and complications when a host becomes infected with influenza virus by killing or suppressing the replication of the virus. To date, antiviral drugs developed have been designed to inhibit the function of an influenza virus gene product. Two classes of antiviral drugs are available for influenza virus; the adamantanes targeted to the M2-ion channel and the more commonly used NA inhibitors such as oseltamivir (Tamiflu) and zanamivir [5, 6]. By targeting a viral function, these drugs put selective pressure on the virus and drive mutations that can lead to viral escape rendering the drugs ineffective. In October of 2008, the CDC began surveying clinical samples for antiviral resistant and found greater than 99% of H1N1 viruses were resistant to oseltamivir [7May 25, 2009]. Thus, an area of increasing interest is switching the focus from viral genes to cellular genes essential for the virus life cycle (but nonessential for the host). There are several examples where partial or complete resistance to pathogens results from loss of expression or function of a critical host gene without severe repercussions to the individual. Individuals homozygous for a dysfunctional allele of CCR5 are protected against HIV transmission, and show delayed disease progression [8, 9]. Also, a mutation of the CFTR gene is known to confer resistance to *Salmonella typhi* [10]. And last, heterozygosity for the mutant sickle hemoglobin protects from *P. falciparum* malaria [11], and complete protection against *P. vivax* malaria is conferred by loss of expression of the erythroid malaria receptor, DARC [12]. Therefore, such an approach may pose an insurmountable barrier against influenza virus replication, as resistance would arise not from relatively simple escape mutations, but rather, adaptation to use a different cellular protein for the same purpose.

RNAi is a natural cellular pathway involving sequence-dependent post-transcriptional silencing of genes [13]. RNAi-based technology has been extensively used in functional genomics studies [14-16] and can be compared to several analogous technologies, including compound screens, microarray and next generation screening and yeast two hybrid screens. The process of exploiting the RNAi pathway to identify the function of particular genes in biological processes can be achieved on a global scale using genome-wide siRNA libraries. The major goal for genome-wide RNAi screening is to carefully dissect host-virus interactions. Genome-wide small interfering RNAs (siRNA) libraries contain multiple siRNAs designed against each gene of interest. Typically a siRNA library consists of 4 pooled siRNAs for each gene. Including multiple siRNAs (pooled) against each gene, provides a cumulative silencing effect which greatly increases the efficacy of silencing compared to that of the use of a single siRNA. siRNA libraries are available for a number of organism including, *H. sapiens*, *M. musculus*, *D. melanogaster*, *C. elegans*, and *T. brucei* and in theory, a library can be generated for any organism whose genome has been sequenced. In terms of the human genome, a whole genome siRNA screen generally consists of a library of over 80,000 different siRNAs which are designed to silence each of the approximately 20,000 human genes. Given the limited size of viral genomes, viruses are highly dependent on host cell machinery for survival and genome-wide screening with siRNA libraries would allow for the study of host genes involved throughout the entire replication cycle [17].

It is well established that the host cell will initiate signaling processes in response to an invading pathogen such as influenza virus, often in a phosphorylation-regulated manner [18, 19]. The signals are then transduced into specific cellular actions, such as changes in gene expression patterns, which aim to defend the host from the pathogen. However, it has also been shown that the virus can hijack these signaling processes to facilitate replication [18, 19]. Recent data reveal host genes involved in protein kinase signaling pathways that are important for influenza virus replication [20-24]. Identifying other host protein kinase genes essential for viral replication will not only provide a framework for better understanding the virus-host interface, but will also provide novel therapeutic targets. To this end, we employ a siRNA library targeting 720 host protein kinase genes in a human alveolar epithelial cell to dissect host kinases modulated during WSN infection. The primary screen data presented here identifies 22 host cofactors of viral replication that likely play an essential role in influenza virus replication.

siGENOME Assay

As way of overview, a key feature of the siRNA genome-wide (siGENOME) silencing studies is reverse transfection of commercially available siRNA libraries into influenza-permissive A549 cells (Figure 3.1A). Following a 48 h incubation to allow target gene silencing, the cells are infected with WSN/33 influenza virus. After 48 h pi, the level of infectious virus is detected in the supernatant and quantified via a modified $TCID_{50}$ assay (Figure 3.1B).

Cell lines and susceptibility to infection

To determine the best model system for investigating host genes required for human influenza virus replication, various cells lines were investigated for susceptibility to infection with WSN (data not shown). For evaluating the impact of host gene silencing on influenza virus replication, a plaque assay was performed where serial 10-fold dilutions of cell supernatants were added to a monolayer of Madin–Darby canine kidney (MDCK) cells using an Avicel overlay [25]. Three days after infection, plaques were visualized by staining with 0.1% crystal violet. The cell lines investigated as candidates for the siGENOME screen were A549 (human lung type II epithelial cells), L929 (mouse fibroblasts), and MDCK cells. The MDCK cell line has been extensively used for influenza studies and is thoroughly documented in the literature [26-29]. Accordingly, this cell line was most susceptible to A/WSN/33 infection and replication (data not shown); however, because this cell line is of canine origin, and the siRNAs used in the siGENOME screen are designed against human targets, A549 cells were chosen as the model cell line as they are susceptible to influenza virus infection [30] and can be readily reverse transfected with siRNA molecules [15, 16]. All studies we performed using A549 human lung epithelial cells. To minimize biological variation between experiments, the A549 cells were grown to a large single batch and 80 aliquots of passage 7 cells, with 3.5×10^5 cells per vial, were frozen down and stored in liquid nitrogen. This allowed us to use the same passage of A549 cells for all studies.

The screen was performed with A/WSN/33 influenza virus because the NA protein of WSN influenza allows viral replication in the absence of exogenous trypsin [31], a feature critical as the addition of trypsin can lead to cell detachment and misinterpretation of results. Another feature of using A/SN/33 virus is that it replicates well in the human lung epithelial cell line A549, and in embryonated chicken eggs [15, 16]. Thus, the virus was propagated in the allantoic cavity of 9-day-old embryonated chicken eggs at 37°C. Allantoic fluid was harvested 48 h after virus inoculation and stored at -80°C. Virus titer was measured by MDCK cell plaque assay (described above) at 5.0×10^7 PFU/mL.

siRNAs, controls, and transfection conditions

The screen used a validated siRNA library (si*GENOME)* that was designed by the manufacturer as SMART-selection siRNA to ensure comprehensive gene silencing [32]. The specific siGENOME library used for these studies included siRNAs targeting all unique human protein kinase (HPK) genes in the NCBI RefSeq database (Appendices Table S1). The HPK library spans 9 plates (a total of 720 gene targets), in 96 well format with the siRNAs lyophilized to the bottom of the designated well. Each well includes four-pooled siRNAs targeting the same kinase gene on a different seed site. All siRNAs were resuspended to 1 µM in Dharmacon 1X resuspension buffer and stored at -80°C. Columns 1 and 12 on each plate are empty to allow room for on plate controls (Figure 3.1A). The mitogen-activated protein kinase kinase 1 positive control (MEK, 5’-GCACAUGGAUGGAGGUUCU-3’, 5’-GCAGAGAGAGCAGAUUUGA-3’,

5’-GAGCAGAUUUGAAGCAACU-3’, 5’-CCAGAAAGCUAAUUCAUCU-3’, siGENOME smartpool, Dharmacon M-003571-01) targets a host gene important for influenza virus replication and its silencing should decrease viral replication compared to the negative control [33]. The negative non-targeting control siRNA (SINEG, 5'-UAGCGACUAAACACAUCAA-3', siCONTROL Non-Targeting siRNA #1, Dharmacon D-001210-01-05) controls for non-sequence-specific effects. The cellular cytotoxicity control (TOX, Dharmacon D-001500-01-05) is used to control for any siGENOME siRNA cytotoxic effects. For our studies, the TOX transfected cells are considered 100% cytotoxic and are used to ensure a decrease in viral replication observed is not due to a decrease in the number of viable cells.

The siGENOME plates are optimized for a reverse transfection format. Therefore, A549 cells were plated onto siRNA-transfection complexes in 96-well flat-bottom plates, 15,000 cells per well, and the cells were transiently transfected for 48 h with 50 nM siRNA. At 48 h post-transfection, cells supernatants were removed and tested to evaluate any toxic effects potentially caused by the siRNA as this could potentially giving false positive results. Specifically, adenylate kinase (AK) release was measured from the cells via luminescence [34]. Luminescence was measured and the readout determined as relative luciferase units (RLU). Cytotoxicity values for each host gene silenced were compared to the TOX cytotoxicity control [35]. Specifically, the percent cytotoxicity was determined after subtracting the background from the TOX positive control and non-targeting negative control, for each experimental siRNA (Appendices Table S2). Any siRNA causing > 20% cytotoxicity was excluded as a hit.

Infection assay

After excluding siRNAs having cytotoxic potential, the A549 cells transfected with siRNAs were rinsed, rested 48h, and subsequently infected at a MOI = 0.001with A/WSN/33 in DMEM supplemented with 5% fetal bovine serum. After 2 h incubation at 37°C, the infection media was removed and cells were rinsed and replenished with fresh media without virus. Then 48 hours pi, a modified $TCID_{50}$ was performed on MDCK cells using the A549 cell supernatants. As shown in Figure 3.1B, twenty milliliters of supernatant was removed from each well of the treated A549 plate and transferred to row A of a new sterile 96 well plate. Subsequently, 1:10 dilutions were performed down to row D discarding the final 20ul. The experiments were performed in duplicate. This whole process was repeated until all rows from the transfection plate were titrated, each well having a final volume of 180 ml. Last, 20 ml of MDCK cells (at a cell suspension of 2×10^6 cell per mL) were added to each well. After a 72 h incubation, a hemagglutination assay (HA) was performed. Specifically, 50 ml of supernatant was mixed with 50 ml of chicken red blood cells to test for agglutination – a feature indicative of the level of virus replication in the supernatant [36].

Validating by proof-of-concept

Once the experimental system was determined and the standard operating procedure finalized, the system was validated using three siRNAs directed to host kinase genes published to be important for influenza virus replication [20, 37-41]. The siRNAs tested were specific for MAP kinase (MEK), a protein involved in host protein and tyrosine kinase activities [20], Protein Kinase C II-beta, a host gene implicated in virus entry processes [41], and lastly Rab5, a host gene thought to regulate the early endosome and trafficking [39]. All siRNAs were effective in decreasing WSN virus replication compared to the positive control siRNA targeting influenza NP (NP-1496) and relative to a non-target negative control siRNA (Figure 3.2).

Screen results

A goal of this study was to identify cellular protein kinase genes required for influenza replication but not essential for host cell survival. The categories of data involved in the screen included, (i) $TCID_{50}$ assay data, (ii) data normalized to controls to allow for across plate comparison, and (iii) Z-score analysis to identify those siRNAs whose silencing target mRNA had the greatest effect on influenza virus replication.

The HPK library contained 9 plates with siRNA SMARTpools that targeted 720 different protein kinase genes (Figure 3.3A-I) where one genes is targeted per SMARTpool well which contains four pooled siRNAs targeting the same gene. A549 cells were reverse transfected in each of the 9 plates, the genes allowed to be silenced by RNAi, and then the cells subsequently infected with A/WSN/33 (WSN). The expected outcomes of host gene silencing on WSN replication was either to increased virus replication through silencing of an innate virus resistance gene, a decreased effect on replication indicative of a host gene required for replication, or no effect compared to siNEG treated cells (Figure 3.3A-I). To facilitate selection of hits, the effect on virus replication was scored 0 to 4 based on how far the virus titrated in an HA assay (Appendices Table S2). A score of zero indicated no detection of virus in any of the wells and a score of 4 indicated influenza virus could be detected in all of the wells. The experiment was done at least in duplicate, independently, by two or more different individuals. All siRNAs with greater that 20% cytotoxicity were excluded.

Normalization, Z-score analysis and hit identification

To allow for across plate comparison, raw data from each plate was normalized to the average score of the non-target negative control siRNA transfected and WSN-infected cells for each plate (Figure 3.4). This was done for all 9 plates, for each duplicate and independent experiment. The result was normalized scores for each of the 720 targets, and for each of the four replicates (Appendices Table S2). For each of the 720 targets, the mean of the four replicates was determined, simplifying to one score per target. Appendices Table S2 states the final 720 normalized scores.

Z-score analysis was performed to identify our the most likely hits. Z-scores are expressed as standard deviations from their means, and as a result, the distribution should have a mean of 0 and a standard deviation of 1 [42, 43]. From the 720 normalized scores, z-scores were calculated to indicate the strength of each score relative to the rest of the sample distribution where z-score = (gene normalized score – mean of the population)/ SD of the population

(Appendices Table S2). The highest positive scores, i.e. those that were furthest from the mean indicated an effect or phenotype that was associated with increased WSN replication. A similarly, negative z-scores furthest from their mean reflected a decrease in WSN replication. A “positive hit” in the primary screen was considered to have a z-score ≥ mean ± 2 standard deviations, thus identifying a total of 22 kinase genes important WSN replication (Figure 3.5; Table 4.1). Those genes whose silencing increased virus replication included NPR2, MAP3K1, DYRK3, suggesting these gene products have a potential role in regulating the innate response towards WSN replication. Those whose silencing decrease virus replication, and have likely have a pro-viral function, included EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5, CALM2, ADK, PKN3, HK2, CDK3, ERBB4.

Conclusions

Limited information is available concerning the importance of host gene expression on resistance to virus replication, or as how the gene products facilitate virus replication. As human influenza A virus, the causative agent of the common flu, encodes a minimal 10-11 proteins, the virus must rely on the host cell and its functions for biological responses linked to infection and replication. Recent studies of influenza virus biology have revealed elaborate mechanisms by which influenza interacts with the host cell affecting various cellular pathways involved in the synthesis of viral and cellular proteins needed to facilitate replication. Of particular interest is the role of host protein kinase genes, as signaling processes can be initiated by the cell as a defense against the pathogen, but can also be used by the virus to support replication. It has been shown that inhibiting the Raf-MEK-ERK signaling cascade resulted in a down regulation of virus production without causing severe cytotoxicity to the host cell [44]. Therefore, the overarching goal of this study was to identify host protein kinase genes that may mediate or have direct involvement in cellular signaling cascades; specifically, targets suspected to have a proviral activity in infected cells. By blocking host signaling cascades required for viral replication, it may be possible to overcome the constant viral mutation and reassortment obstacles that plague current vaccines and therapies and provide a novel disease intervention strategy.

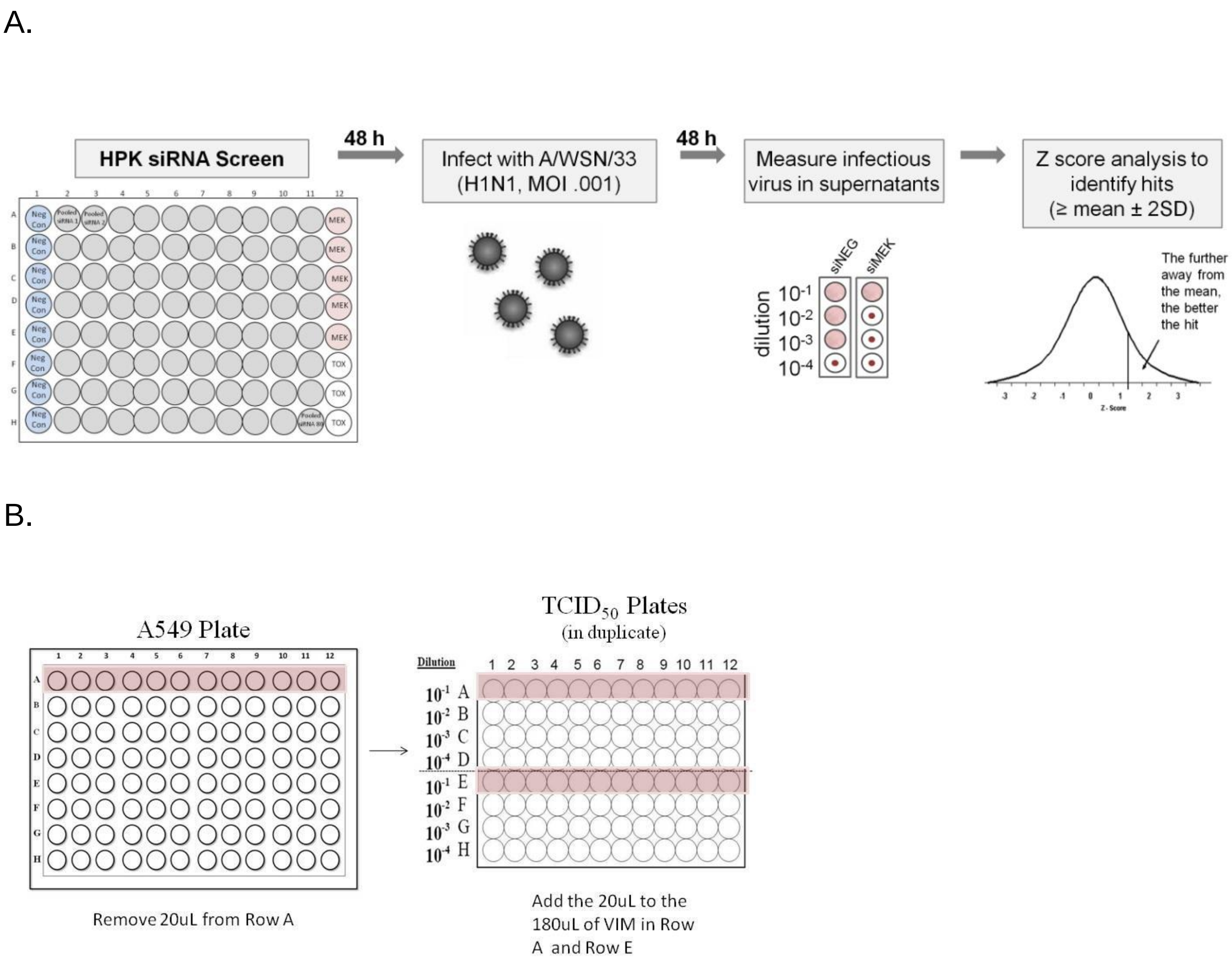

Figure 3.1 RNA interference screen strategy for identification of host factors affecting influenza infection. (A) A549 cells were plated onto lyophilized siRNAs in 96-well flat-bottom plates and transiently transfected for 48h with 50 nM siRNA. At 48 hours post-transfection, cells were infected with influenza virus A/WSN/33 (MOI 0.001). Then 48 hours post-infection, viral replication was assayed by titration of A549 cell supernatant on MDCK cells. Each siRNA is given a score based on the number of wells with detectable virus, and primary hits determined using z-score analysis. (B) Specific layout for virus detection in A549 supernatant quantified via a modified $TCID_{50}$ assay on MDCKs. Neg con, non-target negative control siRNA; MEK, positive control siRNA.

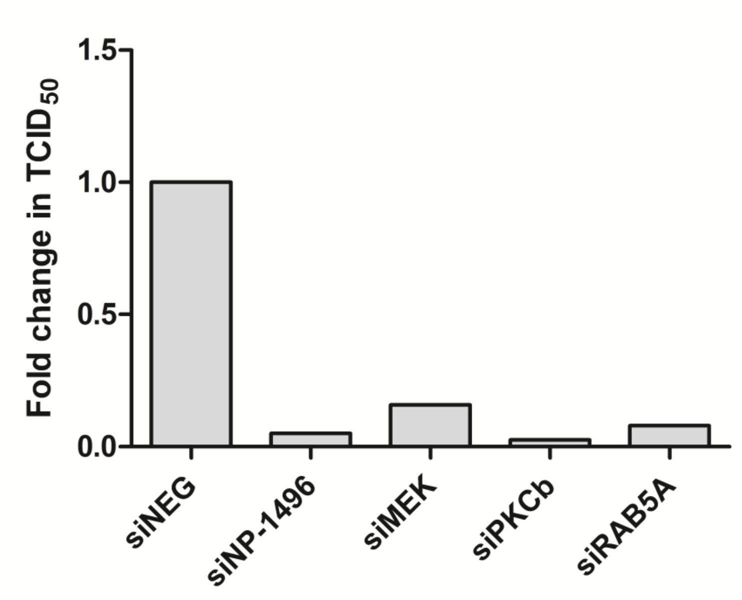

Figure 3.2. Fold change in the level of infectious virus. A549 cells were treated with either siNEG negative control, siNP-1490 positive control, or an siRNA directed to known kinase genes found to be important for influenza virus replication and then infected with WSN and levels of infectious virus measured. All results are reported as a fold change from siNEG treated cells.

A.

Plate 1	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NEG	TTK	SYK	EPHA8	DMPK	COASY	MYO3B	PRKWNK2	PTK2	FER	CAMK2B	MEK
B	NEG	ACVRL1	LYN	PAPSS1	MAPK4	MASTL	TRIB1	BRD3	FLJ34389	HK1	THNSL1	MEK
C	NEG	ALS2CR2	CARKL	ADK	ABL2	PLK1	MINK	CDKL3	MAP3K14	MAP3K7	CKB	MEK
D	NEG	CDC2	PIM2	LRRK2	HK2	FN3K	CRIM1	PIK4CA	SSTK	TAO1	NME3	MEK
E	NEG	PRKAG3	GRK5	LIMK1	UMPK	C7ORF2	KSR	MAP4K4	PRKCN	TTBK1	ROCK1	MEK
F	NEG	TEK	PANK1	CSNK1G3	CKS2	PHKG2	MAPK7	KCNH8	CAMK2D	MAP4K5	PKN3	TOX
G	NEG	ATM	PHKA2	MULK	GRK6	BMPR1B	STK33	MIDORI	RNASEL	STK16	GRK1	TOX
H	NEG	RFP	PIP5KL1	NEK5	PLK2	CSNK1G2	MARK2	TNIK	CALM3	PKM2	CDKL5	TOX

B.

Plate 2	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NEG	PIK3CA	KIAA1804	NAGK	IRAK1	EEF2K	ANKK1	MAP3K5	PRKAA1	RPS6KA4	CALM1	MEK
B	NEG	FLJ32685	ITPKC	EPHA5	MLCK	PSKH1	AURKC	PRKCE	AK1	TRPM6	CLK1	MEK
C	NEG	PAPSS2	RPS6KB2	PFTK1	NME5	SRC	NEK4	CSNK1G1	RIOK1	AKT1	SNRK	MEK
D	NEG	KIAA1811	PDK2	MYLK2	NEK11	KIAA1361	SNF1LK	PTK9	LATS1	PFKP	PRKG1	MEK
E	NEG	RIOK2	FRAP1	PDGFRL	AK7	EPHA10	STK17B	NPR2	GOLGA5	STK24	DGKB	MEK
F	NEG	STK25	CDC7	CAMK2G	URKL1	MAP3K9	GUCY2D	CAMKK2	KIAA1639	MKIINALP	KIT	TOX
G	NEG	PRKCL1	RIPK2	CKMT1B	MKNK2	TNNI3K	ALS2CR7	HCK	CKS1B	BMP2K	PHKB	TOX
H	NEG	TRIO	ETNK1	ERK8	ADP-GK	PIK3CG	FGFR3	IRAK4	PCTK3	SGK2	DYRK4	TOX

C.

Plate 3	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NEG	MAP3K2	LATS2	P101-PI3K	DGKG	MAST3	EFNA3	BCKDK	ITPKA	NEK6	BLK	MEK
B	NEG	DGKK	MPP2	BUB1	UHMK1	PRKCM	PIK4CB	DLG4	BTK	MAP3K15	GSK3A	MEK
C	NEG	CSNK1E	RELA	CCRK	MAPK9	TNK2	PDIK1L	AXL	ANKRD3	RIPK1	CDC42BPB	MEK
D	NEG	JAK3	STK22D	FGFR1	PNKP	ERBB2	PRKAG1	FLJ10761	IGF1R	NEK9	FLJ23356	MEK
E	NEG	FLT3	PIP5K2A	DAPK1	FASTK	PRKG2	MPP1	HSPB8	CSF1R	DYRK2	PRKCL2	MEK
F	NEG	RPS6KA3	EFNA4	ADCK1	MAP3K8	CDK8	MKNK1	PIK3C2G	CDK4	PIM1	DGKQ	TOX
G	NEG	MAP3K12	STK22C	PI4KII	TAF1L	CAMK1G	HSMDPKIN	STK10	AIP1	ABL1	PAK6	TOX
H	NEG	FN3KRP	AKT2	KIAA0999	PLK4	HIPK3	TYRO3	MAPK3	YES1	NYD-SP25	CDADC1	TOX

D.

Plate 4	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	NEG	STK31	AKT3	NLK	PFKFB3	TESK2	EPHA7	STK35	STK11	JAK2	BRD2	MEK
B	NEG	CDKN2C	MAP2K3	LCK	CIB2	AAK1	PIK3R2	IRAK2	ACVR1C	ACVR1B	CPNE3	MEK
C	NEG	CAMK1	MARK3	MGC42105	CHEK1	SCAP1	CDC2L2	STK3	FLT4	CDC2L1	MAP2K7	MEK
D	NEG	EPHB6	MARK1	PRKAR2A	PINK1	MUSK	ULK2	MGC8407	AURKA	PAK4	MAPK1	MEK
E	NEG	MAST4	CKMT2	PIK3R3	ACVR2	PRKWNK3	PRKCZ	ITK	CAMKK1	STK36	MAPK8	MEK
F	NEG	TGFBR1	CDK5R2	LOC91461	FZP761P04	DUSTYPK	FLJ23356	PDK3	DGKA	C9ORF12	PTK6	TOX
G	NEG	PAK1	RYK	PDXK	NEK8	GK	KSR2	PRKAR1A	NUP62	ROR2	PIP5K2C	TOX
H	NEG	ACVR1	PRKACB	STK39	MATK	RPS6KB1	RFK	TTBK2	CDC42BPA	GSG2	HIPK1	TOX

E.

Plate 5	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG	MAP3K11	AURKB	LIMK2	GRK4	LOC340156	PRKX	GNE	PRKAR1B	MVK	PKIB	MEK
B	NEG	CDK5	ADCK4	HRI	MAPKAPK3	ACVR2B	EGFR	ROS1	HK3	DDR2	DAPK2	MEK
C	NEG	ITPK1	ILK-2	EPHA2	PHKG1	FUK	AK3	CSNK2A2	VRK2	KALRN	ARAF1	MEK
D	NEG	BCR	DCK	MGC16169	STK22D	SRP72	DGKI	CSK	IKBKAP	ADRBK2	ICK	MEK
E	NEG	ADCK2	PI4K2B	TRIB3	FLJ23074	HIPK2	PRPS1	CSNK1D	DYRK3	TYK2	MYO3A	MEK
F	NEG	SPHK1	ASK	PDGFRB	FGR	ROR1	CRK7	CDKN1B	PACSIN1	DAPK3	PIK3R1	TOX
G	NEG	PRKY	MAP3K1	ZAP70	STK23	PTK7	RPS6KL1	RPS6KA6	SRPK1	PRKACA	PTK2B	TOX
H	NEG	MERTK	CERK	NUCKS	CIT	CDKL4	KIAA2002	OSR1	RAGE	BMPR2	NRBP2	TOX

F.

Plate 6	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG	MAP2K4	FLJ13052	TLK2	TJP2	ADCK5	MAPK11	PGK2	BUB1B	MAP2K5	LAK	MEK
B	NEG	CDK7	LRRK1	FLJ25006	MAPK14	TBK1	ERN2	PRKCI	IKBKE	CSNK1A1L	PIP5K1A	MEK
C	NEG	STK17A	TEX14	MAPKAPK2	LTK	CASK	DYRK1B	SBK1	PCTK1	MAP3K4	STK38L	MEK
D	NEG	MGC4796	PKIA	RBKS	PHKA1	ERN1	STYK1	ARK5	PIP5K1B	DGUOK	PANK3	MEK
E	NEG	SRPK2	CLK2	TGFBR2	STK22B	GAK	RPS6KA2	MAPK6	GRK7	ADRBK1	CDK10	MEK
F	NEG	CDKN1A	PANK2	EPHA3	PYCS	IHPK1	BRD4	TEC	FRDA	PRPS2	AK5	TOX
G	NEG	MAST2	CDK5R1	WEE1	CDK6	CDKN2D	PRKCQ	PRKAG2	MAP3K3	FGFRL1	NME1	TOX
H	NEG	TNK1	STK29	NTRK3	LMTK2	DTYMK	PIK3R4	GCK	EPHB2	SGKL	DLG3	TOX

G.

Plate 7	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG	RET	MAP2K6	RPS6KC1	NME7	MAP3K10	UCK1	FGFR2	ERBB3	NEK3	COL4A3BP	MEK
B	NEG	CDC2L5	ALK	SPEG	TP53RK	EFNA5	MAPK13	TRIB2	DGKH	FRK	PIK3CB	MEK
C	NEG	KCNH2	CDKL1	NTRK2	AATK	PRKCG	STK38	PMVK	EPHA1	RAF1	PRKCSH	MEK
D	NEG	PRKCD	ATR	CAMK2A	KDR	TAF1	CDK11	SNARK	SCYL1	PRKAB2	MGC4796	MEK
E	NEG	STK32B	MAP3K7IP1	PRKR	RIPK3	ULK4	SPHK2	TESK1	CSNK2B	ERBB4	PFKFB4	MEK
F	NEG	PKMYT1	PACE-1	IKBKB	MAP3K13	CAMK1D	IRAK3	MOS	PIP5K2B	STK4	BAIAP1	TOX
G	NEG	SLK	CDKN1C	PIP5K1C	PFKL	PCK1	CDK9	FGFR4	CRKL	RPS6KA5	STK32C	TOX
H	NEG	PRKD2	CAMKK1	LYK5	COMMD3	TK2	CSNK2A1	SRMS	MAP4K2	KIAA1811	PDK1	TOX

H.

Plate 8	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG	MELK	PRKCH	PAK3	PFKFB1	INSRR	DLG2	PIP5K3	BMPR1A	PRPS1L1	IGF2R	MEK
B	NEG	TGFBR3	UMP-CMPK	CHKA	WNK4	RPS6KA1	IHPK3	BMX	CLK4	PAK2	LOC390226	MEK
C	NEG	CSNK1A1	NME2	MAK	PIK3CD	MAP3K6	BRDT	EIF2AK4	NME6	DYRK1A	CAMK4	MEK
D	NEG	JIK	PKLR	PAK7	VRK1	EPHA6	GUCY2C	PNCK	VRK3	PDGFRA	DDR1	MEK
E	NEG	PTK9L	PIK3C2A	STK32A	GTF2H1	TOPK	ZAK	PDK4	CHUK	TPK1	C14ORF20	MEK
F	NEG	PIM3	LMTK3	CHEK2	JAK1	EIF2AK3	PIK3C3	ROCK2	PIK3C2B	SGK	CLK3	TOX
G	NEG	PXK	HUNK	MAP4K1	PCTK2	INSR	HIPK4	SIK2	MET	FYN	KFZP434C1	TOX
H	NEG	SAST	NEK2	MAPKAPK5	NRK	PFKFB2	MARK4	EPHB1	MAGI-3	SSTK	IKBKG	TOX

I.

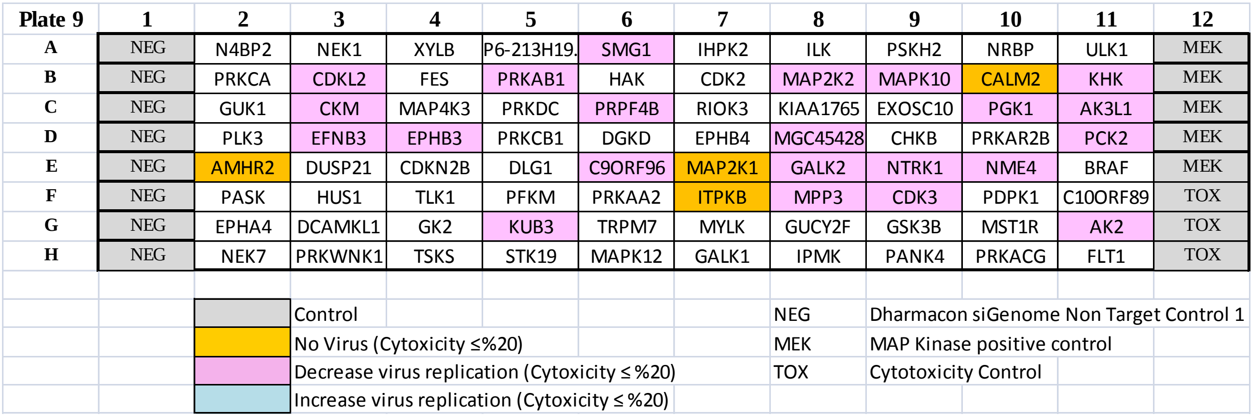

Plate 9	1	2	3	4	5	6	7	8	9	10	11	12
A	NEG	N4BP2	NEK1	XYLB	P6-213H19.	SMG1	IHPK2	ILK	PSKH2	NRBP	ULK1	MEK
B	NEG	PRKCA	CDKL2	FES	PRKAB1	HAK	CDK2	MAP2K2	MAPK10	CALM2	KHK	MEK
C	NEG	GUK1	CKM	MAP4K3	PRKDC	PRPF4B	RIOK3	KIAA1765	EXOSC10	PGK1	AK3L1	MEK
D	NEG	PLK3	EFNB3	EPHB3	PRKCB1	DGKD	EPHB4	MGC45428	CHKB	PRKAR2B	PCK2	MEK
E	NEG	AMHR2	DUSP21	CDKN2B	DLG1	C9ORF96	MAP2K1	GALK2	NTRK1	NME4	BRAF	MEK
F	NEG	PASK	HUS1	TLK1	PFKM	PRKAA2	ITPKB	MPP3	CDK3	PDPK1	C10ORF89	TOX
G	NEG	EPHA4	DCAMKL1	GK2	KUB3	TRPM7	MYLK	GUCY2F	GSK3B	MST1R	AK2	TOX
H	NEG	NEK7	PRKWNK1	TSKS	STK19	MAPK12	GALK1	IPMK	PANK4	PRKACG	FLT1	TOX

▨	Control	NEG	Dharmacon siGenome Non Target Control 1
🟧	No Virus (Cytoxicity ≤%20)	MEK	MAP Kinase positive control
🟪	Decrease virus replication (Cytoxicity ≤ %20)	TOX	Cytotoxicity Control
🟦	Increase virus replication (Cytoxicity ≤ %20)		

Figure 3.4. HPK genes targeted in the siRNA screen and the effect of their silencing on virus replication. A-I represent the 9 plates included in the library. The controls are in columns 1 and 12 (grey) and those highlighted modulated virus replication, either decreasing replication (orange or pink) or increasing replication (blue) compared to a non-target negative control.

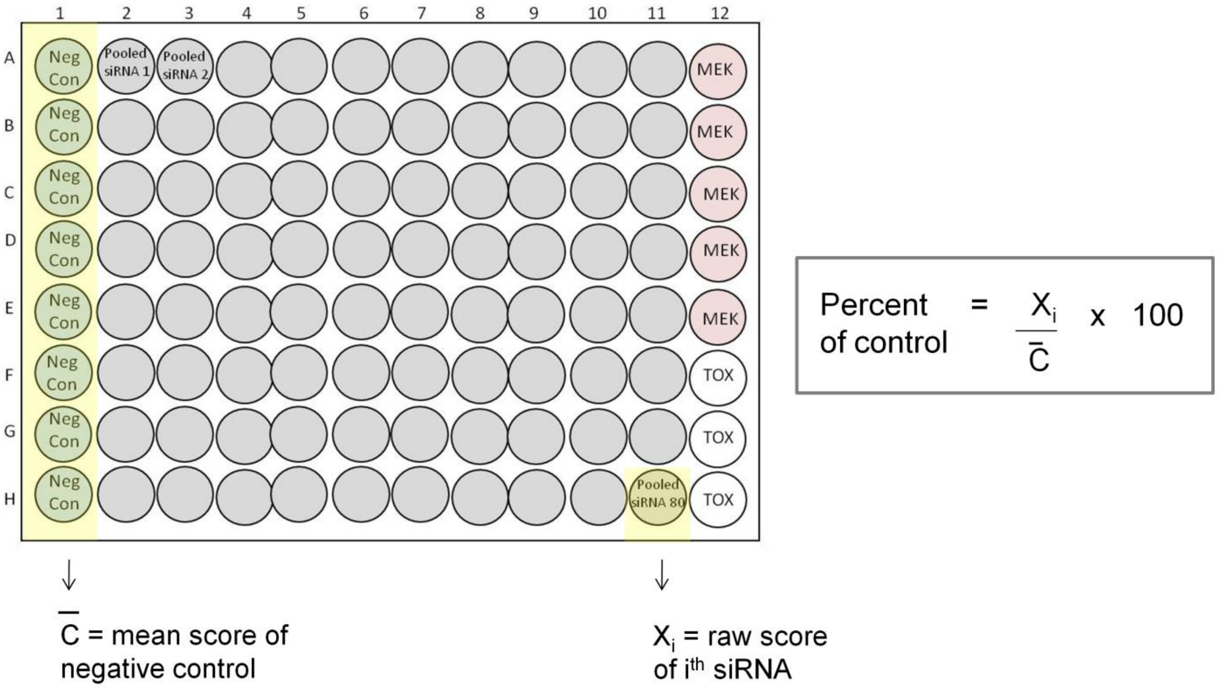

Figure 3.4 Normalization as a percent of the control. To allow for across plate comparison, raw data from each plate is normalized to the average score of the non-target negative control siRNA transfected/infected cells on that plate, resulting in normalized scores for each of the 720 targets. For each of the 720 targets, the mean of the four replicates is determined, simplifying to one normalized score per target.

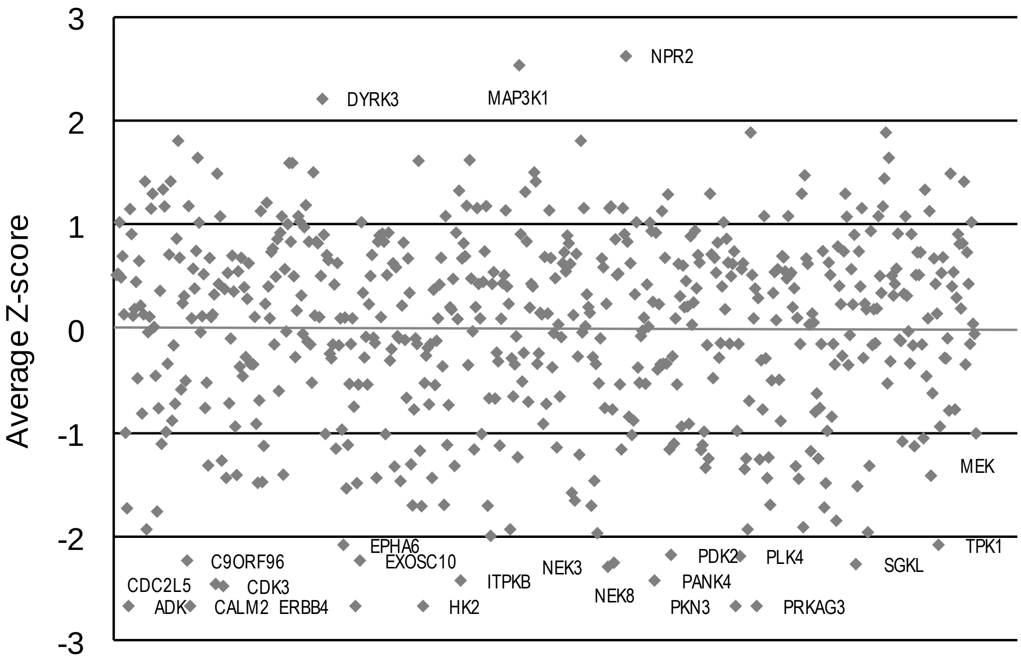

Figure 3.5. Primary screen identified 22 kinase genes associated with influenza infection. Z- score analysis identified primary hits (z scores ≥2 and ≤ -2) whose silencing increased virus replication (≥2) and strongest hits that decreased virus replication (≤2).

References

1. WHO, *Overview of pandemic (H1N1) 2009 in the WHO European Region, retrieved 11th March 2010, <http://www.euro.who.int/influenza/AH1N1/20091026_1>.*

. 2009a.

2. Molinari, N.-A.M., et al., *The annual impact of seasonal influenza in the US: Measuring disease burden and costs.* Vaccine, 2007. **25**(27): p. 5086-5096.
3. Taubenberger, J.K. and D.M. Morens, *Influenza: the once and future pandemic.* Public health reports, 2010. **125 Suppl 3**: p. 16-26.
4. Palese P, S.M., *Fields Virology*. 5th ed, ed. H.P. Knipe DM2007, Philadelphia: Raven.
5. Hay, A.J., et al., *THE MOLECULAR-BASIS OF THE SPECIFIC ANTI-INFLUENZA ACTION OF AMANTADINE.* Embo Journal, 1985. **4**(11): p. 3021-3024.
6. Moscona, A., *Drug therapy - Neuraminidase inhibitors for influenza.* New England Journal of Medicine, 2005. **353**(13): p. 1363-1373.
7. CDC. *Fluview: Antiviral Resistance*. 2009 May 25 2009].
8. Huang, Y.X., et al., *The role of a mutant CCR5 allele in HIV-1 transmission and disease progression.* Nature Medicine, 1996. **2**(11): p. 1240-1243.

9. Michael, N.L., et al., *The role of CCR5 and CCR2 polymorphisms in HTV-1 transmission and disease progression.* Nature Medicine, 1997. **3**(10): p. 1160-1162.

10. Pier, G.B., et al., *Salmonella typhi uses CFTR to enter intestinal epithelial cells.* Nature, 1998. **393**(6680): p. 79-82.

11. Friedman, M.J., *ERYTHROCYTIC MECHANISM OF SICKLE-CELL RESISTANCE TO MALARIA.* Proceedings of the National Academy of Sciences of the United States of America, 1978. **75**(4): p. 1994-1997.

12. Miller, L.H., et al., *RESISTANCE FACTOR TO PLASMODIUM-VIVAX IN BLACKS - DUFFY-BLOOD-GROUP GENOTYPE, FYFY.* New England Journal of Medicine, 1976. **295**(6): p. 302-304.

13. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.

14. Brass, A.L., et al., *The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus.* Cell, 2009. **139**(7): p. 1243-54.

15. Karlas, A., et al., *Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication.* Nature, 2010.

16. Konig, R., et al., *Human host factors required for influenza virus replication.* Nature, 2009. **463**(7282): p. 813-7.

17. Cherry, S., *What have RNAi screens taught us about viral-host interactions?* Current opinion in microbiology, 2009. **12**(4): p. 446-52.

18. Ludwig, S., et al., *Influenza-virus-induced signaling cascades: targets for antiviral therapy?* Trends Mol Med, 2003. **9**(2): p. 46-52.

19. Ludwig, S., et al., *Ringing the alarm bells: signalling and apoptosis in influenza virus infected cells.* Cell Microbiol, 2006. **8**(3): p. 375-86.

20. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* Febs Letters, 2004. **561**(1-3): p. 37-43.

21. Ehrhardt, C., et al., *Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence.* Cell Microbiol, 2006. **8**(8): p. 1336-48.

22. Ehrhardt, C., et al., *Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses.* Journal of Virology, 2007. **81**(7): p. 3058-67.

23. Marjuki, H., et al., *Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Calpha-mediated activation of ERK signaling.* J Biol Chem, 2006. **281**(24): p. 16707-15.

24. Wurzer, W.J., et al., *NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation.* J Biol Chem, 2004. **279**(30): p. 30931-7.

25. Matrosovich, M., et al., *New low-viscosity overlay medium for viral plaque assays.* Virology Journal, 2006. **3**: p. 63.

26. Li, I.W.S., et al., *Differential susceptibility of different cell lines to swine-origin influenza A H1N1, seasonal human influenza A H1N1, and avian influenza A H5N1 viruses.* J Clin Virol, 2009. **46**(19801200): p. 325-330.
27. Primetzhofer, D., et al., *Resonant charge transfer in low-energy ion scattering: Information depth in the reionization regime.* Surf Sci, 2011. **605**(22053118): p. 1913-1917.
28. Reina, J., et al., *Comparison of Madin-Darby canine kidney cells (MDCK) with a green monkey continuous cell line (Vero) and human lung embryonated cells (MRC-5) in the isolation of influenza A virus from nasopharyngeal aspirates by shell vial culture.* J Clin Microbiol, 1997. **35**(9196221): p. 1900-1901.
29. Schepetiuk, S.K. and T. Kok, *The use of MDCK, MEK and LLC-MK2 cell lines with enzyme immunoassay for the isolation of influenza and parainfluenza viruses from clinical specimens.* J Virol Methods, 1993. **42**(8390473): p. 241-250.
30. Zou, S.M., et al., *[The proliferation of H1N1 subtype influenza viruses in A549 and BEAS-2B cells].* Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 2011. **25**(3): p. 205-7.
31. Li, S., et al., *Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus.* J Virol, 1993. **67**(11): p. 6667-73.
32. Dharmacon, *siGENOME siRNA*, 2004.

33. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* FEBS Lett, 2004. **561**(1-3): p. 37-43.

34. Lonza, *ToxiLight BioAssay Kit: a non-destructive bioluminescent cytotoxicity assay*, I. Cambrex Bio Science Rockland, Editor 2005.

35. Dharmacon, *siCONTROL TOX Transfection Control, D-001500-01-05, Thermo Fisher Scientific, Lafayette, CO*, 2006.

36. Hirst, G.K., *Adsorption of Influenza Hemagglutinins and Virus by Red Blood Cells.* J Exp Med, 1942. **76**(2): p. 195-209.

37. Pinto, R., et al., *Inhibition of influenza virus-induced NF-kappaB and Raf/MEK/ERK activation can reduce both virus titers and cytokine expression simultaneously in vitro and in vivo.* Antiviral Res, 2011. **92**(1): p. 45-56.

38. Pleschka, S., et al., *Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade.* Nature cell biology, 2001. **3**(3): p. 301-5.

39. Sieczkarski, S.B. and G.R. Whittaker, *Differential requirements of Rab5 and Rab7 for endocytosis of influenza and other enveloped viruses.* Traffic, 2003. **4**(5): p. 333-343.

40. Mitzner, D., et al., *Phosphorylation of the influenza A virus protein PB1-F2 by PKC is crucial for apoptosis promoting functions in monocytes.* Cell Microbiol, 2009. **11**(10): p. 1502-16.

41. Sieczkarski, S.B., H.A. Brown, and G.R. Whittaker, *Role of protein kinase C beta II in influenza virus entry via late endosomes.* Journal of Virology, 2003. **77**(1): p. 460-469.

42. Zhang, J.H., T.D. Chung, and K.R. Oldenburg, *A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays.* J Biomol Screen, 1999. **4**(2): p. 67-73.

43. Birmingham, A., et al., *Statistical methods for analysis of high-throughput RNA interference screens.* Nat Methods, 2009. **6**(8): p. 569-75.

44. Pleschka, S., et al., *Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade.* Nat Cell Biol, 2001. **3**(3): p. 301-5.

CHAPTER 4

MICRORNA REGULATION OF HUMAN PROTEIN KINASE GENES REQUIRED FOR INFLUENZA VIRUS REPLICATION[1]

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[1] Lauren E. Andersen, Abhijeet Bakre, Victoria Meliopoulos, Keegan Coleman, Xiuzhen Yan, Paula Brooks, Jackelyn Crabtree, S. Mark Tompkins, and Ralph A. Tripp. To be submitted to *PLoS Pathogens*.

Abstract

Human protein kinases (HPKs) have profound effects on cellular responses. To better understand the role of HPKs, and the signaling networks that influence influenza virus replication, a small interfering RNA (siRNA) screen of 720 HPKs was performed. From the screen, 17 HPKs (NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5, CALM2, PKN3, and HK2) were validated as important for A/WSN/33 influenza virus replication, and 6 HPKs (CDC2L5, HK2, NEK8, PANK4, PLK4 and SGK3) were identified as important for both A/WSN/33 and A/New Caledonia/20/99 influenza virus replication. These HPKs were found to affect multiple host pathways and to be governed by several microRNAs (miRNAs). Using a panel of miRNA agonists and antagonists, miR-148a and miR-181b were identified as regulators of CDC2L5 and SGK3 expression, respectively, and were also shown to inhibit A/WSN/33 replication. The data presented here advances our understanding of miRNA regulation of genes critical for virus replication and is important for developing novel disease intervention strategies.

Author Summary

Influenza A virus causes substantial disease in humans and animals every year. Identifying the host genes required for virus replication and the miRNAs governing their expression would provide novel targets for antiviral therapy. Using a siRNA library targeting 720 HPKs and a human respiratory epithelial (A549) cell line model, 17 host HPK genes were validated to be modulated during influenza virus replication. Bioinformatic pathway analysis indicated that several HPK genes were integrated into previously identified host signaling pathways; namely p53, PI3K/Akt, and PKC/CA^{++} signaling pathways. In addition, a panel of miRNA agonists and antagonists was used to validate miRNA regulation of the 17 HPK genes. The results show that miRNAs governance of HPK genes can influence influenza virus replication. This study has identified and validated several novel HPK genes and miRNAs that may offer therapeutic disease intervention targets.

Introduction

Influenza A viruses are ubiquitous, causing acute respiratory disease and substantial morbidity and mortality each year [1-3]. Although vaccination is the best means for protection, prophylaxis with licensed antiviral drugs are available and have shown >80% efficacy against the development of illness during interpandemic influenza [4]. However, influenza virus has rapidly developed resistance to most antiviral drugs [5-8]. Given this and the difficulties with seasonal and pandemic influenza vaccine development [9,10], there is clearly a need for new disease intervention strategies.

Influenza A viruses belong to the family *Orthomyxoviridae*. These are enveloped viruses having an eight segmented, negative-sense, single-stranded RNA genome that encodes up to 11 proteins [11]. The viral envelope contains hemagglutinin (HA) and neuraminidase (NA) glycoproteins, and the membrane ion channel protein, M2. Within the virion, the matrix protein (M1) provides structure and secures the viral ribonucleoprotein (vRNP) complexes consisting of viral RNA coupled to nucleoprotein (NP) and the three polymerase proteins (PB1, PB2 and PA). The remaining viral proteins include the nonstructural proteins, NS1 and NS2, and the recently identified PB1-F2 protein found in some virus species. Alone, these 11 proteins are not sufficient to facilitate viral replication and the virus must co-opt host cell pathways at each stage of its life cycle. These pathways that are critical for influenza virus replication provide potential targets for disease intervention strategies. Therefore, identification of these host-virus interactions is important for accelerating the rationale design of novel anti-viral drugs.

RNA interference (RNAi) has become a powerful experimental tool used to suppress a gene of interest to better understand its biological function. Five recent RNAi-based studies have addressed the host genes important for influenza virus infection and replication [12-16], and meta-analysis of these findings indicates that many of the genes identified are in shared pathways associated with virtually every step in the virus life cycle [13,16-19]. Despite differences in methodology used in these studies, such as different viruses and cell types used, a level of accord is evident among the host cell pathways affected. Overlap was identified in pathways used for virus entry [16-19], fusion of the endosomal and viral membrane [13,17-19], transport of the viral components to the nucleus [14,15], as well as late events including export of the vRNP complex and RNA into the cytoplasm [12,13,16-18]. Among the known signaling components which are induced upon influenza infections are the protein kinase C (PKC) [20,21], the extracellular signal-regulated kinase ERK [22,23], and the phosphatidylinositol-3 kinase (PI3K) [24]. Early binding of influenza virus HA to the host cell sialic acid receptor, rapidly activates PKC [20]. This is likely in a pro-viral manner, as blocking this activity prevents entry and infection of the influenza virus [25]. Late accumulation of influenza virus HA on the host cell membrane activates the Raf/MEK/ERK mitogenic kinase cascade via PKCα, subsequently regulating RNP export [24]. Inhibition of this cascade, results in nuclear retention of the vRNP and a significant decrease in influenza virus propagation [22,26]. These findings show that host cell kinases are largely involved in influenza virus replication and their inhibition can successfully reduce the replication of influenza virus within its host. Therefore, host protein kinases may be particularly good targets for disease intervention.

Host miRNAs are highly conserved small non-coding RNAs that negatively regulate messenger RNAs (mRNAs) by specifically binding to their 3′ untranslated regions (UTRs) [27-29]. It has been predicted that miRNAs may post-transcriptionally repress up to half of all human genes through translational arrest or mRNA degradation, thereby regulating diverse cellular functions [28]. Since viral infection alters cellular miRNA expression during infection and replication, it is likely that host factors responding to infection are regulated by miRNAs. Accordingly, there is a growing range of viruses shown to affect miRNA regulation of host responses. For example, herpes simplex virus-1 (HSV-1) replication has been shown to be suppressed when miRNA-101 (miR-101) targets a subunit of mitochondrial ATP synthase (ATP5B) [30]. Alternatively, miR-132 has been shown to be highly upregulated after HSV-1 and human cytomegalovirus (HCMV) infection, and to have a negative effect on the expression of interferon-stimulated genes, thereby facilitating viral replication [31]. Human immunodeficiency virus type 1 (HIV-1) down regulates the expression of many cellular miRNAs [32], and for miR-17/92, miRNA suppression is required for efficient viral replication [33]. With regard to influenza virus, cellular miRNAs miR-323, miR-491, and miR-654 have been shown to inhibit replication by binding to the viral PB1 gene [34], while miR-507 and miR-136 have potential binding sites within the viral PB2 and HA genes [35]. Additionally, miR-26a and miR-939 regulate the replication of H1N1 influenza virus in MDCK cells [34]. These data show that miRNAs are important for governing aspects of the host response to influenza virus replication and those regulating host kinase genes identified important for influenza virus replication are particularly promising therapeutic targets. Compared to other antiviral approaches, targeting miRNAs could potentially have fewer off-target or side effects because they are endogenously expressed within the cell and may trigger lower immunogenic responses [36,37].

To better understand the host kinase genes regulating intracellular signaling events affecting influenza virus replication, we performed a directed screen focusing on human protein kinases. Using a siRNA library, we evaluated 720 human kinase genes for their role in host signal transduction pathways linked to influenza virus replication. The kinase genes identified as positive hits were validated as important for influenza virus replication, and these 17 genes were then computationally analyzed for putative miRNA binding sites. The putative miRNAs predicted to target the 3’-UTR of the validated kinase genes were confirmed using miRNA agonists or antagonists to affect influenza virus replication. These results provide strong evidence that the kinase genes identified are important for influenza virus replication, and show that miRNAs govern their expression and consequently affect influenza virus replication.

Materials and Methods

Cell culture, viruses and infection conditions

To minimize biological variation, a single passage of A549 human lung epithelial cells (CCL-185, ATCC) and Madin–Darby canine kidney cells (MDCK, CCL-34, ATCC) were used for all assays from a frozen stock stored in 10% DMSO and 90% FBS in liquid nitrogen. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, ThermoScientific) supplemented with 5% heat-inactivated fetal bovine serum (FBS, HyClone, Thermo Scientific) at 37^{o}C and 5% CO_2. All cells were confirmed to be free of mycoplasma using a PlasmoTest kit (InvivoGen, San Diego, CA).

Influenza A/WSN/33 (H1N1; WSN) was obtained from Richard Webby (St Jude's Children Research Hospital, Memphis, TN) and A/New Caledonia/20/99 (H1N1) was obtained from the Centers for Disease Control and Prevention, Atlanta, GA. The influenza viruses were grown in the allantoic cavities of 9-day-old embryonated chicken eggs as previously described [38]. Virus stocks were titrated in MDCK cells as previously described [39], and a 50% tissue culture infectious dose ($TCID_{50}$) determined using the method described by Reed and Muench [40]. Prior to infection, cells were rinsed with phosphate buffered saline (PBS) and then infected with the multiplicity of infection (MOI) as indicated. For infection with WSN, DMEM supplemented with 5% fetal bovine serum and viruses were incubated on cells for 1 h at 37^{o}C. For infection with A/New Caledonia/20/99, DMEM supplemented with 0.2% bovine serum albumin (Sigma

Aldrich) and 1ug/mL of TPCK trypsin (Worthington) were incubated on cells for 1 h at 37^{o}C.

siRNA arrayed library, controls, and conditions

A siRNA arrayed library containing four pooled siRNAs per target gene for 720 different human protein kinase genes (Dharmacon siARRAY siRNA Library (G-003505 Human Protein Kinase Lot 08105), Thermo Scientific) was used. Controls included siRNA targeting mitogen-activated protein kinase kinase 1 positive control (siMEK, 5’-GCACAUGGAUGGAGGUUCU-3’, 5’-GCAGAGAGAGCAGAUUUGA-3’, 5’-GAGCAGAUUUGAAGCAACU-3’, 5’-CCAGAAAGCUAAUUCAUCU-3’, siGENOME smartpool, Dharmacon M-003571-01), a negative non-targeting control siRNA (siNEG, 5'-UAGCGACUAAACACAUCAA-3', siCONTROL Non-Targeting siRNA #1, Dharmacon D-001210-01-05), and a control for cellular cytotoxicity (TOX, Dharmacon D-001500-01-05). All siRNAs were resuspended in Dharmacon siRNA buffer to a concentration of 1 µM and stored at -80°C. The sequences for all siRNA duplexes provided with Dharmacon’s siGENOME libraries are proprietary and confidential. The siRNA screens incorporated a primary screen using 4 pooled siRNAs per well per one of the 720 HPK genes followed by a validation step using a single, novel siRNA (Dharmacon) targeting a different seed site on the same gene to confirm the phenotype from the primary screen (Appendices Table S1). For all transfections in both the primary and validation screens siRNAs were reverse transfected into A549 cells at a 50 nM final concentration using 0.4% Dharmafect 1 (Dharmacon), and incubated at 37°C and 5% CO_2 for 48 h. Cell cytotoxicity was evaluated in all siRNA transfected cells and cytotoxicity was determined to be >20% based on the bioluminescent measurement of adenylate kinase (ToxiLight BioAssay Kit, Lonza) (Appendices Table S2). A Safire_2 luminometer (Tecan U.S., Durham, NC) was used for the luminescence readout. All RNAi experiments were carried out according to the Minimum Information for an RNAi Experiments (MIARE) guidelines [41].

RNAi screen, normalization, and validation.

To identify HPK genes important for influenza infection in the primary screen, the levels of infectious virus in the supernatant of siRNA-transfected and WSN-infected A549 cells was determined by TCID_{50} [40]. Briefly, serial ten-fold dilutions of culture supernatant from treated A549 cells in DMEM + 5% FBS was transferred to a 96-well plate containing a confluent monolayer of MDCK cells and incubated at 37^{o}C and 5% CO_2 for 72 h. All wells were evaluated and scored for hemagglutination, a feature indicative of the presence virus in the supernatant [42]. To adjust for across plate comparison, the percent inhibition of infectious virus was calculated for each experimental siRNA such that the difference of the experimental HPK siRNA treated well (X_{siHPK}) was subtracted from the mean of the negative control well values ($\overline{X}_{\text{siNEG}}$) and then divided by the difference of the means of the negative control and the TOX control ($\overline{X}_{\text{siTOX}}$) for each plate, i.e.,

$$\text{Percent Inhibition} = \frac{\overline{X}_{\text{siNEG plate A}} - X_{\text{siHPK plate A}}}{\overline{X}_{\text{siNEG plate A}} - \overline{X}_{\text{siTOX plate A}}}$$

The primary screen was performed as two independent experiments and each experiment was performed in duplicate. The average of normalized scores from all 4 replicates was used to obtain a single Z'-score for each of the 720 kinase targets (Appendices Table S2). To aid validation of the positive kinase gene hits discovered by the primary screen, a novel siRNA targeting a different seed site for each gene was synthesized (Dharmacon) and evaluated to emulate the same phenotype. The validation screen included three different endpoint assays i.e. $TCID_{50}$ as described above, quantitative real-time PCR (qPCR), and immunofluorescence, as described below.

Quantitative Real-time PCR

The RNA from A549 cells was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of total RNA was determined using a NanoDrop ND-1000 Fluorospectrometer (NanoDrop Technologies, Wilmington, DE). Equal amounts of RNA were then reverse transcribed to cDNA using random hexamers and MuLV reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) in accordance to the manufacturer's protocol.

For quantification of influenza M gene expression, qPCR was performed using 200 nM internal probe (M + 64, 5'-FAM-TCA GGC CCC CTC AAA GCC GA-BHQ-1-3'), 400 nM forward primer (M + 25, 5'-AGATGAGTCTTCTAACCGAGGTCG-3'), and 400 nM reverse primer (M-124, 5'-TGCAAAAACATCTTCAAGTCTCTG-3') following a previously described

TaqMan assay [43]. The cycling conditions for qPCR were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 15 sec. The qPCR was carried out and analyzed with a Stratagene Mx3005P instrument and software (La Jolla, CA). Copy numbers were determined by generation of a standard curve using plasmid DNA encoding influenza M gene [43]. Results reported for these studies were the averages of at least three replicates.

To determine the gene silencing efficiency associated with siRNA treatment, qPCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer’s instructions with the primer sequences described in Appendices Table S3. Relative expression level was calculated using the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Fold changes were calculated against the mean of negative control siRNA treated cells. Methodology and data analysis for all qPCR experiments were carried out according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [44].

Indirect immunofluorescence to detect influenza NP

A549 cells were fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 (Sigma), 10% FBS (Hyclone) in PBS. A549 cells were incubated with a primary antibody against the viral NP (25 μg/mL, H16-L10, HB65; ATCC) diluted in PBS with 10% FBS for 1 h at room temperature. Following this, the cells were incubated with Alexa-488 goat anti-mouse secondary antibody (A11001, Invitrogen) diluted 1:500 in PBS with 10% FBS for

1 h in the dark, and the cells were subsequently stained for 20 min with 1 µg/mL of 4'-6-Diamidino-2-phenylindole stain (DAPI, Invitrogen). The cells were imaged using the EVOS digital inverted fluorescent microscope (Advanced Microscopy Group, Bothell, WA) at two wavelengths, 488 nm to detect Influenza infected cells expressing NP, and 350 nm for nuclear DNA bound by DAPI. For quantification of NP immunofluorescence, cells were fixed, permeabilized and stained as above and 20X images were acquired and analyzed using Cellomics ArrayScan VTI High Content Imager and Cellomics ArrayScan software (Thermo Fisher Scientific).

Network analysis and computational miRNA target predictions

Kinase gene networks were constructed using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). The validated kinase genes were overlaid onto a global molecular network developed from direct and indirect interactions. To identify the connectivity between pathways, all direct upstream and downstream interactions were expanded. To better understand how these networks are modulated by influenza replication, an extensive review of the literature and data mining from Ingenuity, as well as other databases including Database for Annotation Visualization and Integrated Discovery (DAVID) [45,46], Kyoto Encyclopedia of Genes and Genomes (KEGG) database [47,48], the Human Protein Reference Database (HPRD) [49], and Biomolecular Interaction Network Database (BIND) [50-52] was performed.

To investigate miRNA regulation of the validated kinase genes affecting influenza virus replication, bioinformatic target gene predictions were performed as described [53] using several algorithms, specifically miRanda (http://www.microrna.org/miranda.html) [54], TargetScan (http://genes.mit.edu/targetscan) [55], PicTar (http://pictar.bio.nyu.edu) [56], miRBase (http://microrna.sanger.ac.uk/targets/v2) [57], DIANA-microT 3.0 (http://diana.cslab.ece.ntua.gr/), miRDB (http://mirdb.org/miRDB/), miRWalk (http://www.ma.uniheidelberg.de/apps/zmf/mirwalk/), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). To reduce the chance of false positives, the results were overlaid to identify the genes commonly predicted by four or more of the methods.

miRNA validation

A549 cells (2.0 x 10^4) were transfected with either a miRIDIAN miRNA antagonist (25 nM), a miRIDIAN miRNA agonist (12.5 nM) (Dharmacon), or a siRNA targeting the miRNA-predicted target using Lipofectamine 2000 (Invitrogen) per the manufacturer’s protocol. The miRIDIAN miRNA antagonists are chemically modified dsRNAs consisting of a central region complementary to the mature miRNA flanked by 0-16 nt long sequences with reverse complement to pri-miRNA to enhance specificity and reduce RISC incorporation [58-60]. Conversely, agonists are chemically modified dsRNAs that increased the guide strand concentration in RISC complex, thus increasing native miRNA mediated repression [60,61]. Cells treated with an equal concentration of a non-targeting control sequence (mock) were used to control for non-sequence-specific effects in miRNA experiments. After 24 h post-transfection, the cells were infected (MOI = 0.01) with WSN/33 for 48 h. Virus replication was assayed by TaqMan qPCR for M gene (described above) and knock-down efficiency for CDC2L5, NEK8, PLK4, and SGK3 was determined using SYBR Green and primers (described above) (Appendices Table S3). Relative expression level was calculated using the endogenous control human 18S rRNA or GAPDH. Fold changes were calculated against the mean of mock treated cells.

Statistical Analysis

The separation between positive and negative controls was assessed using a Z’ factor [62,63]. A Z-score was used to evaluate the chance of including siRNAs with no specific impact in the evaluation between plates and was calculated to indicate the strength of each score relative to the rest of the sample distribution where Z-score = (gene normalized score – mean of the population)/ (standard deviation (SD) of the population). Strongest scores were those furthest from the mean (μ) where positive Z-scores reflect an increase in virus levels and the negative Z-scores reflect a resulting decrease in virus levels. A “positive hit” in the primary screen was considered to have a Z-score $\geq \mu \pm 2$ standard deviations. Less robust hits had a Z-score $\geq \mu \pm 1$ standard deviation. Statistical analysis for validation screen, pathway analysis, and miRNA studies were performed using Student’s t test with the GraphPad Prism 5 software. Statistical significance ($p<0.05$) is indicated by a single asterisks or double asterisks if highly significant ($p<0.001$).

Results

RNAi screen identifies host kinase genes important for influenza virus replication.

The HPK genes affecting influenza virus replication were identified by an RNAi screen performed using a validated siRNA library targeting individual HPK genes, as well as kinase-interacting proteins, thereby covering an annotated ‘kinome’ (Appendices Table S1). All validated siRNAs transfected A549 cells at >85% efficiency (data not shown). Of the 720 HPK genes examined in primary screens, 22 genes were initially scored as positive hits (z-scores ≥ 2 and ≤ -2) affecting influenza virus replication (Appendices Figure S2). Levels of infectious virus as measured by $TCID_{50}$ showed that siRNA silencing of 3 of the 22 positive hits increased influenza virus replication, while siRNA silencing 19 of the 22 positive hits decreased influenza virus replication compared to siNEG transfected cells (Table 4.1). Cells transfected with the positive control, i.e. siMEK had consistently lower influenza virus titers compared to siNEG transfected cells (Appendices Table S2). As false positives caused by off-target effects can occur in siRNA screens [64], the positive hits were retested using novel synthetic siRNAs targeting the same gene but at a different seed site (Appendices Table S1). Following siRNA transfection, knockdown of the target mRNA was confirmed by qPCR (Appendices Figure S3). The novel siRNAs targeting genes

CDK3, PRKAG3, ERBB4, and C9ORF96 did not silence mRNA expression and were excluded from the validation screen. The remaining positive gene hits were validated, i.e. where two siRNAs yield the same phenotype, using the novel siRNAs targeting NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5, CALM2, ADK, PKN3, and HK2 where > 80% silencing of the target mRNA was evident (Appendices Figure S3).

The 18 validated HPK genes that were silenced with the novel siRNAs targeting a different seed site were re-assessed for their contribution to influenza virus replication. The endpoint analysis was expanded to include influenza nucleoprotein high content analysis (Figure 4.1A), and viral genome replication as determined by qPCR measurement of influenza M gene expression (Figure 4.1B). Of the 18 genes that were considered validated, 17 of 18 repeated the phenotype of the original screen, e.g. increased or decreased virus replication, with the exception being the ADK gene. Identifying 22 hits in the primary screen yielded a positive hit rate of 3.1% which is consistent with related published findings [12-16]. The majority of primary hits identified were novel compared to the related studies [12-16]; however, there was some overlap specifically with PANK4, NEK8, PLK4, ITPKB, CALM2 and HK2 genes (Table 4.1; Appendices Table S4).

For hit selection, it is important not to miss siRNAs which after silencing have a large effect on influenza virus replication, nor miss those with smaller effects. Thus, false-negatives in which siRNAs with larger effects are not selected as hits, and false-positives in which siRNAs with negligible effects are selected as hits were determined. Assessment of the false-negative rate in these studies using a Z- score ≥ μ ± 2 SD revealed a rate of 87.5%. If we expand potential hits to those fitting a Z-score ≥ μ ± 1SD, the false-negative rate decreases to 23% because 37 HPK genes can be identified that overlap with HPK genes discovered in related published screens [12-16]. The false-positive rate in the primary screen was 1/18 HPK genes, or 5%, since only the ADK gene was not validated. This excellent false-positive rate is linked to the assay conditions that evaluated four pooled siRNA duplexes which has been reported to reduce the rate of false-positives [64].

Positive hit kinase genes also affect H1N1 A/New Caledonia/20/99 replication

To determine if the HPK genes identified as positive hits in the WSN/33 influenza virus screen were also important for replication by other influenza virus strains, the siRNA screen for the HPK genes was repeated for a more contemporary influenza virus strain, i.e. A/New Caledonia/20/99 (Figure 4.2). Of the 17 siRNAs previously evaluated for HPK genes that were validated as important for WSN/33 replication, six genes when silenced emulated the WSN/33 phenotype of the original screen, i.e. increased or decreased virus replication, as measured by influenza NP localization (Figure 4.2A) and influenza M gene levels (Figure 4.2B). Specifically, the following kinase genes were identified and validated as important for A/WSN/33 and A/New Caledonia/20/99 influenza virus replication: CDC2L5, HK2, NEK3, PANK4, PLK4, and SGK3.

Kinase genes and pathways implicated in influenza virus replication

To understand the complex biological interactions associated with HPK genes in the host cell that are linked to influenza virus replication, a bioinformatic meta-analysis was performed to identify major networks integrating the validated HPK genes in this study to those in previous functional and proteomic studies (Figure 3; Table 2). A combination of pathway analysis software as well as an extensive literature review led to the identification of p53/DNA damage apoptosis, PI3K/Akt, and calcium signaling pathways common among the validated HPK genes (Figure 4.3; Table 4.2). A combination of pathway analysis software as well as an extensive literature review led to the identification of p53/DNA damage apoptosis, PI3K/Akt, and calcium signaling pathways common among the validated HPK genes (Table 4.2; Appendices Table S5). HPK genes EPHA6, NEK8, CALM2, DYRK3, HK2, ITPKB, MAP3K1, NPR2, PANK4, PDK2, and TPK1 were linked to p53/DNA damage pathways, a feature that may be related to the potential hijacking of these genes by influenza virus to regulate host cell survival in a virus-supporting manner. Other HPK genes were found associated with PI3K/Akt signaling, specifically NEK8, CALM2, HK2, ITPKB, MAP3K1, NPR2, and PKN3, which may be linked to mechanisms to aid efficient entry of the virus into the host cell. The remaining HPK genes, specifically EPHA6, NEK8, CALM2, DYRK3, HK2, ITPKB, MAP3K1, PKN3, PANK4, PDK2, and TPK1 were found to be associated with calcium signaling, a feature that is likely modulated by influenza virus to alter transcriptional regulation processes in a virus-supportive manner. Taken together, the HPK genes identified here have both overlapping and relevant biological function in influenza virus replication.

miRNAs govern HPK genes and affect influenza virus replication

Approximately 60% of the human transcriptome is believed to be regulated post-transcriptionally by miRNAs causing either a transcript decay or translation block [55]. Influenza infection has been shown to deregulate the expression of multiple miRNAs, so next we asked what miRNAs were regulating the hit HPKs identified in our screen, and subsequently asked what effect they had on influenza virus replication by degrading PB1 transcripts [34]. The miRNAs that may potentially govern the expression of the HPK genes identified as crucial for influenza virus replication in this study were predicted by host pathway analysis, and by a thorough literature review (Figure 4.3; Figure 4.4). A subset of miRNAs predicted to target the CDC2L5 gene (miR-148a), PLK4 gene (miR-375, miR-34c, and miR-214), NEK8 gene (miR-512-3p, miR-93, miR183, miR-373), and SGK3 gene (miR-367, miR-155, miR-181b) were determined to be out strongest candidate miRNAs (Figure 4.4). These pairings were evaluated using miRNA agonists or antagonists to affect the transcript levels of SGK3, NEK8, PLK4 and CDC2L5 relative to a housekeeping gene, GAPDH or 18S rRNA (Figures 4.5A-D), and consequently influenza virus replication as measured by influenza M gene copy numbers (Figures 4.5E-H). Expression was normalized against GAPDH for NEK8, CDC2L5 and PLK4 and 18S rRNA for SGK3 levels since miR-155 is predicted to affect GAPDH expression [66]; and then the values were compared to mock transfected cells (Figures 4.5A-D). Transfection of a 25nM miRNA antagonist concentration generally reduced miRNA expression by >85%, while 25nM miRNA agonists led to an increase in miRNA levels. Thus, for veritable agonist/antagonist matches to the target genes, one would expect to observe an anti-correlation between the effect of antagonist or agonist on gene expression.

For the CDC2L5 gene, the miR-148a antagonist had little to no effect on CDC2L5 transcript expression compared to the mock negative control (Figure 4.5A). However, the miR-148a agonist decreased both CDC2L5 transcript expression and influenza M gene levels (Figure 4.5A and Figure 4.5E). In addition, differences in influenza M gene copy numbers were statistically ($p<0.001$) significant between miR-148a antagonist and agonist transfected cells (Figure 4.5E). Taken together, these findings suggest that miR-148a regulation of CDC2L5 can affect influenza virus replication.

The only miRNA agonist to reduced PLK4 expression was miR-654 (Figure 4.5B), but a miR-654 antagonist also reduced PLK4 expression. This data, along with Figure 4.5F, show miR-654-reduced PLK4 levels were associated with an increase in influenza M gene levels for both the agonist and antagonist, and therefore nullify PLK4 as a target for miR-654. The finding for miR-654 contradicts an earlier finding [34], but may be explained by the differences in cell lines tested (A549 vs. MDCK) or variations in assay methods. Anti-correlation between miR-214 antagonist and agonist transfected cells was observed for PLK4 (Figure 4.5B); however, treatment with the miR-214 agonist significantly ($p<0.0079$) increased virus replication (Figure 4.5F). Since PLK4 silencing was found to decrease influenza virus levels, PLK4 is also not likely being targeted by miR-214 to decrease influenza replication.

For the NEK8 gene, none of the miRNA agonist had a significant effect on NEK8 expression (Figure 4.5C); although they all decreased influenza M gene levels compared to the mock negative control (Figure 4.5G). Differences in M gene copy numbers were not statistically different between any of the three miRNA antagonist/agonists, suggesting that they did not detectably effect influenza virus replication (Figure 4.5G).

Compared to the negative control mock cells, transfection with miR-367, miR-155 and miR-181b agonists led to a reduction in SGK3 transcript levels relative to 18S rRNA, while cells transfected with antagonists increased SGK3 levels compared to mock transfected cells (Figure 4.5D). A siRNA against SGK3 was used as a positive control for SGK3 knockdown. The greatest agonist/antagonist effect occurred for miR-155 which dramatically affected SGK3 transcript levels; however, the less-dramatic miR-367 and miR-181b are still relevant as the inhibitory effects induced by miRNAs on any particular target may be mild and may merely lead to a subtle reduction of expression. If this analysis is tied to the effect of miRNA agonist and antagonists, only miR-181b shows a significant difference between the miR-181b agonist and antagonist treatment (Figure 4.5H). This suggests that miR-181b has a role in influenza virus replication; either by directly acting on SGK3 or indirectly by influencing multiple genes involved in a SGK3-related pathway. Together, these studies show that miR-148a and miR-181b target CDC2L5 and SGK3, respectively, and likely have a role in regulating influenza virus replication. Although not significant, miR-373 and miR-512-3p may act in a similar fashion by targeting NEK8 and miR-155 by targeting SGK3. This needs to be investigated further.

Discussion

The study of influenza virus biology has revealed complex mechanisms by which the influenza virus co-opts host cellular pathways to facilitate virus replication and evade the antiviral response. With the application of genome-wide RNAi screens, interactions between the virus and specific host cell components are now being identified. Identification and characterization of these virus-host interactions, including those at the miRNA level, will provide insight into the mechanisms by which viruses complete their life cycle. Moreover, such knowledge would potentially provide novel targets for therapeutic intervention. In this study, we investigate the role of human kinase genes involved in host signal transduction pathways linked to influenza virus replication, and then evaluate miRNA regulation of these genes using a panel of miRNA agonists and antagonists.

To date, five independent genome-wide screens in mammalian cells have been published characterizing host factors important for influenza virus replication [13,16-19]. Minimal overlap of critical host genes has been observed among the studies, in part because under different experimental settings, assay conditions, and tempos of response, it is likely that different host genes are involved in influenza virus replication. A meta-analysis of these screens has shown that although specific genes were not commonly identified, many genes are in shared cell pathways including PI3K/AKT signaling, NFKB, PKC/CA^{++} signaling, and p53/DNA damage pathways [65]. The host response to influenza infection is mediated by intracellular signaling cascades that transduce signals into stimulus specific actions such as altered gene expression, shift in the metabolic state, or even apoptosis. Functional signal transduction pathways within the host cell induced, modulated, and/or co-opted by RNA viruses are not well understood. It is therefore of primary interest to identify and characterize these host cell pathways, and understand the host genes involved to provide an avenue for the rationale design of disease intervention strategies related to the development of novel therapeutics.

Based on our findings and those in the meta-analysis of the five other influenza screens [13,16-19], we predict that influenza virus in general interfaces and co-opts host genes late during replication. Briefly, influenza virus carries all the necessary proteins to infect a cell, and once a host cell is infected, the virus can deliver its vRNP into the nucleus where it acts as a template for mRNAs encoding virus-specific proteins. Ultimately, viral proteins are synthesized in the cytoplasm, and NP and RNA polymerases are imported back into the nucleus to form new vRNP, a process that requires host proteins, as this is not encoded for by the virus. Likewise, the assembly of influenza viral components occurs at the plasma membrane, again requiring host proteins, and new vRNP must be exported from the nucleus into the cytoplasm for viral offspring production

[11,66]. The M1 and NS2/NEP are known to be necessary for the nuclear export of vRNP [67-72], however it remains unclear what host proteins are involved in transport and assembly of influenza virus proteins at the cell surface.

To begin to address this, in this study we used a 48h time-point post-infection with a low MOI of influenza virus to allow the virus to undergo multiple rounds of replication. We validated the screen using three endpoints to determine the effect of host gene silencing on influenza virus replication, including infectious virus titers as measured in MDCK cells, viral genome replication as determined by qPCR measurement of influenza M gene expression and influenza NP as determined by high content analysis. The screen and validation steps identified 17 HPK genes in the human kinome essential for influenza virus replication (NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5, CALM2, PKN3, and HK2). One primary screen hit, ADK, showed reduced M1 levels measured by qPCR but did not validate in our NP localization analysis. Any genomic changes would be expected to be subtle compared to changes in NP localization and could account for these findings. It should be noted that other primary screen kits, i.e. CDK3, PRKAG3, ERBB4, and C9ORF96, were excluded from validation studies only because their expression was not silenced with the novel siRNA, and they may also have a role in virus replication.

Of the 17 validated hits, 6 HPK genes (CDC2L5, HK2, NEK8, PANK4, PLK4, and SGK3) were found to be important for both A/WSN/33 and A/New Caledonia/20/99 influenza virus replication. Interestingly, these genes were key nodes for both FOXO and p53 signaling among our pathway analysis and CDC2L5, NEK3, PLK4, and SGK3 were largely associated with miRNA regulation (Figure 3). The minimal overlap between different virus strains is expected since the tempo of signal transduction and host gene expression can be differentially induced by different virus strains because of differences in replication dynamics and virus yields [73]. This may be particularly relevant as WSN/33 is a mouse-adapted virus and A/New Caledonia is a human virus. Also, differential activation of the PI3K/Akt pathway is affected by serum contained in the culture medium [74] and may reduce the ability of WSN to induce apoptosis [75,76], resulting in a stronger phosphorylation or activation of PI3K-Akt [77]. Ultimately, this would result in a stronger association of PI3K/Akt related genes necessary for viral replication.

Among the known signaling components induced upon influenza binding are PKC [20,21], ERK [22,23], PI3K [24], and p53/DNA damage pathways [78-84]. Early binding of the viral HA to the host cell sialic acid receptor rapidly activates PKC [20], likely in a pro-viral manner, as blocking this activity prevents entry and infection of the virus [25]. Late accumulation of viral HA on the host cell membrane activates the RAF/MEK/ERK mitogenic kinase cascade via PKCα, subsequently regulating RNP export [24]. In addition, influenza competes with the host to control apoptotic signaling and has been described *in vivo* [78-80,85] and *in vitro* [81-84,86,87]. Meta-analysis of the important HPK genes identified in this study combined with information from the five other influenza virus screens [12-16] identified a level of overlap most notably with the p53/DNA damage pathway. This is not surprising as cellular stress caused by infection has been shown to activate p53/DNA damage pathways and induce the expression of genes involved in cell cycle arrest and apoptosis [88]. The voltage-dependent anion channel (VDAC) protein, also termed porin, is regulated by its interaction with bound proteins, including Bcl-2 family members, mitochondrial creatine kinase, and HK2 [89]. Through these direct interactions, VDAC regulates the outer mitochondrial membrane permeability and the transport of solutes such as Ca^{2+} and ATP across it [90,91]. Importantly, the influenza virus proapoptotic PB1-F2 protein is known to localize to the mitochondria of infected cells and associate with VDAC to promote the permeabilization and release of mitochondrial products that trigger cell death or apoptosis [92].

As host genes are governed by miRNAs that regulate their magnitude and tempo of expression [28,54], we investigated miRNA regulation of the HPK genes that were identified as essential for influenza virus replication. Previous studies have shown that viral infection also alters cellular miRNA expression during infection and replication [30,33,93-96]. For influenza virus, cellular miRNAs miR-323, miR-491, and miR-654 have been shown to inhibit replication by binding to the viral PB1 gene [34], while miR-507 and miR-136 have potential binding sites within the viral PB2 and HA genes [35]. Additionally, miR-26a and miR-939 regulate the replication of H1N1influenza virus in MDCK cells [34]. Importantly, infections with HPAI and 1918 pandemic H1N1influenza virus are characterized by uncontrolled local and systemic inflammation, and it has recently been suggested that the virulence associated with these viruses may be mediated by host miRNA through dysregulation of genes critical to the inflammatory process [97]. A recent study profiling host miRNAs in lung tissue from HPAI or 1918 H1N1 infected macaques identified 23 miRNAs associated with virulence. The expression patterns of the miRNAs was found to be inversely correlated with that of predicted gene targets and pathway analyses identified the miRNA targets as genes commonly associated with aberrant and uncontrolled inflammatory responses and increased cell death [97].

In this study, the miRNA analysis was limited to those miRNAs that were predicted to target the validated HPK genes based on miRNA seed site predictions from computational analysis, and validated miRNA regulation of these genes using a panel of miRNA agonists and antagonists. The results showed that miRNAs regulated HPK genes that were important for influenza virus replication, specifically miR-181b regulated SGK3 and 148a regulated CDC2L5. It is also possible that miR-214 regulates PLK4, miR-375 and miR-654 regulates NEK8, but this needs to be further investigated. Since a single miRNA can regulate multiple perhaps hundreds of genes, and multiple miRNAs can regulate a single gene, it is likely that other relevant miRNAs were not identified in this study. An additional caveat to note reflects the underlying assumption that over-expression of a miRNA agonist increases repression of its target; however, this depends on the concentrations of miRNA agonist, and the temporal regulation of the gene, and the associated variability of expression in different cell types. Examining the involvement of miRNA HPK gene regulation and the impact on influenza virus replication showed that a number of miRNAs affected influenza virus replication by targeting different HPK genes, or multiple miRNAs targeted a single HPK gene. Both miR-155 and miR-181b were predicted to target SGK3. SGK3 is a member of the Ser/Thr protein kinase family, and is one of three serum/glucocorticoid regulated kinases isoforms [98]. All isoforms contain a similar nuclear localization signal sequence, indicating both nuclear and cytoplasmic subcellular distribution in all three [99]. The functional significance of SGK and the three isoforms is still not understood. Notably, all three kinases are potent regulators of ion channel activity, transport, and transcription [100-102]. SGK3 are activated by oxidation, insulin, and IGF-I through a signaling cascade involving PI3K as well as PDK1 and another hit kinase identified in our screen, PDK2 [103]. miR-155, predicted to target and govern SGK3, is implicated in regulating hematopoiesis, the innate and acquired immune response, and autoimmune disorders [104-109]. Accordingly, miR-155 is highly expressed in immune cell subsets, namely T cells, and regulates their differentiation and proliferation by targeting a negative regulator of IFN signaling, i.e. suppressor of cytokine signaling 1 (SOCS1) [110]. Also, miR-155 targets transcripts implicated in DNA repair [111]. For miR-181b, which also is implicated here to regulate the SGK3 gene, IL-6 induction of STAT3 transcription factor has been shown to directly activate miR-181b which in turn inhibits PTEN leading to increased NF-κB activity [112], and NF-κB activity affects influenza virus replication through induction of antiviral cytokines and the expression of other important genes related to host defense mechanisms [113,114]. In influenza virus infection NF-κB acts by inducing pro-apoptotic factors [115], and by activating caspases [116], thereby enhancing the nuclear export of viral RNPs.

The miRNA, miR-148a, was shown to target CDC2L5 in this study. It has been shown that members of the miRNA-148 family act to regulate the innate immune response and affect the antigen presenting capacity of dendritic cells, thus having a role in immune homeostasis and immune regulation [117]. The protein encoded by CDC2L5 is a member of the cyclin-dependent serine/threonine protein kinase family. Members of this family are well known for their roles as master switches in cell cycle control. They can be broadly sub-divided into two groups: the SR proteins, characterized by one or two N-terminal RNA recognition motifs and a C-terminal RS domain and the SR-related proteins [118]. The SR-related protein family is functionally associated with different nuclear processes, chromatin structure, mRNA transcription, coordination between pre-mRNA splicing, and 3-end processing [118]. Interestingly, the SR splicing factor SF2/ASF has been shown to control the level of the influenza virus M2 ion channel protein [119]. The M2 mRNA that encodes the ion channel protein is produced by alternative splicing from influenza M1 mRNA. The production of M2 mRNA is controlled in two ways. First, a 5' splice site in M1 mRNA is blocked by the complex of viral polymerase proteins synthesized during infection, allowing the cellular splicing machinery to switch to the M2 5' splice site. Second, utilization of M2 5' splice site requires its activation by the cellular SF2/ASF protein [119]. This activation is mediated by the binding of the SF2/ASF protein to a purine-rich splicing enhancer sequence that is located in the 3' exon of M1 mRNA [119]. Therefore, the findings from this study support the notion that SF2/ASF and associated CDC2L5 are modulated by influenza virus to support viral splicing events, or possibly, CDC2L5 may regulate the M2 splice event and loss of this gene reduces virus replication.

Given the large number of potential miRNA targets, perhaps in the hundreds [28], it is unlikely that one miRNA governing one HPK gene could sufficiently explain a phenotype, e.g. increased or decreased influenza virus replication. The RNAi screen in this study yielded 17 candidate HPK genes based on a Z-score ≥ μ ± 2SD (Appendices Table S2); however, lowering the threshold to a Z- score ≥ μ ± 1SD would have added an additional 37 HPK genes that overlapped with HPK genes discovered in related published screens [12-16]. Thus, it is likely that other HPK genes not identified in this study may be required or contribute to influenza virus replication.

The features of the virus-host interface are becoming clearer, and this study together with others has dissected some of the host components that affect influenza virus replication. Notably, there remains a gap in our understanding of the role of miRNA regulation of host genes, and how this interaction affects intracellular signaling pathways used during viral infection and replication; however, this study provides a framework for future studies. The findings reported here contribute toward a better understanding of host-pathogen interactions which may help in accelerating the rational design of therapeutics aimed to control influenza infection and disease pathogenesis.

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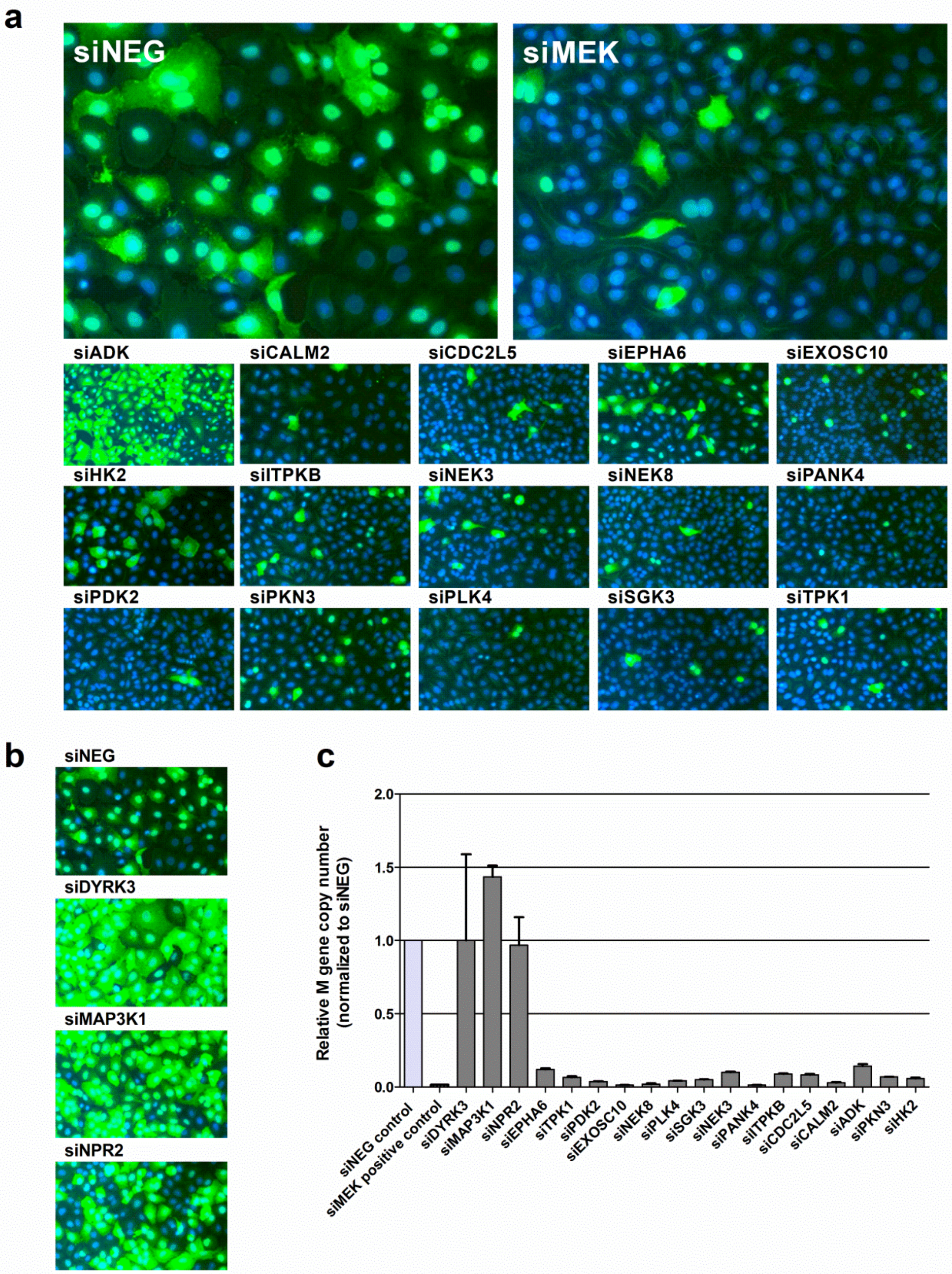

Figure 4.1. Validation of human protein kinase genes affecting influenza virus replication. A549 cells were reverse transfected with 50 nM of a non-target negative control siRNA (siNEG) or with siHPK and infected at a MOI =

0.01 with A/WSN/33. Cells were then fixed, permeabilized and incubated with an anti-influenza virus NP monoclonal antibody and subsequently with an Alexa-488 goat anti-mouse secondary antibody and DAPI. The intracellular distribution of the viral RNPs (NP, green) and cellular nuclei (DAPI, blue) for HPK gene whose silencing (A) decreased influenza virus levels or (B) increased levels. The intracellular distribution of the viral RNPs (NP, green) and cellular nuclei (DAPI, blue) are shown. (C) The effect of siRNA silencing on influenza virus replication was measured by quantifying the levels of influenza M gene expression. The RNA from siRNA-transfected and WSN-infected A549s was isolated and used for quantification with an influenza M-specific primer/probe set. Light gray bars indicate controls. Data show mean + SEM independent experiments.

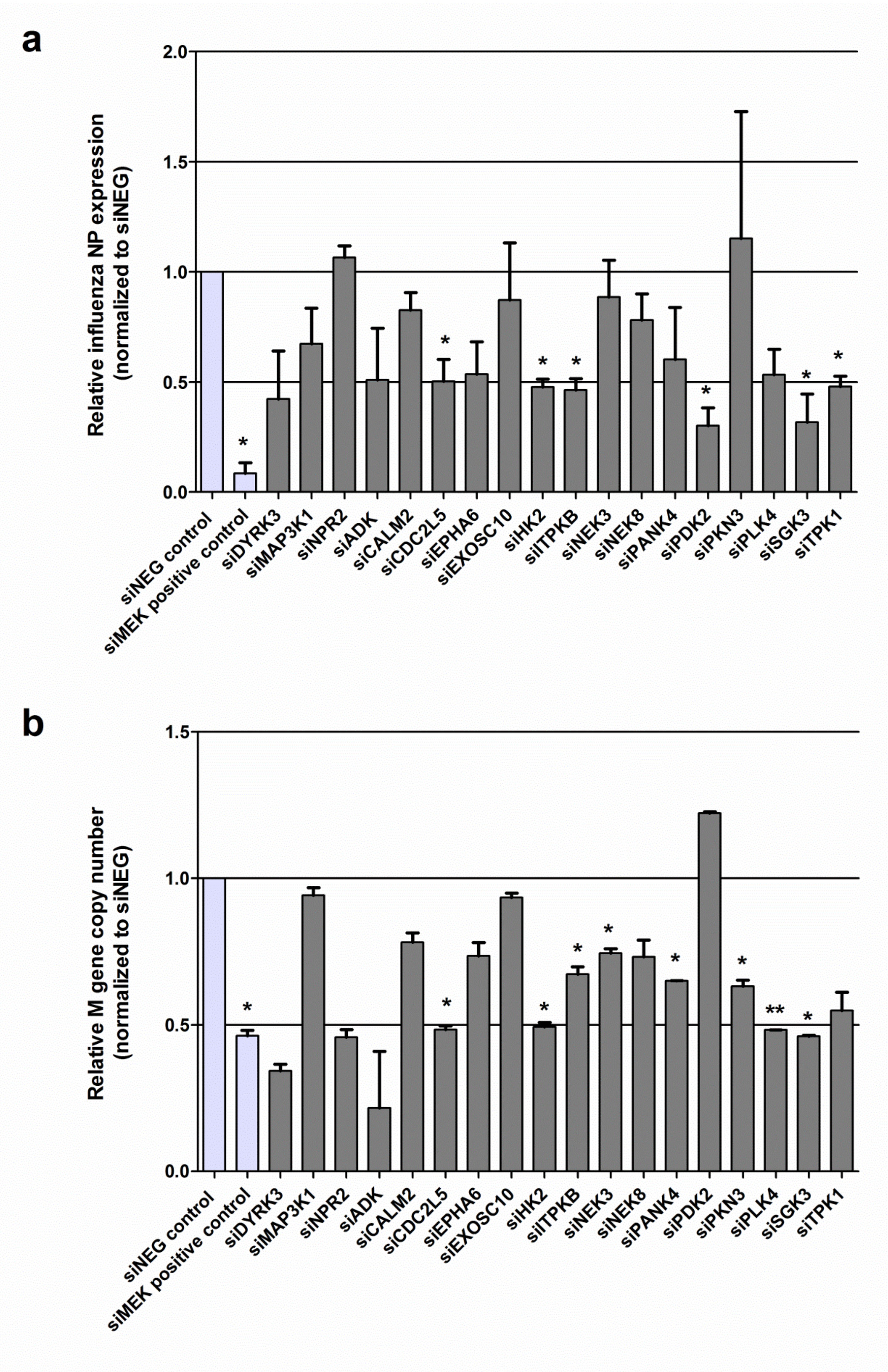
a
Relative influenza NP expression (normalized to siNEG)
2.0
1.5
1.0
0.5
0.0
siNEG control
siMEK positive control
siDYRK3
siMAP3K1
siNPR2
siADK
siCALM2
siCDC2L5
siEPHA6
siEXOSC10
siHK2
siITPKB
siNEK3
siNEK8
siPANK4
siPDK2
siPKN3
siPLK4
siSGK3
siTPK1
*
b
Relative M gene copy number (normalized to siNEG)
1.5
1.0
0.5
0.0
siNEG control
siMEK positive control
siDYRK3
siMAP3K1
siNPR2
siADK
siCALM2
siCDC2L5
siEPHA6
siEXOSC10
siHK2
siITPKB
siNEK3
siNEK8
siPANK4
siPDK2
siPKN3
siPLK4
siSGK3
siTPK1
*
**

Figure 4.2. Human protein kinase genes affect H1N1 A/New Caledonia/20/99 virus replication. A549 cells were reverse transfected with 50 nM of siRNA specific for validated HPK genes and after 48h, the cells were infected with A/New Caledonia/20/99 at an MOI of 0.01 in the presence of 1 µg/ml TPCK-trypsin. After 48 h of infection, RNA was extracted and the effect of siRNA silencing of HPK genes on viral genome replication was measured by quantifying the levels of influenza M gene. Data show mean + SEM of independent experiments. * $p < 0.05$ and ** $p < 0.001$ compared to control.

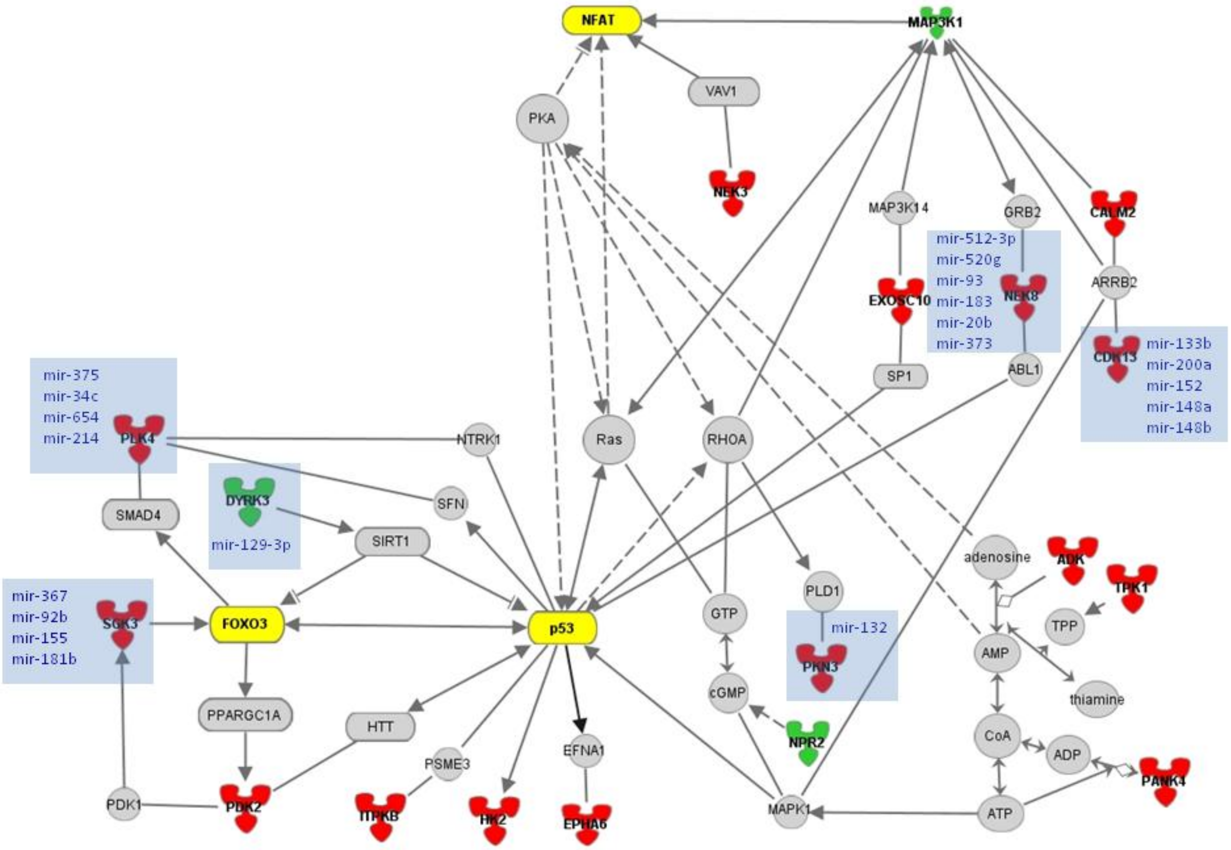

Figure 4.3. Network relationships among human protein kinase genes identified as important for influenza virus replication. Pathway analysis (Ingenuity, STRING, DAVID), miRNA regulation (TargetScan, PicTar, miRANDA) and a review of the literature functionally classified and embedded hits into known pathways and protein-protein interaction networks. The genes have been classified into kinases whose silencing increases influenza (shaded green), kinases whose silencing decreases influenza (red), predicted miRNAs targeting the hit kinases (blue), and others (grey). Solid lines are direct interactions and dashed lines are indirect interactions established by Ingenuity.

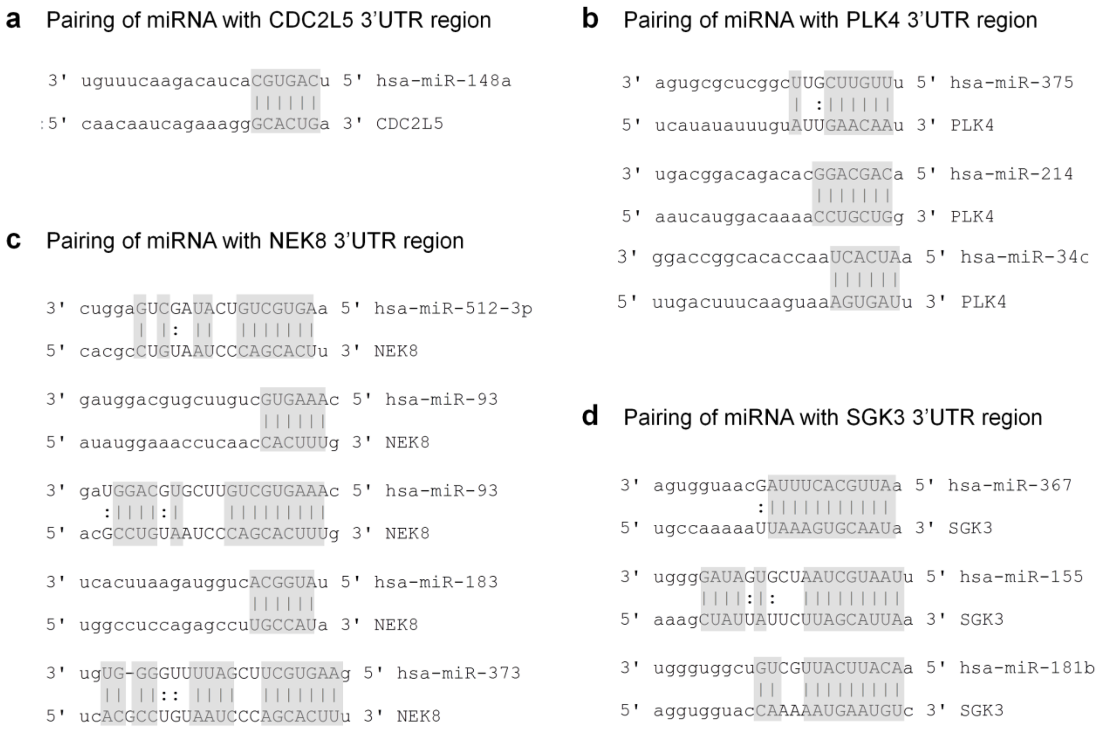

Figure 4.4. Predicted miRNA target sites in the 3'UTR of host kinase genes identified important for influenza virus replication. The locations of the putative target sites are indicated with capital letters and unpaired bases are in lower case for A) CDC2L5, B) PLK4, C) NEK8, and D) SGK3. Analysis utilized TargetScan, PicTar, and miRANDA.

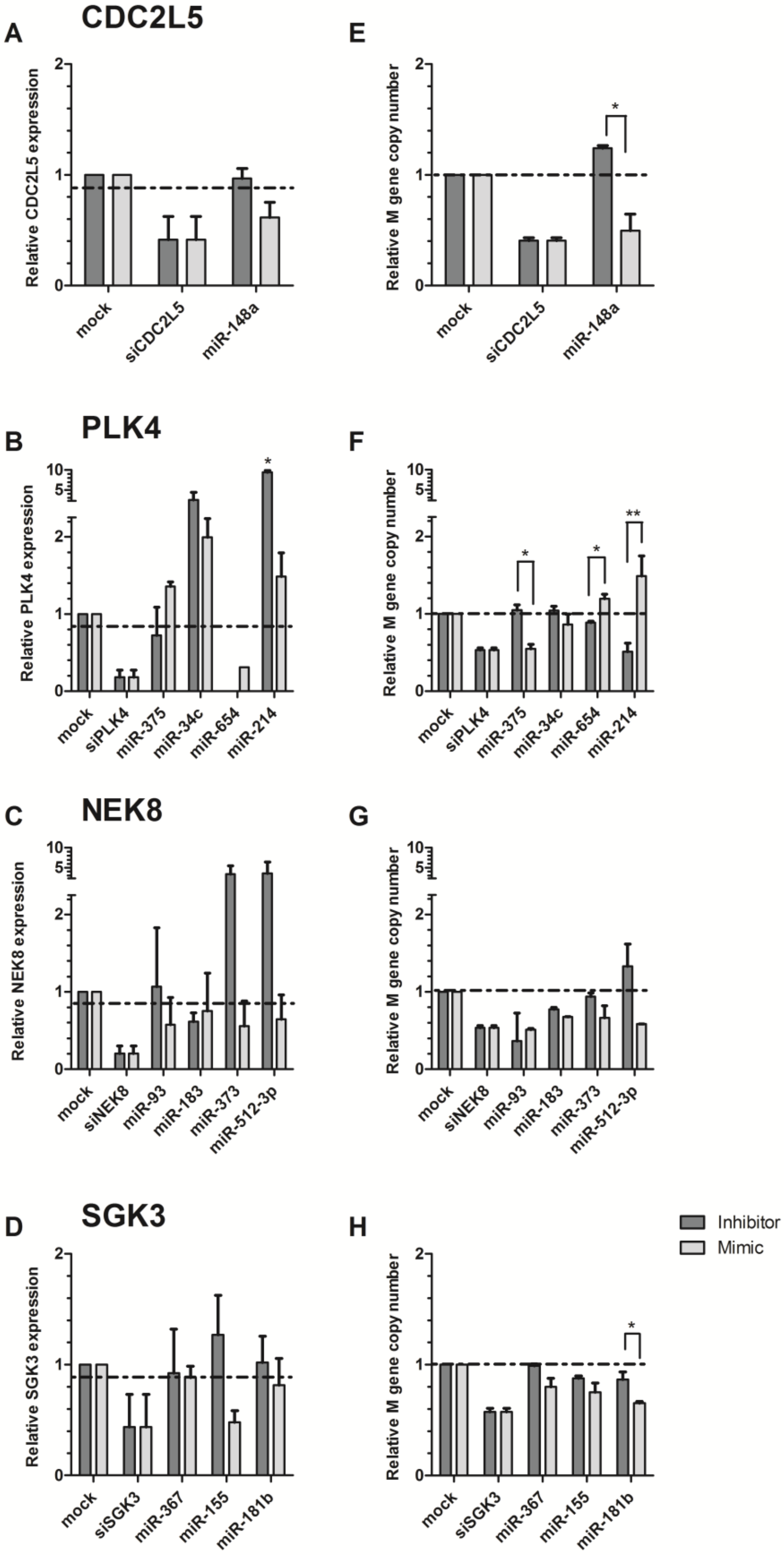
A
CDC2L5
Relative CDC2L5 expression
2
1
0
mock
siCDC2L5
miR-148a
E
Relative M gene copy number
2
1
0
*
mock
siCDC2L5
miR-148a
B
PLK4
Relative PLK4 expression
10
5
2
1
0
*
mock
siPLK4
miR-375
miR-34c
miR-654
miR-214
F
Relative M gene copy number
10
5
2
1
0
*
*
**
mock
siPLK4
miR-375
miR-34c
miR-654
miR-214
C
NEK8
Relative NEK8 expression
10
5
2
1
0
mock
siNEK8
miR-93
miR-183
miR-373
miR-512-3p
G
Relative M gene copy number
10
5
2
1
0
mock
siNEK8
miR-93
miR-183
miR-373
miR-512-3p
D
SGK3
Relative SGK3 expression
2
1
0
mock
siSGK3
miR-367
miR-155
miR-181b
H
Relative M gene copy number
2
1
0
*
mock
siSGK3
miR-367
miR-155
miR-181b
Inhibitor
Mimic

Figure 4.5. miRNA affect on human protein kinase gene mRNA expression and influenza virus replication. (A-D) Host cell miRNAs predicted to target and regulate HPK kinases CDC2L5, PLK4, NEK8, and SGK3 were evaluated by qPCR. A549 cells were transfected with 12.5 nM of the appropriate miRNA agonist and 25 nM of the appropriate miRNA antagonist for 24 h. RNA was isolated and evaluated for host gene expression using a SYBR green assay with gene-specific primers. Gene expression was compared to mock transfected cells. Data is normalized to GAPDH expression and shown as mean + SEM of independent experiments. * $p < 0.05$ compared to control. **(E-G)** To determine the effect of specific miRNAs on influenza replication, A549 cells were transfected with 12.5 nM of the appropriate miRNA agonist and 25 nM of the appropriate miRNA antagonist for 48 h, followed by infection with A/WSN/33 (MOI = 0.01). The RNA from siRNA-infected A549s was isolated and used for quantification with an influenza M gene specific primer/probe set for CDC2L5, PLK4, NEK8, and SGK3. Data show mean + SEM of independent experiments. * $p < 0.05$ and ** $p < 0.001$ compared to control.

Table 4.1 Human kinase genes identified important for influenza virus replication in primary screen

Symbol	Name	Z-score	Log_{10} $TCID_{50}$/mL
NPR2	natriuretic peptide receptor B/guanylate cyclase B	2.64	6.5
MAP3K1	mitogen-activated protein kinase kinase kinase 1	2.55	5.7
DYRK3	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	2.23	5.9
EPHA6	EPH receptor A6	-2.01	3.0
TPK1	thiamin pyrophosphokinase 1	-2.01	3.0
PDK2	pyruvate dehydrogenase kinase, isozyme 2	-2.03	2.7
C9ORF96	chromosome 9 open reading frame 96	-2.16	2.7
EXOSC10	exosome component 10	-2.16	2.7
NEK8*	never in mitosis gene a- related kinase 8	-2.18	2.7
PLK4*	polo-like kinase 4	-2.18	1.7
SGK3	serum/glucocorticoid regulated kinase family, member 3	-2.19	2.7
NEK3	never in mitosis gene a-related kinase 3	-2.22	2.7
PANK4*	pantothenate kinase 4	-2.35	1.7
ITPKB*	inositol 1,4,5-trisphosphate 3-kinase B	-2.35	1.7
CDC2L5	cell division cycle 2-like 5	-2.38	1.7
CDK3	cyclin-dependent kinase 3	-2.40	1.7
CALM2*	calmodulin 2	-2.59	n.v.
PRKAG3	protein kinase, AMP-activated, gamma 3 non-catalytic subunit	-2.59	n.v.
ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4	-2.59	n.v.
ADK	adenosine kinase	-2.59	n.v.
PKN3	protein kinase N3	-2.59	n.v.
HK2*	hexokinase 2	-2.59	n.v.

* Genes identified important for influenza in a previous screen; n.v., no detectable virus

Table 4.2 **Host cells pathways predicted for validated genes**

Functional category	Genes	Cellular function	Proposed role in influenza virus replication	Replication block
p53/DNA damage pathways	EPHA6, NEK8, ADK, CALM2, DYRK3, HK2, ITPKB, MAP3K1, NPR2, PANK4, PDK2, TPK1	Cell cycle arrest, cellular senescence or apoptosis	Down regulated by the virus to extend host cell survival	Late stages of viral replication
PI3K/AKT signaling	NEK8, CALM2, HK2, ITPKB, MAP3K1, NPR2, PKN3	Signaling for cell growth, proliferation, differentiation, survival or intracellular trafficking	Important for efficient viral entry, protein yields and nuclear export of vRNPs; involved in inhibition of apoptosis	Entry
PKC/CA^{++} signaling	EPHA6, NEK8, ADK, CALM2, DYRK3, HK2, ITPKB, MAP3K1, PKN3, PANK4, PDK2, TPK1	Transport, catabolism, motility, growth, communication or apoptosis	Transcriptional regulation	Post-entry

References for the genes and proposed role in virus replication are provided in Supplementary Table S6.

References

1. Thompson, W.W., et al., *Influenza-associated hospitalizations in the United States.* JAMA, 2004. **292**(11): p. 1333-40.
2. Thompson, W.W., et al., *Mortality associated with influenza and respiratory syncytial virus in the United States.* JAMA, 2003. **289**(2): p. 179-86.
3. MMWR, *Estimates of Deaths Associated with Seasonal Influenza - United States, 1976–2007*, 2010, Centers for Disease Control and Prevention: Atlanta, GA. p. 1057-1062.
4. Regoes, R.R. and S. Bonhoeffer, *Emergence of drug-resistant influenza virus: population dynamical considerations.* Science, 2006. **312**(5772): p. 389-91.
5. Beigel, J. and M. Bray, *Current and future antiviral therapy of severe seasonal and avian influenza.* Antiviral Res, 2008. **78**(1): p. 91-102.
6. Conly, J. and B. Johnston, *Ode to oseltamivir and amantadine?* Can J Infect Dis Med Microbiol, 2006. **17**(1): p. 11-4.
7. Hsieh, H.P. and J.T. Hsu, *Strategies of development of antiviral agents directed against influenza virus replication.* Curr Pharm Des, 2007. **13**(34): p. 3531-42.
8. Lackenby, A., C.I. Thompson, and J. Democratis, *The potential impact of neuraminidase inhibitor resistant influenza.* Curr Opin Infect Dis, 2008. **21**(6): p. 626-38.

9. Osterhaus, A., R. Fouchier, and G. Rimmelzwaan, *Towards universal influenza vaccines?* Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1579): p. 2766-73.
10. Schultz-Cherry, S. and J.C. Jones, *Influenza vaccines: the good, the bad, and the eggs.* Adv Virus Res, 2010. **77**: p. 63-84.
11. Palese P, S.M., *Fields Virology*. 5th ed, ed. H.P. Knipe DM2007, Philadelphia: Raven.
12. Brass, A.L., et al., *The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus.* Cell, 2009. **139**(7): p. 1243-54.
13. Hao, L., et al., *Drosophila RNAi screen identifies host genes important for influenza virus replication.* Nature, 2008. **454**(7206): p. 890-3.
14. Karlas, A., et al., *Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication.* Nature, 2010.
15. Konig, R., et al., *Human host factors required for influenza virus replication.* Nature, 2009. **463**(7282): p. 813-7.
16. Shapira, S.D., et al., *A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection.* Cell, 2009. **139**(7): p. 1255-67.
17. Karlas, A., et al., *Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication.* Nature.
18. Konig, R., et al., *Human host factors required for influenza virus replication.* Nature. **463**(7282): p. 813-7.

19. Brass, A.L., et al., *Identification of host proteins required for HIV infection through a functional genomic screen.* Science, 2008. **319**(5865): p. 921-6.

20. Arora, D.J. and N. Gasse, *Influenza virus hemagglutinin stimulates the protein kinase C activity of human polymorphonuclear leucocytes.* Arch Virol, 1998. **143**(10): p. 2029-37.

21. Kunzelmann, K., et al., *Influenza virus inhibits amiloride-sensitive Na+ channels in respiratory epithelia.* Proc Natl Acad Sci U S A, 2000. **97**(18): p. 10282-7.

22. Pleschka, S., et al., *Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade.* Nature cell biology, 2001. **3**(3): p. 301-5.

23. Pleschka, S., *RNA viruses and the mitogenic Raf/MEK/ERK signal transduction cascade.* Biol Chem, 2008. **389**(18713014): p. 1273-1282.

24. Marjuki, H., et al., *Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Calpha-mediated activation of ERK signaling.* J Biol Chem, 2006. **281**(24): p. 16707-15.

25. Root, C.N., et al., *Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor.* J Gen Virol, 2000. **81**(Pt 11): p. 2697-705.

26. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* Febs Letters, 2004. **561**(1-3): p. 37-43.

27. Filipowicz, W., S.N. Bhattacharyya, and N. Sonenberg, *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nat Rev Genet, 2008. **9**(2): p. 102-14.

28. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Res, 2009. **19**(1): p. 92-105.

29. Ambros, V., *The functions of animal microRNAs.* Nature, 2004. **431**(7006): p. 350-5.

30. Zheng, S.Q., et al., *MiR-101 regulates HSV-1 replication by targeting ATP5B.* Antiviral Res. **89**(3): p. 219-26.

31. Lagos, D., et al., *miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator.* Nature cell biology. **12**(5): p. 513-9.

32. Yeung, M.L., et al., *Changes in microRNA expression profiles in HIV-1-transfected human cells.* Retrovirology, 2005. **2**: p. 81.

33. Triboulet, R., et al., *Suppression of microRNA-silencing pathway by HIV-1 during virus replication.* Science, 2007. **315**(5818): p. 1579-82.

34. Liu, H., L. Song, and W. Huang, *MiR26a and miR939 regulate the replication of H1N1 influenza virus in MDCK cell.* Wei Sheng Wu Xue Bao, 2010. **50**(10): p. 1399-405.

35. Scaria, V., et al., *Host-virus interaction: a new role for microRNAs.* Retrovirology, 2006. **3**: p. 68.

36. Bell, J.C. and D. Kirn, *MicroRNAs fine-tune oncolytic viruses.* Nat Biotechnol, 2008. **26**(12): p. 1346-8.

37. Cullen, B.R., *Viruses and microRNAs.* Nat Genet, 2006. **38 Suppl**: p. S25-30.

38. Woolcock, P.R., *Avian influenza virus isolation and propagation in chicken eggs.* Methods Mol Biol, 2008. **436**: p. 35-46.

39. Reed LJ, M.H., *A simple method of estimating fifty percent endpoints.* The American Journal of Hygiene, 1938. **27**: p. 493-497.

40. Haney, S.A., *Increasing the robustness and validity of RNAi screens.* Pharmacogenomics, 2007. **8**(8): p. 1037-49.

41. Hirst, G.K., *Adsorption of Influenza Hemagglutinins and Virus by Red Blood Cells.* J Exp Med, 1942. **76**(2): p. 195-209.

42. Spackman, E., et al., *Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes.* J Clin Microbiol, 2002. **40**(9): p. 3256-60.

43. Bustin, S.A., et al., *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.

44. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.

45. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists.* Nucleic Acids Res, 2009. **37**(1): p. 1-13.

46. Kanehisa, M. and S. Goto, *KEGG: kyoto encyclopedia of genes and genomes.* Nucleic Acids Res, 2000. **28**(1): p. 27-30.

47. Kanehisa, M., et al., *From genomics to chemical genomics: new developments in KEGG.* Nucleic Acids Res, 2006. **34**(Database issue): p. D354-7.

48. Prasad, T.S., K. Kandasamy, and A. Pandey, *Human Protein Reference Database and Human Proteinpedia as discovery tools for systems biology.* Methods Mol Biol, 2009. **577**: p. 67-79.

49. Alfarano, C., et al., *The Biomolecular Interaction Network Database and related tools 2005 update.* Nucleic Acids Res, 2005. **33**(Database issue): p. D418-24.

50. Bader, G.D., D. Betel, and C.W. Hogue, *BIND: the Biomolecular Interaction Network Database.* Nucleic Acids Res, 2003. **31**(1): p. 248-50.

51. Willis, R.C. and C.W. Hogue, *Searching, viewing, and visualizing data in the Biomolecular Interaction Network Database (BIND).* Curr Protoc Bioinformatics, 2006. **Chapter 8**: p. Unit 8 9.

52. Rajewsky, N., *microRNA target predictions in animals.* Nat Genet, 2006. **38 Suppl**: p. S8-13.

53. John, B., et al., *Human MicroRNA targets.* PLoS Biol, 2004. **2**(11): p. e363.

54. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets.* Cell, 2005. **120**(1): p. 15-20.

55. Krek, A., et al., *Combinatorial microRNA target predictions.* Nat Genet, 2005. **37**(5): p. 495-500.

56. Enright, A.J., et al., *MicroRNA targets in Drosophila.* Genome Biol, 2003. **5**(1): p. R1.

57. Zhang, J.H., T.D. Chung, and K.R. Oldenburg, *A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays.* J Biomol Screen, 1999. **4**(2): p. 67-73.

58. Birmingham, A., et al., *Statistical methods for analysis of high-throughput RNA interference screens.* Nat Methods, 2009. **6**(8): p. 569-75.

59. *Current Topics in RNAi: Why Rational Pooling of siRNAs is SMART*, 2010: Thermo Fisher Scientific Inc.

60. Dweep, H., et al., *miRWalk - Database: Prediction of possible miRNA binding sites by "walking" the genes of three genomes.* Journal of biomedical informatics, 2011. **44**(5): p. 839-47.

61. Min, J.Y. and K. Subbarao, *Cellular targets for influenza drugs.* Nat Biotechnol, 2010. **28**(3): p. 239-40.

62. Ito, T., et al., *Virulent influenza A viruses induce apoptosis in chickens.* Virus Res, 2002. **84**(1-2): p. 27-35.

63. Mori, I., et al., *In vivo induction of apoptosis by influenza virus.* J Gen Virol, 1995. **76 (Pt 11)**: p. 2869-73.

64. Technau-Ihling, K., et al., *Influenza A virus infection of mice induces nuclear accumulation of the tumorsuppressor protein p53 in the lung.* Arch Virol, 2001. **146**(9): p. 1655-66.

65. Fesq, H., et al., *Programmed cell death (apoptosis) in human monocytes infected by influenza A virus.* Immunobiology, 1994. **190**(1-2): p. 175-82.

66. Schultz-Cherry, S., et al., *Influenza virus ns1 protein induces apoptosis in cultured cells.* J Virol, 2001. **75**(17): p. 7875-81.

67. Takizawa, T., et al., *Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells.* J Gen Virol, 1993. **74 (Pt 11)**: p. 2347-55.

68. Xing, Z., et al., *Host immune and apoptotic responses to avian influenza virus H9N2 in human tracheobronchial epithelial cells.* Am J Respir Cell Mol Biol. **44**(1): p. 24-33.

69. Mori, I. and Y. Kimura, *Apoptotic neurodegeneration induced by influenza A virus infection in the mouse brain.* Microbes Infect, 2000. **2**(11): p. 1329-34.

70. Ludwig, S., et al., *Ringing the alarm bells: signalling and apoptosis in influenza virus infected cells.* Cell Microbiol, 2006. **8**(3): p. 375-86.

71. Mitzner, D., et al., *Phosphorylation of the influenza A virus protein PB1-F2 by PKC is crucial for apoptosis promoting functions in monocytes.* Cell Microbiol, 2009. **11**(10): p. 1502-16.

72. Polyak, K., et al., *A model for p53-induced apoptosis.* Nature, 1997. **389**(6648): p. 300-5.

73. Tsujimoto, Y. and S. Shimizu, *The voltage-dependent anion channel: an essential player in apoptosis.* Biochimie, 2002. **84**(2-3): p. 187-93.

74. Colombini, M., *Voltage gating in the mitochondrial channel, VDAC.* J Membr Biol, 1989. **111**(2): p. 103-11.

75. Bernardi, P., *Mitochondrial transport of cations: channels, exchangers, and permeability transition.* Physiol Rev, 1999. **79**(4): p. 1127-55.

76. Zamarin, D., et al., *Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1.* PLoS Pathog, 2005. **1**(1): p. e4.

77. Umbach, J.L. and B.R. Cullen, *The role of RNAi and microRNAs in animal virus replication and antiviral immunity.* Genes Dev, 2009. **23**(10): p. 1151-64.

78. Lecellier, C.H., et al., *A cellular microRNA mediates antiviral defense in human cells.* Science, 2005. **308**(5721): p. 557-60.

79. Jopling, C.L., et al., *Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA.* Science, 2005. **309**(5740): p. 1577-81.

80. Lu, F., et al., *Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence.* J Virol, 2008. **82**(21): p. 10436-43.

81. Li, Y., et al., *Differential microRNA expression and virulence of avian, 1918 reassortant, and reconstructed 1918 influenza A viruses.* Virology, 2011.

82. Lang, F., et al., *(Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms.* Physiol Rev, 2006. **86**(4): p. 1151-78.

83. Firestone, G.L., J.R. Giampaolo, and B.A. O'Keeffe, *Stimulus-dependent regulation of serum and glucocorticoid inducible protein kinase (SGK) transcription, subcellular localization and enzymatic activity.* Cell Physiol Biochem, 2003. **13**(1): p. 1-12.

84. Bhargava, A. and D. Pearce, *Mechanisms of mineralocorticoid action: determinants of receptor specificity and actions of regulated gene products.* Trends Endocrinol Metab, 2004. **15**(4): p. 147-53.

85. Fillon, S., et al., *Serum- and glucocorticoid-dependent kinase, cell volume, and the regulation of epithelial transport.* Comp Biochem Physiol A Mol Integr Physiol, 2001. **130**(3): p. 367-76.

86. Lang, F., et al., *Regulation of channels by the serum and glucocorticoid-inducible kinase - implications for transport, excitability and cell proliferation.* Cell Physiol Biochem, 2003. **13**(1): p. 41-50.

87. Virbasius, J.V., et al., *Activation of the Akt-related cytokine-independent survival kinase requires interaction of its phox domain with endosomal phosphatidylinositol 3-phosphate.* Proc Natl Acad Sci U S A, 2001. **98**(23): p. 12908-13.

88. Tili, E., C.M. Croce, and J.J. Michaille, *miR-155: on the crosstalk between inflammation and cancer.* Int Rev Immunol, 2009. **28**(5): p. 264-84.

89. Tili, E., et al., *Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock.* J Immunol, 2007. **179**(8): p. 5082-9.

90. O'Connell, M.J., M.J. Krien, and T. Hunter, *Never say never. The NIMA-related protein kinases in mitotic control.* Trends Cell Biol, 2003. **13**(5): p. 221-8.
91. O'Connell, R.M., et al., *MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development.* Immunity. **33**(4): p. 607-19.
92. O'Connell, R.M., et al., *Physiological and pathological roles for microRNAs in the immune system.* Nat Rev Immunol. **10**(2): p. 111-22.
93. Sonkoly, E. and A. Pivarcsi, *Advances in microRNAs: implications for immunity and inflammatory diseases.* J Cell Mol Med, 2009. **13**(1): p. 24-38.
94. Iorio, M.V., et al., *MicroRNA gene expression deregulation in human breast cancer.* Cancer Res, 2005. **65**(16): p. 7065-70.
95. Valeri, N., et al., *Modulation of mismatch repair and genomic stability by miR-155.* Proc Natl Acad Sci U S A. **107**(15): p. 6982-7.
96. Iliopoulos, D., et al., *STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer.* Mol Cell. **39**(4): p. 493-506.
97. Hiscott, J., H. Kwon, and P. Genin, *Hostile takeovers: viral appropriation of the NF-kappaB pathway.* J Clin Invest, 2001. **107**(11160127): p. 143-151.
98. Pinto, R., et al., *Inhibition of influenza virus-induced NF-kappaB and Raf/MEK/ERK activation can reduce both virus titers and cytokine*

expression simultaneously in vitro and in vivo. Antiviral Res, 2011. **92**(1): p. 45-56.

99. Wurzer, W.J., et al., *NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation.* J Biol Chem, 2004. **279**(15143063): p. 30931-30937.

100. Wurzer, W.J., et al., *Caspase 3 activation is essential for efficient influenza virus propagation.* EMBO J, 2003. **22**(12773387): p. 2717-2728.

101. Liu, X., et al., *MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha.* J Immunol, 2010. **185**(12): p. 7244-51.

102. Boucher, L., et al., *A genome-wide survey of RS domain proteins.* RNA, 2001. **7**(12): p. 1693-701.

103. Shih, S.R. and R.M. Krug, *Novel exploitation of a nuclear function by influenza virus: the cellular SF2/ASF splicing factor controls the amount of the essential viral M2 ion channel protein in infected cells.* EMBO J, 1996. **15**(19): p. 5415-27.

CHAPTER 5

HOST GENE TARGETS FOR NOVEL INFLUENZA THERAPIES ELUCIDATED BY HIGH-THROUGHPUT RNA INTERFERENCE SCREENS[1]

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Introduction

Many emerging infectious diseases (EIDs) in humans are zoonotic: deriving from animals or animal products. EIDs are broadly defined to include 1) new agents, 2) existing yet previously undetected agents, 3) the re-emergence of known agents, and/or 4) expansion of a known agent into a new geographic range [1]. Influenza viruses are well-documented examples of EIDs. In 2009, the H1N1 pandemic virus erupted from the swine population and quickly spread to over 200 countries, overtaking pre-existing H1N1 and H3N2 viruses in the human population and becoming the dominant circulating strain [2]. Similarly, outbreaks of highly pathogenic H5N1 influenza viruses continue to emerge and exhibit up to 60% mortality rates in humans [3]. These outbreaks highlight the persistent and devastating nature of influenza infections, and increase the risk of new pandemics.

Despite scientific advancements over the last three decades, EIDs continue to inflict substantial social and economic costs. For example, influenza is a leading cause of morbidity and mortality in the world [4] with seasonal viruses affecting up to 15% of the human population - causing severe illness in 3-5 million people and fatality of approximately 500,000 individuals per year [5]. Coupled with this is the economic burden that is associated with widespread influenza infection. In the United States alone, financial losses resulting from seasonal influenza infection are estimated to exceed 87 billion dollars annually [6].

The ability of influenza viruses to continuously mutate has made control strategies based on vaccination difficult. Simultaneously, resistance to current antivirals is increasing. For these reasons, new strategies to combat EIDs such as influenza are required and an expanded knowledge of host-virus interactions is a crucial first step. Despite the complexity of influenza biology, viruses of this class contain only 8 gene segments [7] and therefore lack the full complement of proteins required to produce infectious virus. As such, influenza, like all viruses, must co-opt a wide array of host proteins, non-coding RNAs, and cellular processes (for example, transport vesicles) to generate infectious viral particles. The replicative cycle of orthomyxoviruses can be divided into early, middle and late stages [8]. Early stages involve events linked to virus binding via hemagglutinin (HA) to sialic acids moieties found on the host cell membrane and internalization of the viral particles [9]. Mid-stage events include the expression and translation of viral genes [10], while late events comprise virus protein trafficking to the cell membrane, virion assembly, and budding from the cell [11, 12]. Understanding the host contribution to viral replication and immune evasion is essential for discovering new therapeutic strategies. Genome screening technologies that utilize RNA interference (RNAi), together with bioinformatics provide the ability to rapidly identify the complement of essential host functions and pathways that are essential to the virus.

The RNAi pathway

RNAi is a natural, sequence-specific post-transcriptional gene silencing pathway (Figure 5.1) present in most eukaryotes [13, 14]. Components of the RNAi pathway serve multiple roles that can vary in different organisms. On the one hand, elements of the pathway participate in the innate immune response by recognizing and degrading long double-stranded (ds) RNAs typically associated with viral infection [15-17]. Simultaneously, the RNAi pathway has a recognized role in regulating host gene expression related to a myriad of cell functions by guiding the maturation and transcript targeting capabilities of a unique class of non-coding RNAs called microRNAs (miRNAs) [18, 19]. This aspect of RNAi is particularly intriguing given that some viruses, in particular herpesviruses, encode miRNAs that regulate host gene expression and therefore have a critical role in the dynamics of host-pathogen interactions [20-22].

Outside the important role in gene modulation, the RNAi pathway is now recognized as a preeminent means for studying gene function [23, 24]. In this regard, researchers have created entire libraries of gene-specific targeting reagents that silence individual gene function by entering the pathway at one of two distinct positions. In one instance, synthetic double-stranded RNA reagents referred to as small interfering RNAs (siRNA) have been generated. When introduced into cells, siRNAs associate with the RNA Induced Silencing Complex (RISC) where the siRNA guide strand anneals to its complimentary target mRNA [25, 26] and facilitates cleavage by Argonaute 2, a core endonuclease of RISC (Figure 5.1). In an alternative approach researchers have developed collections of gene-targeting short hairpin RNAs (shRNAs) that can be generated from plasmid or viral expression constructs [27-29]. Like their endogenous miRNA counterparts, shRNAs are expressed in the nucleus and are predicted to associate with RISC only after being processed by two separate endonucleases, Drosha and Dicer (Figure 5.1)[18, 19].

General workflow of host-pathogen screening using RNAi technology

The complexity of RNAi screens for host-pathogen interactions requires thoughtful consideration of the silencing reagent, the cell line, and virus to be used. RNAi screening typically begins with the plating of the target cells and introduction of the silencing reagent. For siRNA-based studies, screens are generally performed in an arrayed format where individual genes are targeted with anywhere from 1-4 siRNAs per well. In most cases, pooled reagents are preferred as they provide a cumulative silencing effect that greatly increases the efficacy of gene knockdown. Synthetic silencing reagents can be introduced via forward or reverse transfection protocols [30-33] that in many cell types leads to efficient intracellular delivery of the siRNA. In cases where shRNAs are utilized and expressed from a viral construct, one of two approaches can be employed. In the first, shRNA workflows can parallel siRNA screening processes by adopting a one-gene-one-well arrayed platform. Alternatively, shRNAs screens often make use of pools of viral particles that contain expression constructs targeting hundreds to thousands of genes. In this scenario, cell plating is followed by virus transduction at low multiplicities of infection, thus ensuring that each infected cell receives only one viral particle. Drug selection follows transduction to eliminate cells that do not carry a gene targeting construct [27-29]. Pooled shRNA screens are best suited to output assays that permit selection or outgrowth of cells that exhibit the phenotype of interest, where specific shRNAs which influence the phenotype (and are consequently enriched or depleted from the population) can be identified by either microarray or next generation sequencing. For an overview of the comparative workflows for siRNA versus shRNA see Table 5.1.

Regardless of the type of silencing reagent employed, the cells are subsequently cultured for a period of time that: 1) allows for gene knockdown, and 2) accounts for any unique attributes of the screen. For instance, if the intent of the screen is to study viral uptake, infection may be delayed to avoid lipid-mediated siRNA transfection affecting the ability of the virus to bind the cell. Cells are then infected with the virus of choice, cultured for an additional period of time, and assayed by one or more techniques (e.g. transcriptional reporters, viral titers) to determine the effect of gene knockdown on various aspects of the viral lifecycle. It is worth noting that different silencing reagents permit different types of assays to be incorporated in the workflow. Synthetic siRNAs generally provide 3-7 days of gene knockdown. As such, these reagents are compatible with assays that are performed within this brief window. In contrast, shRNAs expression constructs delivered by a lentivirus stably integrate into the host genome and therefore permit assays that extend beyond the transient (siRNA) window to be performed.

RNAi screens that utilize siRNA technology are greatly aided by automation, where extreme accuracy, reproducibility, and to some extent speed are necessary for managing the siRNA library resource, dispensing and aspirating cells and media, and performing lipid-mediated transfection. For screens that involve infectious reagents, liquid handling platforms that are modular are attractive as they facilitate the separation of steps that require high-level bio-containment from those that are common to all RNAi screens.

Both screening processes are iterative and full genome wide screens can take anywhere between weeks to months depending upon the assay complexity, the level of automation, the particulars of the pathogen being investigated, and the logistics used to divide and screen the gene silencing collection. With regards to hit identification, a term used to identify genes that when silenced have a significant effect on the function being assayed, a number bioinformatic approaches have been described. In one instance, a Z' factor is calculated to determine how distinct the positive and negative controls are from each other, and subsequently, a Z score which is a method of normalization to the mean value of the samples on each plate, is commonly used to rank genes [34]. Candidate genes are then categorized for defining features before being validated. A statistical enrichment of targets relative to a reference population is then ranked for validation. Many parameters may be captured in a screen and these are weighted on importance to the principle screen phenotype and classified into distinct bins to refine the gene list prior to validation. For example, for a shRNA screen, following Next Generation Sequence analysis, data are subjected to a statistical enrichment of targets relative to a reference population, which results in a ranked list to identify targets for validation.

siRNAs and shRNAs

siRNA libraries have been constructed with a variety of designs, and while these reagents have been used in a range of studies and cell types, it is important to consider which of these tools is best suited for host-pathogen screens. One potentially relevant consideration centers on activation of cellular immunity pathways. It is well documented that viral infection is monitored by pathogen recognition receptors (PRRs) that trigger an innate immune response upon identifying an evolutionarily conserved pathogen-associated molecular pattern (PAMP) [35]. For example, recognition of influenza single-stranded RNA genome by the endosomal PRR, Toll-like receptor 7 (TLR7), leads to a strong anti-viral cytokine response [36-38]. Previous studies have also shown that transfection of short dsRNA (such as siRNA) can also perturb cellular immunity. For example, it has been demonstrated that siRNAs longer than ~23-25 base pairs strongly upregulate interferon (IFN) stimulated genes in HeLa cells [39]. Other groups have shown that siRNAs can activate a Type I interferon response, particularly through TLR7 and TLR8 [40, 41]. Thus, if both siRNA and virus can independently stimulate TLR pathways, the question arises of how one can effectively screen for host genes that play a role in virus replication. One solution may lie in reagent selection. For example, while most siRNAs are designed to have complete complementarity to the target transcript, one design variable is duplex length. Short (19 base pair) silencing molecules effectively silence genes but are weaker activators of TLR signaling pathways. As such, these reagents minimize the immune stimulatory effects that would otherwise complicate RNAi screens involving pathogens.

Separately, another noteworthy caveat of all RNAi screening is siRNA specificity. Extensive studies have shown that in addition to targeting the mRNA of interest, siRNAs can act in a similar fashion to miRNAs and attenuate the translation of dozens of genes [42, 43]. False positives (referred to as “off-targets”) induced by this mechanism are frequently mediated by the seed region of the siRNA (nucleotides 2-8), and may result in a 2-4 fold down regulation of unintended targets [43]. Importantly, while off-targets are generally thought to be genes encoded by the cell, in the case of RNAi screens designed to identify host-pathogen interactions, off-targets can also include genes encoded by the pathogen.

Multiple approaches have been developed to minimize the effects of off-targets including chemically modified siRNAs that reduce seed-mediated gene silencing. These siRNAs are frequently used as primary screening reagents and/or as secondary validation tools. Alternatively, in cases where pools of siRNA were used in the primary screen, deconvolution of the pools and demonstration that multiple individual siRNAs (having different seed sequences) give the same phenotype increases the level of confidence that the phenotype is related to the knockdown of the targeted gene. Lastly, bioinformatics can be effectively used to identify off-target effects. Cross-checking the seed sequence of siRNAs that induce a phenotype with the seeds of known miRNAs (host and pathogen) can often identify instances where one or more siRNAs are mimicking a miRNA. While these hits should not be excluded from further study (due to potentially interesting host miRNA-pathogen interactions) they may indicate that the observed phenotype(s) are the result of events unrelated to the knockdown of the intended target gene.

Similar considerations may be relevant when performing screens with shRNA expression cassettes. In most cases shRNAs are generated from DNA-based expression constructs and the predominant collections of shRNA libraries use either lentiviral or retroviral delivery systems to facilitate the entry and integration of the cassette into the host genome [27, 28]. Previous genome profiling studies of lentiviral (HIV) infected CD4+ T cells have shown that infection leads to differential expression of multiple gene categories including those related to complement activation, actin filaments, and proteasome cores [44]. Thus, while the current shRNA platforms are essential for performing host-pathogen studies in cases where 1) the cell type of choice is refractory to lipid mediated transfection, or 2) an extended period of gene knockdown is required, one must consider the potential contributions that delivery platform has on the outcome of host-pathogen screens.

miRNAs

miRNAs have emerged as essential regulators of eukaryotic gene expression [45-48]. Mature miRNA sequences have been found in over 150 species, including viruses, with over 1,000 identified in man [45, 47, 49, 50]. Recent studies have identified both host and viral encoded miRNAs as critical elements regulating virus replication. For example, human miR-122 has been identified as an essential component affecting the biology of hepatitis C virus replication [51]. Interestingly, both pro- and anti-cytomegalovirus (CMV) miRNAs have been identified as encoded by the mouse genome [52]. These and other studies emphasize the need for miRNA screening to accompany siRNA/shRNA screens that target protein coding genes.

Identifying host-encoded miRNAs that are relevant to viral infection generally involves a two-pronged approach. Synthetic miRNAs mimics (or equivalent sets of reagents expressed from e.g., a plasmid or viral-based vector) can be used to increase the cellular concentration of any given miRNA. In contrast, miRNA inhibitors are designed against mature miRNAs and can act as artificial targets to prevent the endogenous miRNA from interacting with its natural targets. [53]. A library of mimics and inhibitors was recently used to identify miRNAs that play a role in diverse herpes virus infections (MCMV, MHV-68, and HSV-1) [52]. By evaluating the phenotype effect induced by mimics and corresponding inhibitors, the study was able to identify four antiviral and three proviral miRNAs that acted across diverse β-herpesviruses. Further analysis implicated the miRNAs in a variety of host signaling networks, including ERK/MAPK and PI3K/AKT signaling, among others [52]. Since these pathways are also implicated in influenza infection [54, 55], these findings suggest that miRNAs might have broad therapeutic potential in prospective disease intervention strategies.

Cell Types

As the scope of many screens is translational, the cell type chosen often has qualities consistent with evaluating druggable, pharmacokinetic, and cytotoxicity properties. As far as is practical, researchers try to use cell types that are most representative of their biology of interest (for example a lung cell line to investigate influenza infection), but full genome wide RNAi screens with primary cells is often impractical. For this reason, most screens utilize immortalized cell lines. In general, these cells are amenable to the standard transfection/transduction procedures used in RNAi screening, but it is recognized that cell lines maintained in culture for long periods of time have significantly altered genomes, epigenomes, and transcriptomes. In truth, these differences may be one of the largest contributors to screen-to-screen variation observed across host-pathogen studies. For this reason, putative hits identified in the primary screen are often validated using counter-screens that employ alternative cell types, related viruses, siRNAs (or shRNAs) targeting different seed sites on the same gene, or small molecule inhibitors known to target the gene of interest. This iterative process is designed to identify candidate genes for in vivo testing and (ultimately) clinical studies.

Finally, it is worth noting that the QA/QC of cell lines is particularly important in host-pathogen screening. It is recognized that cell lines derived from a range of sources can be contaminated with pathogens such as mycoplasma. Given the focus of host-pathogen screens and the possibility that contaminant pathogens can augment the cellular physiology, frequent testing of cell cultures over the course of screening and validation is necessary to minimize the possibility that underlying contamination alters screen outputs.

Viruses

Viruses grow rapidly and are capable of accumulating mutations (e.g. point mutations, deletions) in very short period of time. While DNA viruses are generally more genetically stable than RNA viruses (due to the cellular error correction mechanism for DNA) mutations in either viral class can result in defective progeny (referred to as defective interfering (DI) particles) that lack the necessary complement of genes for infection and replication. As these features can dramatically alter the output and interpretation of an RNAi screen, studies designed to identify host-pathogen interactions should utilize low-passage viral particles from recently isolated stocks.

In one scenario, the virus under investigation may be of serious concern to human and/or animal health, and is therefore subject to high-level biosafety containment. In this case, the virus may require enhanced containment, such as biosafety (BSL)-3 or BSL-4 that utilizes biosafety cabinets, isolators and personal protection equipment including HEPA-filtered exhaust air. To minimize the impact of these restrictions on assay workflow, RNAi screens that utilize high-level pathogens limit the amount of work performed at the highest level of containment by performing siRNA transfections and cell handling in regular BSL-2 laboratories, and moving cells to high bio-containment for the infection phases only. Once the infection period is complete, protocols allow for virus-infected plates to be decontaminated for removal allowing functional readouts to be performed outside containment. Such an approach has been taken to screen chemical compound libraries for inhibitors of Hendra virus [56].

In the absence of bio-containment facilities, one way to study high-risk viruses at lower biosafety levels, e.g. BSL-2 is to use pseudotype viruses. These recombinant model viruses are constructed by replacing the native envelope glycoprotein of a BSL-2 level virus (e.g. vesicular stomatitis virus) with the envelope glycoprotein of the high-risk virus of interest (e.g. Ebola). Pseudotype viruses mimic the viral entry process of the original virus and are competent for a single cycle of infection. The shortcomings associated with using pseudoviral particles are that not all envelope proteins can be incorporated in the carrier virus and their value in investigating post-entry processes is limited. As such, researchers can gain valuable information regarding host-encoded viral receptors and endocytic pathways that facilitate viral entry, but subsequent steps in replication may be masked. Such viruses have been used in vaccine development for highly pathogenic H5N1 influenza virus, Ebola and Lassa hemorrhagic fever virus as means to work around biosafety issues in working with wild type viruses [57-59].

Meta-analysis of RNAi screens for influenza virus

To date, six RNAi screens incorporating variable methodologies and endpoints have been performed to detect host contributions to influenza replication. Some screens focused on early replication events only [31, 33] while others included both early and late events [30, 32, 60]. Furthermore, each screen examined different subsets of host genes and utilized unrelated cell models ranging from known permissive cells [32, 33] to cells that influenza does not infect naturally [30, 31, 60]. The six screens generated a list of potentially relevant host genes that represented approximately 2% of the screened genes (Note: the exception to this was the Shapira study which focused on genes previously implicated in a yeast two-hybrid study, and therefore observed a significantly higher hit rate (35%)[60]. As has been observed in several host-pathogen RNAi screens, the candidates identified across all of the screens had very little overlap, a feature that might be expected given the different methods and reagents employed among the studies. Overall, only three genes were independently validated in four of the six screens, nine genes were validated in three of six screens, and eighty-six genes were validated across two of the screens (Table 5.2).

While the lack of overlap between hits identified across screens appears troubling at first glance, a meta-analysis shows significant overlap in the cellular pathways identified from screen to screen (Table 5.2). This suggests that the identity of specific cellular factors involved in the response to an influenza virus infection is context dependent and influenced by screen-specific experimental variability factors. However, the more significant pathway overlap suggests that a conserved set of core processes are co-opted when an influenza virus infects a mammalian cell, and that the identification of these processes is more robust to experimental variables. These core processes generally involve many of the host cell signaling cascades illustrated in Figure 5.2 and below.

Receptor tyrosine kinases (RTK) signaling: Within the host cell, a major mechanism that transmits extracellular signals to intracellular signaling is the engagement of receptor tyrosine kinases (RTKs) (Figure 5.2). Among the family of RTKs are the group of epidermal growth factor receptors (EGFR), consisting of four members (EGF-, ErbB2-, ErbB3 and ErbB4-receptors) [61]. ErbB expression has been associated with a multitude of cellular functions and responses, including proliferation, cell migration, differentiation and apoptosis [62-65]. Cellular endocytosis of influenza co-opts pathways used by EGFRs, resulting in protein ubiquitinylation and sorting into the vacuolar pathway [66]. In addition, influenza virus particles are sorted into the same population of late endosomes as EGFRs [67]. Specific inhibition of tyrosine kinases by small molecule inhibitors as well as specific EGFR inhibition via RNAi reduces virus uptake and subsequent virus titers [68]. Furthermore, attachment of influenza virus to the host cell causes clustering of plasma membrane lipids, similar to that seen following EGF stimulation. Therefore, upon influenza virus binding to host cell sialic acids, it is able to cluster and activate EGFR and other RTKs to form a lipid raft-based signaling platform [68]. This leads to receptor-mediated signaling events, which enhance influenza virus uptake and subsequent viral replication. It is thought that this activation is not mediated by viral engagement of a particular receptor kinase but is a more general phenomenon that affects several RTKs [68]. This is additionally supported by results of a recent siRNA screening study, which identified the involvement of fibroblast growth factor receptors FGFR 1, 2 and 4 as RTKs in the very early steps of viral infection [33]. Therefore, influenza virus entry accompanied by down-regulation of signaling receptors promotes co-endocytosis of the virus into the host cell.

Protein kinase C signaling: Protein kinase C (PKC) belongs to large family of serine/threonine kinases involved in a multitude of physiological processes [69]. PKC plays an integral role in sodium ion transport, important for maintaining the low pH in the endosome [70-73]. PKC has also been shown to be critical for the entry of enveloped viruses via receptor-mediated endocytosis [74]. Upon influenza virus infection, the hemagglutinin rapidly activates PKC [71, 74] and it has been shown that a specific inhibitor of PKC prevents influenza virus replication by inhibiting the entry of the virus. Similarly, influenza virus replication has also been reported in cells expressing a phosphorylation-deficient form of PKC [75].

Phosphatidylinositol 3-kinase signaling: The family of phosphatidylinositol 3-kinases (PI3Ks) regulates various cellular events, such as cell metabolism, proliferation and survival [76, 77]. The major function of the PI3K is to phosphorylate membrane phospholipids. Upon PI3K activation phosphatidylinositol-3,4,5-triphosphate (PIP3) is generated by phosphorylation of phosphatidylinositol-4,5-bisphosphate which functions as a second messenger through interaction with pleckstrin homology domain-containing proteins such as Akt/PKB and phosphoinositide-dependent kinase (PDK)-1 [76]. Cells treated with inhibitors of PI3K or PIP3 show significantly decreased influenza virus titers [78], suggesting that PI3K performs influenza-supportive functions.

Raf/MEK/ERK signaling: Infection with influenza virus leads to activation of a variety of different MAP-kinase (MAPK) cascades [79-82]. They are activated by a variety of extracellular stimuli such as growths factors, cytokines, and environmental stress factors like osmotic stress or ultraviolet light. Downstream substrates for MAPKs are transcription factors (e.g., ATF2, ELK-1, or c-Jun) and other protein kinases such as the MAP kinase-activated protein kinases MK2 and MK3. Thereby, MAPK pathways regulate a variety of cellular responses such as gene expression, proliferation, differentiation, apoptosis, and immune responses [55, 83, 84].

Influenza ribonucleoprotein (RNP) formation and nuclear export are important steps in the life cycle of influenza virus and data indicate that Raf/MEK/ERK cascade is required for an efficient nuclear RNP export as indicated by several studies [82, 85]. Inhibition of Raf signaling results in nuclear retention of viral RNP and the concomitant inhibition of virus production [82]. Influenza virus HA membrane accumulation and its tight association with lipid-raft domain trigger the activation of MAPK cascades via PKC-α activation and RNP export [86]. HA membrane accumulation is enhanced by the higher polymerase activity of influenza virus, resulting in up-regulation of the MAPK cascade and more efficient nuclear RNP-export, along with virus production [87]. In addition, p38 MAPK and JNK have been shown to regulate the expression of pro-inflammatory cytokines in influenza virus infected cells [79, 88-90].

NF-kB signaling: An important influenza virus-induced signaling mediator is the transcription factor nuclear factor κB (NF-κB). This factor regulates expression of a variety of antiviral cytokines, including IFN-β, which is the initiator of a strong type I IFN defense program [91]. Although NF-κB is generally regarded as a central factor in the innate immune defense [92], two independent studies demonstrated that replication of influenza viruses was impaired rather than enhanced in cells where this pathway was blocked [93, 94]. NF-κB acts via induction of pro-apoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL) or FasL [93], and subsequent activation of caspases [95]. This results in an enhanced export of viral RNPs from the nucleus, presumably by specific cleavage of nuclear pore proteins, resulting in an enhanced diffusion of the RNP through the pores [96, 97]. Lastly, NF-κB differentially regulates viral RNA synthesis [10]. Each of these mechanisms may contribute to a different extent to the enhancing effect of NF-κB on virus propagation, thereby identifying the factor as a potential target for antiviral intervention. Besides the direct antiviral action, NF-κB inhibition may also indirectly influence and in fact exacerbate the pathogenesis of influenza virus, since the majority of cytokines/chemokines induced during infection with highly pathogenic viruses are regulated by NF-κB [91].

To conclude, the host cell pathway overlap identified among RNAi screens for influenza virus (Table 5.2), indicates that core cell signaling processes are co-opted by influenza virus. Variability in specific gene hit lists from different screens is likely explained by variations in the efficacy of knockdown of specific gene targets by different RNAi libraries, variation in the timing of individual screen assays, and other factors related to the assays.

Challenges of RNAi Screening

While RNAi screening has demonstrated enormous potential in improving our understanding of host-pathogen interactions, several challenges remain. As just described, one issue focuses on the lack of overlap associated with hits identified across different screens. Differences in viral subtypes, host cells, assays types and reagents may be responsible for some of the differences, but to date there has been no definitive study to address these discrepancies. Several practices could offset the challenges associated with disparate hit lists. One would be to follow the direction of researchers in the microarray field who have developed a consistent set of minimum information standards complemented with a centralized, publically accessible portal for data submission and review [98, 99]. Minimum Information About an RNAi Experiment (MIARE, http://miare.sourceforge.net/) is a standards concept for RNAi screening that advocates all published screening data include extensive experimental details regarding target cell sources, delivery methods, assay design, plate layout, reagent composition, controls, and metrics of data analysis [100]. In 2008, a database was established for submission of MIARE-compliant RNAi screens. While the ongoing development of the MIARE standards and the co-development of the PubMed database for RNAi screens represents important progress, community wide adoption of MIARE standards and submission policies have not yet been adopted. A revitalized collaborative effort by academic, government, and industrial partners would greatly accelerate the acceptance and implementation of MIARE and thereby facilitate the community's ability to address questions regarding screen reproducibility.

A second practice that could address the issue of disparate hit lists pertains to efforts in hit validation. In cases where siRNA pools are used in the primary screen, deconvolution of the pool and identification of instances where two or more individual siRNAs from a single pool induce the same phenotype have been used in validation [30]. In other cases, validation employs the use of completely different sets of siRNAs targeting a different seed site on the same gene [32, 33], the use shRNAs [28], multiple screening assays [60], multiple viral strains [32, 33, 60], bioinformatics [29], and completely different gene silencing technologies, for example small molecules [101]. Whether hits validated by each of these procedures should be considered equal is open to debate, however adopting a standard validation method may bring greater parity between screens.

Related to techniques employed for hit validation is the criteria used to identify a primary hit. Most screens to date have defined a hit based on a plate mean, or standard deviation from the mean, or relative to a negative control [102]. However in a recent study where 15 separate parameters were used to compile a 'hit signature', there was no improvement in the concordance with previous screens or increase in validation of the hits during secondary experiments [103, 104].

Comparing RNAi screening to analogous technologies

Since a major goal of RNAi host-pathogen screens is to identify host genes and pathways that facilitate viral replication, it is important to note other analogous technologies that have been used to address this question, such as compound screens, microarrays and next generation sequencing and yeast two hybrid screens.

Compound library screens: Small molecule inhibitor and drug compound library screens involve high-throughput analysis of potential pharmacological agents that modulate biological pathways and sometimes specific host genes. A major advantage of compound library screens is the large number of candidate compounds, sometimes in the millions, which come from bioactive, commercial, academic and natural extract libraries which can be screened for anti-viral potential, often without the need for delivery reagents. Although a major limitation is often the lack of information that compound screens provide on the cellular target and mechanisms linked to anti-viral activity, advances in screening technology platforms can compensate. For example, high-content analysis methods that use images of living cells as the basic unit for molecule discovery can track, quantitate and provide qualitative information of the proteins of interest present in the cells using fluorescent tags, such as the green fluorescent protein, or by fluorescent antibodies. Image analysis is then used to measure changes in properties of the cells caused by treatment with candidate compounds such as chemical inhibitors or RNA interference. However, it is important to note that compound library screens are often complicated by the need to perform analyses at multiple compound concentrations to address toxicity concerns as cell death could be mistakenly identified as efficacy. Other problems associated with compound and small molecule library screens include poor solubility of drugs, poor and/or variable cellular uptake, and drug specificity issues [105]. Despite these concerns, a number of compound library screens have been conducted on Madin-Darby canine kidney (MDCK) cells to identify influenza inhibitors and these results have led to follow-up validation studies in human cells [105, 106].

Microarrays and next-generation sequencing: Microarrays have been used extensively to study host pathways implicated in virus life cycles and to inform our understanding of viral pathogenesis [107]. In the first reported microarray study of host-influenza virus interaction, the expression levels of over 4600 genes were measured in HeLa cells in response to live or heat-inactivated influenza A/WSN/33 (H1N1) exposure [108]. Of the 329 differentially expressed genes identified within 8 h of influenza infection, the majority were down-regulated, and could be classified into 5 main groups – protein synthesis, cytokine signaling, ubiquitin pathway factors, mRNA processing and export proteins, and transcription factors. Broad comparisons of the gene families and pathways show a reasonable level of complementarity with those identified more recently by RNAi screening. Experiments performed using microarray technology have identified a number of host pathways that are impacted by a range of viruses, including HIV [109], human papillomavirus [110] and herpes simplex virus [111], and by treatments with IFNs -α, -β and -γ [112]. These studies have provided valuable information regarding host pathways and have provided new candidates for anti-viral therapies.

Despite the large amount of microarray data and infrastructure available to researchers, it appears that next generation sequencing (NGS) may supersede this technology due mainly to improvements in transcriptome coverage, sensitivity, and resolution. Since microarrays are limited by the genome coverage of their probe sets, NGS has the advantage of identifying expression changes of poorly characterized transcripts, particularly those from the small non-coding RNA families. NGS-generated genome-wide expression profiles of host cells are emerging for a number of viruses, including Mimivirus [113], vaccinia virus [114] and HIV [115]. These studies provide a global view of how the host genome is impacted by viral infection, in addition to changes in virus gene expression levels during infection. NGS analyses of host cells in response to influenza infection are eagerly anticipated. An important distinction is the information based on loss-of-function resulting from RNAi-mediated gene silencing. Microarray and sequencing approaches, while providing detailed readouts on fold changes in gene expression, may not determine the importance of specific genes in biological processes, and may ignore important genes and pathways if low level changes in expression are observed following infection.

Yeast two-hybrid screens: Yeast two-hybrid (Y2H) technology allows screening of protein-protein and protein-DNA interactions [116]. Y2H screens have identified a protein-protein “interactome” network for Epstein-Barr virus [117], SARS-like coronavirus [118] and Hepatitis C virus [119] infections. A comprehensive study has recently been conducted where intra-viral protein-protein interactions were analyzed by Y2H technology for 10 influenza virus (A/PR/8/34) proteins, in addition to interactions between these viral proteins with 12,000 human proteins from the Human ORFeome [60]. While the influenza NS1 gene segment is known to interact with host pathways to disrupt antiviral actions (reviewed in [17], numerous novel contacts of host proteins with PB1, PB2 and NP influenza gene segments were identified. This study elegantly combined Y2H and RNAi technology to identify host proteins and pathways involved in physical recognition of influenza proteins and the resultant pathways mediating anti-viral responses.

Concluding remarks

The importance of influenza virus to global human and animal health, coupled with the current limitations associated with vaccine production and anti-viral therapeutic pipelines, underscores the need for novel solutions to accelerate the development of virus intervention strategies. Genome-wide RNAi screening is an emerging technology with the power to detail host-virus interactions, furthering our understanding of virus pathogenesis and thereby driving the development of next-generation antivirals. Given the scale of information generated by RNAi screens it is critically important and beneficial to collaborate in this research arena. There are admirable examples of how scientific consortia and centers composed of dispersed networks of laboratories have successfully worked together to achieve significant goals. The Centers for Excellence in Influenza Research and Surveillance and the Human Genome Project provides two examples where intervention strategies are tackled by academic, government, and industrial entities worldwide and represent good examples as to how disparate entities can work in partnership toward a common goal. Similarly, the efforts by organizations such as the RNAi Global Initiative are designed to tackle the challenges represented by EIDs such as influenza to benefit human health. It will be vital to the success of this emerging field that such collaborative initiatives promote the development of data standards and best practices that facilitate the comparison of data from complementary screens. This should lead to a better understanding of the factors that most strongly influence screen reproducibility and data quality, and ultimately improve the potential of RNAi technology to identify optimal antiviral-drug candidates.

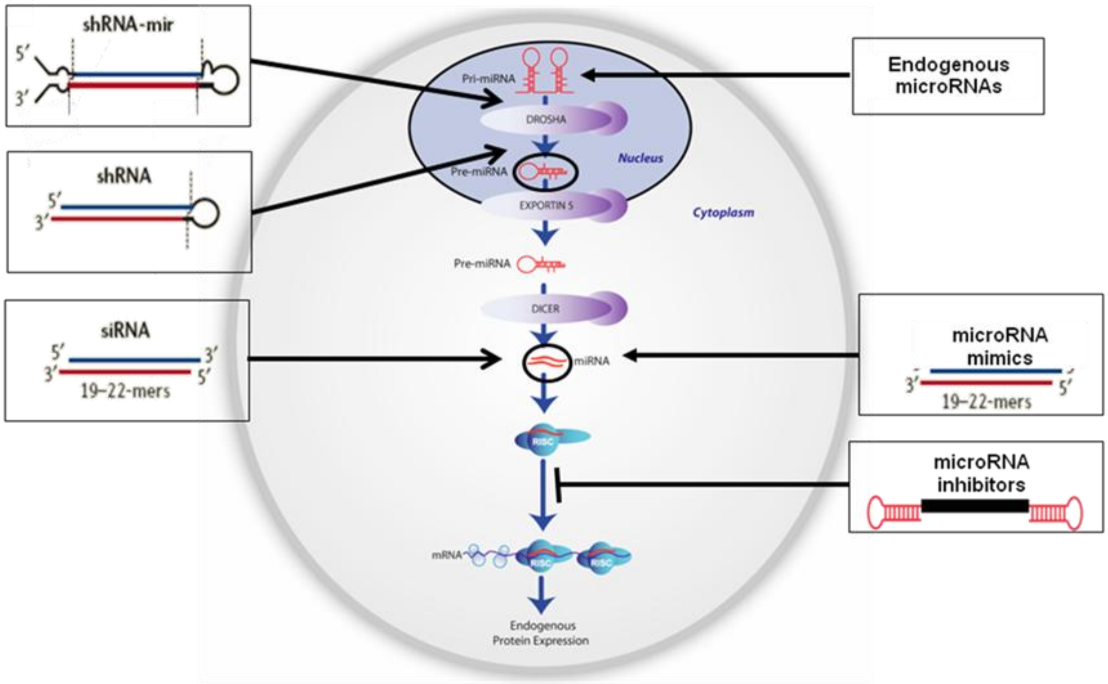

Figure 5.1. The RNAi Pathway. Endogenous miRNAs are transcribed as Pri-miRNAs that are subsequently processed by Drosha and Dicer to generate mature miRNAs. The guide strand is then incorporated into the RNA Induced Silencing Complex (RISC) to facilitate gene silencing. Reagents that have been developed for screening include expressed constructs (shRNA and shRNA-miR), as well as synthetic constructs: siRNA, miRNA mimics, and miRNA inhibitors.

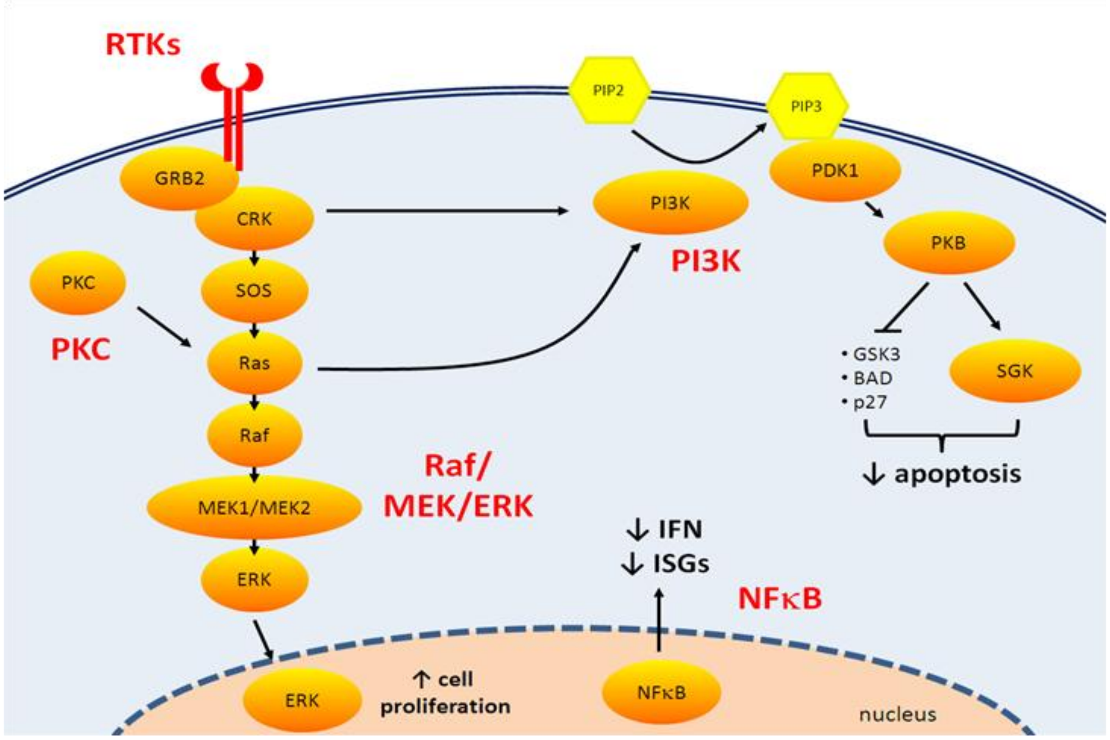

Figure 5.2. Host signaling factors. A: Upon binding to host cell sialic acids, influenza virus activates receptor tyrosine kinases (RTKs) to form lipid raft-based signaling platforms that enhance influenza uptake and cell proliferation. HA-mediated activation of protein kinase C (PKC) also aids viral entry and cellular proliferation. RTKs activate phosphatidylinositol 3-kinase (PI3K) signaling, inhibiting cellular apoptosis. The Raf/MEK/ERK cascade downstream of RTKs is required for efficient nuclear export of viral ribonucleoproteins. Viral manipulation of NF-kB inhibits anti-viral type I interferon production and resultant interferon-stimulated gene (ISG) production.

Table 5.1 Comparison of the screen stages for shRNA and siRNA screens.

Screen stages	shRNA	siRNA
1. Cell-based optimization	•Define MOI for cell line •Identify +ve, -ve controls •Make virus for individual shRNAs	•Establish transfection conditions •Identify +ve, -ve controls •siGLO and non-targeting control
2. Assay development	•Establish screen phenotype •Verify phenotype of controls in dilution of pools	•Define assay parameters •Robustness, Z'factor •Develop automation •Identify analysis 'rules'
3. Screen	•Transduce cells with library pool •Select with GFP/puromycin •Assay – select reference population •Freeze cells, extract genomic DNA •Amplify gDNA and NGS analysis	•Primary SMARTpool screen •Duplicate or triplicate technical replicates •Analysis ongoing
4. Bioinformatics	•Process NGS data •Statistically rank shRNAs	•Statistical analysis •Define hit list
5. Validation	•Identify individual shRNA hits •Make virus for all constructs per target •Rescreen using same assay •Verify knockdown	•Secondary validation screen •Deconvolute SMARTpools •Same assay or different assay •Additional cell lines
6. Bioinformatics	•Pathway analysis •Data mining	•Pathway analysis •Data mining •Tertiary analysis, more cell lines, different assays

shRNA and siRNA screen stages broken into chronological order. Stages can take different times depending on the assay and quantitation method. Bioinformatics analysis is an ongoing effort that intervenes in the screen process at several points. Central to each screening platform is identification of robust positive (+ve) and negative controls (-ve). For the siRNA platform, siGLO, a fluorescent reporter is used to indicate transfection efficiency.

Table 5.2 Comparison of genome screens for influenza virus.

Screen	Cell line	Influenza virus	Readout	Genes screened	Validated hits	Validation	Reference
siRNA screen (Ambion)	*Drosophila* D-Mel2	Recombinant A/WSN/33	Luciferase activity	13,071	121 (110 ↓[a] and 11 ↑[b])	Decreased luciferase expression in two replicates, inhibiting ≥ mean ± 2.5 s.d. in at least one replicate, and phenotype consistent when targeted with an alternate dsRNA amplicon	[31]
siRNA screen (Dharmacon)	Human U2OS	A/PR/8/34 H1N1	HA immunostain	17,877	260 (250 ↓[a] and 10 ↑[b])	Rescreen with individual siRNAs from pool	[30]
virus-host direct interactions (Y2H), transcriptional responses (microarray), and pathway association (IPA)	Human HBEC	A/PR/8/34, ΔNS1[c], or vRNA	Infectious virus or IFN□□production	1745	616	siRNA to candidate gene affected the phenotype in ≥ 1 of 3 functional assays	[60]
siRNA screen (Qiagen, Invitrogen, IDT)	Human A549	Recombinant A/WSN/33, SOIV A/NL/602/09	Luciferase activity	19,628	295 (295 WSN, 12 SOIV)	≥ 2 unique siRNAs to candidate gene reduced viral infection by 35% or more	[33]
siRNA screen (Qiagen)	Human A549	A/WSN/33, SOIV A/Hamburg/04, HPAI A/VN/1203/04	Infectious virus quantified using a 293T cell reporter system and NP immunostain; viral replication measured by titrating A549 supernatant on MDCK cells.	22,843	168 (119 WSN, 121 SOIV, 6 HPAI)	≥ 2 unique siRNAs to candidate gene decreased virus replication by more than fivefold	[32]
siRNA screen (Dharmacon)	Human A549	A/WSN/33	Amount of infectious virus, NP expression, M gene levels	1201	28 (25 ↓[a] and 3 ↑[b])	Phenotype is emulated using a novel siRNA targeting a different seed region of the hit gene	[120, 121]

[a] ↓, hits that decreased virus replication. [b] ↑, hits that increased virus replication. [c] ΔNS1, PR8 virus lacking the non-structural gene.

HA, hemagglutinin; NA, neuraminidase; VSV-G, vesicular stomatitis virus glycoprotein G; eGFP, enhanced green fluorescence protein; HBEC, human bronchial epithelial cells; NP, influenza virus nucleoprotein; M, influenza virus matrix protein; Y2H, yeast two-hybrid; IPA, Ingenuity Pathway Analysis; vRNA, viral RNA; IFN, interferon; SOIV, swine-origin influenza virus; HPAI, highly pathogenic avian-origin influenza virus; MDCK, Madin-Darby canine kidney.

References

1. Daszak, P., A.A. Cunningham, and A.D. Hyatt, *Emerging infectious diseases of wildlife--threats to biodiversity and human health.* Science, 2000. **287**(5452): p. 443-9.
2. Taubenberger, J.K. and D.M. Morens, *Influenza: the once and future pandemic.* Public health reports, 2010. **125 Suppl 3**: p. 16-26.
3. Komar, N. and B. Olsen, *Avian influenza virus (H5N1) mortality surveillance.* Emerging infectious diseases, 2008. **14**(7): p. 1176-8.
4. WHO, *Overview of pandemic (H1N1) 2009 in the WHO European Region, retrieved 11th March 2010, <http://www.euro.who.int/influenza/AH1N1/20091026_1>.*

. 2009a.

5. WHO, *Seasonal influenza, retrieved 10th March 2010, <http://www.euro.who.int/influenza/20080618_1>*. 2009b.
6. Molinari, N.-A.M., et al., *The annual impact of seasonal influenza in the US: Measuring disease burden and costs.* Vaccine, 2007. **25**(27): p. 5086-5096.
7. Nagata, K., A. Kawaguchi, and T. Naito, *Host factors for replication and transcription of the influenza virus genome.* Reviews in Medical Virology, 2008. **18**(4): p. 247-60.

8. Zhou, Z., et al., *Lysosome-associated membrane glycoprotein 3 is involved in influenza A virus replication in human lung epithelial (A549) cells.* Virology Journal, 2011. **8**: p. 384.
9. Su, B., et al., *Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA).* PLoS One, 2009. **4**(12): p. e8495.
10. Kumar, N., et al., *NF-kappaB signaling differentially regulates influenza virus RNA synthesis.* Journal of Virology, 2008. **82**(20): p. 9880-9.
11. Bruce, E.A., P. Digard, and A.D. Stuart, *The Rab11 Pathway Is Required for Influenza A Virus Budding and Filament Formation.* Journal of Virology, 2010. **84**(12): p. 5848-5859.
12. Rossman, J.S. and R.A. Lamb, *Influenza virus assembly and budding.* Virology, 2011. **411**(2): p. 229-36.
13. Leung, R.K. and P.A. Whittaker, *RNA interference: from gene silencing to gene-specific therapeutics.* Pharmacol Ther, 2005. **107**(2): p. 222-39.
14. Tripp, R.A. and S.M. Tompkins, *Therapeutic applications of RNAi for silencing virus replication.* Methods Mol Biol, 2009. **555**: p. 43-61.
15. Bagasra, O. and K.R. Prilliman, *RNA interference: the molecular immune system.* Journal of molecular histology, 2004. **35**(6): p. 545-53.
16. Blair, C.D., *Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission.* Future microbiology, 2011. **6**(3): p. 265-77.

17. Hale, B.G., et al., *The multifunctional NS1 protein of influenza A viruses.* J Gen Virol, 2008. **89**(Pt 10): p. 2359-76.

18. Kim, D.H. and J.J. Rossi, *Strategies for silencing human disease using RNA interference.* Nat Rev Genet, 2007. **8**(3): p. 173-84.

19. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 126-39.

20. Held, K., et al., *Expression of Herpes Simplex Virus 1-Encoded MicroRNAs in Human Trigeminal Ganglia and Their Relation to Local T-Cell Infiltrates.* Journal of Virology, 2011. **85**(19): p. 9680-5.

21. Tirabassi, R., et al., *Human Cytomegalovirus US7 is Regulated Synergistically by Two Virally-Encoded miRNAs and by Two Distinct Mechanisms.* Journal of Virology, 2011.

22. Pfeffer, S., et al., *Identification of virus-encoded microRNAs.* Science, 2004. **304**(5671): p. 734-6.

23. Hughes, R.A., A.E. Miklos, and A.D. Ellington, *Gene synthesis methods and applications.* Methods in enzymology, 2011. **498**: p. 277-309.

24. Zhang, W. and R.A. Tripp, *RNA interference inhibits respiratory syncytial virus replication and disease pathogenesis without inhibiting priming of the memory immune response.* Journal of Virology, 2008. **82**(24): p. 12221-31.

25. Sakurai, K., P. Chomchan, and J.J. Rossi, *Silencing of gene expression in cultured cells using small interfering RNAs.* Current protocols in cell

biology / editorial board, Juan S. Bonifacino ... [et al.], 2010. **Chapter 27**: p. Unit 27 1 1-28.

26. Snead, N.M. and J.J. Rossi, *Biogenesis and function of endogenous and exogenous siRNAs.* Wiley Interdiscip Rev RNA, 2010. **1**(1): p. 117-31.
27. Blakely, K., T. Ketela, and J. Moffat, *Pooled Lentiviral shRNA Screening for Functional Genomics in Mammalian Cells.* Methods in molecular biology, 2011. **781**: p. 161-82.
28. Gobeil, S., et al., *A genome-wide shRNA screen identifies GAS1 as a novel melanoma metastasis suppressor gene.* Genes Dev, 2008. **22**(21): p. 2932-40.
29. Yeh, M.H., et al., *Lentiviral short hairpin RNA screen of human kinases and phosphatases to identify potential biomarkers in oral squamous cancer cells.* International journal of oncology, 2011. **39**(5): p. 1221-31.
30. Brass, A.L., et al., *The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus.* Cell, 2009. **139**(7): p. 1243-54.
31. Hao, L., et al., *Drosophila RNAi screen identifies host genes important for influenza virus replication.* Nature, 2008. **454**(7206): p. 890-3.
32. Karlas, A., et al., *Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication.* Nature, 2010.
33. Konig, R., et al., *Human host factors required for influenza virus replication.* Nature, 2009. **463**(7282): p. 813-7.

34. Birmingham, A., et al., *Statistical methods for analysis of high-throughput RNA interference screens.* Nat Methods, 2009. **6**(8): p. 569-75.

35. Wu, S., J.P. Metcalf, and W. Wu, *Innate immune response to influenza virus.* Current opinion in infectious diseases, 2011. **24**(3): p. 235-40.

36. Barchet, W., et al., *Dendritic cells respond to influenza virus through TLR7- and PKR-independent pathways.* European journal of immunology, 2005. **35**(1): p. 236-42.

37. Lund, J.M., et al., *Recognition of single-stranded RNA viruses by Toll-like receptor 7.* Proc Natl Acad Sci U S A, 2004. **101**(15): p. 5598-603.

38. Hayden, F.G., et al., *Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense.* The Journal of clinical investigation, 1998. **101**(3): p. 643-9.

39. Kim, D.H., et al., *Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase.* Nature biotechnology, 2004. **22**(3): p. 321-5.

40. Hornung, V., et al., *Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7.* Nature medicine, 2005. **11**(3): p. 263-70.

41. Sioud, M., *Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization.* Journal of molecular biology, 2005. **348**(5): p. 1079-90.

42. Hirsch, A.J., *The use of RNAi-based screens to identify host proteins involved in viral replication.* Future microbiology. **5**(2): p. 303-11.

43. Sigoillot, F.D. and R.W. King, *Vigilance and validation: Keys to success in RNAi screening.* ACS chemical biology, 2011. **6**(1): p. 47-60.

44. Wu, J.Q., et al., *Genome-wide analysis of primary CD4+ and CD8+ T cell transcriptomes shows evidence for a network of enriched pathways associated with HIV disease.* Retrovirology. **8**: p. 18.

45. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**: p. 843-854.

46. Lee, R.C. and V. Ambros, *An extensive class of small RNAs in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 862-4.

47. Lau, N.C., et al., *An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans.* Science, 2001. **294**(5543): p. 858-62.

48. Lagos-Quintana, M., et al., *Identification of novel genes coding for small expressed RNAs.* Science, 2001. **294**(5543): p. 853-8.

49. Griffiths-Jones, S., et al., *miRBase: microRNA sequences, targets and gene nomenclature.* Nucleic Acids Res, 2006. **34**(Database issue): p. D140-4.

50. Griffiths-Jones, S., *The microRNA Registry.* Nucleic Acids Res, 2004. **32**(Database issue): p. D109-11.

51. Jopling, C.L., et al., *Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA.* Science, 2005. **309**(5740): p. 1577-81.

52. Santhakumar, D., et al., *Combined agonist-antagonist genome-wide functional screening identifies broadly active antiviral microRNAs.* Proc Natl Acad Sci U S A, 2010. **107**(31): p. 13830-5.
53. Vermeulen, A., et al., *Double-stranded regions are essential design components of potent inhibitors of RISC function.* RNA, 2007. **13**(5): p. 723-30.
54. Ehrhardt, C., et al., *Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses.* Journal of Virology, 2007. **81**(7): p. 3058-67.
55. Pleschka, S., *RNA viruses and the mitogenic Raf/MEK/ERK signal transduction cascade.* Biol Chem, 2008. **389**(10): p. 1273-82.
56. Porotto, M., et al., *Simulating henipavirus multicycle replication in a screening assay leads to identification of a promising candidate for therapy.* Journal of Virology, 2009. **83**(10): p. 5148-55.
57. Bredenbeek, P.J., et al., *A recombinant Yellow Fever 17D vaccine expressing Lassa virus glycoproteins.* Virology, 2006. **345**(2): p. 299-304.
58. Geisbert, T.W., et al., *Recombinant adenovirus serotype 26 (Ad26) and Ad35 vaccine vectors bypass immunity to Ad5 and protect nonhuman primates against ebolavirus challenge.* Journal of Virology, 2011. **85**(9): p. 4222-33.
59. Zhang, W., et al., *Increase in viral yield in eggs and MDCK cells of reassortant H5N1 vaccine candidate viruses caused by insertion of 38 amino acids into the NA stalk.* Vaccine, 2011.

60. Shapira, S.D., et al., *A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection.* Cell, 2009. **139**(7): p. 1255-67.

61. Prenzel, N., et al., *The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification.* Endocr Relat Cancer, 2001. **8**(1): p. 11-31.

62. Weiss, F.U., et al., *Distinct characteristics of heregulin signals mediated by HER3 or HER4.* J Cell Physiol, 1997. **173**(2): p. 187-95.

63. Jones, F.E., et al., *ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation.* J Cell Biol, 1999. **147**(1): p. 77-88.

64. Kainulainen, V., et al., *A natural ErbB4 isoform that does not activate phosphoinositide 3-kinase mediates proliferation but not survival or chemotaxis.* J Biol Chem, 2000. **275**(12): p. 8641-9.

65. Long, W., et al., *Impaired differentiation and lactational failure of Erbb4-deficient mammary glands identify ERBB4 as an obligate mediator of STAT5.* Development, 2003. **130**(21): p. 5257-68.

66. Khor, R., L.J. McElroy, and G.R. Whittaker, *The ubiquitin-vacuolar protein sorting system is selectively required during entry of influenza virus into host cells.* Traffic, 2003. **4**(12): p. 857-68.

67. Lakadamyali, M., M.J. Rust, and X. Zhuang, *Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes.* Cell, 2006. **124**(5): p. 997-1009.

68. Eierhoff, T., et al., *The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells.* PLoS Pathog. **6**(9).

69. Toker, A., *Signaling through protein kinase C.* Front Biosci, 1998. **3**: p. D1134-47.

70. Hoffmann, H.H., P. Palese, and M.L. Shaw, *Modulation of influenza virus replication by alteration of sodium ion transport and protein kinase C activity.* Antiviral Res, 2008. **80**(2): p. 124-34.

71. Kunzelmann, K., et al., *Influenza virus inhibits amiloride-sensitive Na+ channels in respiratory epithelia.* Proc Natl Acad Sci U S A, 2000. **97**(18): p. 10282-7.

72. Lazrak, A., et al., *Influenza virus M2 protein inhibits epithelial sodium channels by increasing reactive oxygen species.* FASEB J, 2009. **23**(11): p. 3829-42.

73. Sieczkarski, S.B. and G.R. Whittaker, *Characterization of the host cell entry of filamentous influenza virus.* Arch Virol, 2005. **150**(9): p. 1783-96.

74. Constantinescu, S.N., C.D. Cernescu, and L.M. Popescu, *Effects of protein kinase C inhibitors on viral entry and infectivity.* FEBS Lett, 1991. **292**(1-2): p. 31-3.

75. Root, C.N., et al., *Entry of influenza viruses into cells is inhibited by a highly specific protein kinase C inhibitor.* J Gen Virol, 2000. **81**(Pt 11): p. 2697-705.

76. Neri, L.M., et al., *The nuclear phosphoinositide 3-kinase/AKT pathway: a new second messenger system.* Biochim Biophys Acta, 2002. **1584**(2-3): p. 73-80.

77. Vanhaesebroeck, B., et al., *Signalling by PI3K isoforms: insights from gene-targeted mice.* Trends Biochem Sci, 2005. **30**(4): p. 194-204.

78. Ehrhardt, C., et al., *Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence.* Cell Microbiol, 2006. **8**(8): p. 1336-48.

79. Kujime, K., et al., *p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells.* J Immunol, 2000. **164**(6): p. 3222-8.

80. Ludwig, S., et al., *Ringing the alarm bells: signalling and apoptosis in influenza virus infected cells.* Cell Microbiol, 2006. **8**(3): p. 375-86.

81. Ludwig, S., et al., *Influenza-virus-induced signaling cascades: targets for antiviral therapy?* Trends Mol Med, 2003. **9**(2): p. 46-52.

82. Pleschka, S., et al., *Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade.* Nat Cell Biol, 2001. **3**(3): p. 301-5.

83. Pearson, G., et al., *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev, 2001. **22**(2): p. 153-83.

84. Dong, C., R.J. Davis, and R.A. Flavell, *MAP kinases in the immune response.* Annu Rev Immunol, 2002. **20**: p. 55-72.

85. Ludwig, S., et al., *MEK inhibition impairs influenza B virus propagation without emergence of resistant variants.* Febs Letters, 2004. **561**(1-3): p. 37-43.

86. Marjuki, H., et al., *Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Calpha-mediated activation of ERK signaling.* J Biol Chem, 2006. **281**(24): p. 16707-15.

87. Marjuki, H., et al., *Higher polymerase activity of a human influenza virus enhances activation of the hemagglutinin-induced Raf/MEK/ERK signal cascade.* Virol J, 2007. **4**: p. 134.

88. Guillot, L., et al., *Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus.* J Biol Chem, 2005. **280**(7): p. 5571-80.

89. Karin, M., Z. Liu, and E. Zandi, *AP-1 function and regulation.* Curr Opin Cell Biol, 1997. **9**(2): p. 240-6.

90. Ludwig, S., et al., *The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors.* J Virol, 2002. **76**(21): p. 11166-71.

91. Pahl, H.L., *Activators and target genes of Rel/NF-kappaB transcription factors.* Oncogene, 1999. **18**(49): p. 6853-66.

92. Chu, W.M., et al., *JNK2 and IKKbeta are required for activating the innate response to viral infection.* Immunity, 1999. **11**(6): p. 721-31.

93. Wurzer, W.J., et al., *NF-kappaB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation.* J Biol Chem, 2004. **279**(30): p. 30931-7.

94. Nimmerjahn, F., et al., *Active NF-kappaB signalling is a prerequisite for influenza virus infection.* J Gen Virol, 2004. **85**(Pt 8): p. 2347-56.

95. Wurzer, W.J., et al., *Caspase 3 activation is essential for efficient influenza virus propagation.* EMBO J, 2003. **22**(11): p. 2717-28.

96. Kramer, A., et al., *Apoptosis leads to a degradation of vital components of active nuclear transport and a dissociation of the nuclear lamina.* Proc Natl Acad Sci U S A, 2008. **105**(32): p. 11236-41.

97. Faleiro, L. and Y. Lazebnik, *Caspases disrupt the nuclear-cytoplasmic barrier.* J Cell Biol, 2000. **151**(5): p. 951-9.

98. Brazma, A., et al., *Minimum information about a microarray experiment (MIAME)-toward standards for microarray data.* Nature genetics, 2001. **29**(4): p. 365-71.

99. Brazma, A., *Minimum Information About a Microarray Experiment (MIAME)--successes, failures, challenges.* TheScientificWorldJournal, 2009. **9**: p. 420-3.

100. Haney, S.A., *Increasing the robustness and validity of RNAi screens.* Pharmacogenomics, 2007. **8**(8): p. 1037-49.

101. Schulte, J., et al., *High-Content Chemical and RNAi Screens for Suppressors of Neurotoxicity in a Huntington's Disease Model.* PLoS One, 2011. **6**(8): p. e23841.

102. Pache, L., R. Konig, and S.K. Chanda, *Identifying HIV-1 host cell factors by genome-scale RNAi screening.* Methods, 2011. **53**(1): p. 3-12.

103. Genovesio, A., et al., *Visual genome-wide RNAi screening to identify human host factors required for Trypanosoma cruzi infection.* PLoS One, 2011. **6**(5): p. e19733.

104. Genovesio, A., et al., *Automated Genome-Wide Visual Profiling of Cellular Proteins Involved in HIV Infection.* J Biomol Screen, 2011. **16**(9): p. 945-58.

105. Severson, W.E., et al., *High-throughput screening of a 100,000-compound library for inhibitors of influenza A virus (H3N2).* J Biomol Screen, 2008. **13**(9): p. 879-87.

106. Shih, S.R., et al., *Pyrazole compound BPR1P0034 with potent and selective anti-influenza virus activity.* J Biomed Sci. **17**: p. 13.

107. Borjabad, A., et al., *Significant Effects of Antiretroviral Therapy on Global Gene Expression in Brain Tissues of Patients with HIV-1-Associated Neurocognitive Disorders.* PLoS pathogens, 2011. **7**(9): p. e1002213.

108. Geiss, G.K., et al., *Global impact of influenza virus on cellular pathways is mediated by both replication-dependent and -independent events.* J Virol, 2001. **75**(9): p. 4321-31.

109. Geiss, G.K., et al., *Large-scale monitoring of host cell gene expression during HIV-1 infection using cDNA microarrays.* Virology, 2000. **266**(1): p. 8-16.

110. Chang, Y.E. and L.A. Laimins, *Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31.* J Virol, 2000. **74**(9): p. 4174-82.

111. Khodarev, N.N., et al., *Accumulation of specific RNAs encoding transcriptional factors and stress response proteins against a background of severe depletion of cellular RNAs in cells infected with herpes simplex virus 1.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 12062-7.

112. Der, S.D., et al., *Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15623-8.

113. Legendre, M., et al., *mRNA deep sequencing reveals 75 new genes and a complex transcriptional landscape in Mimivirus.* Genome Res. **20**(5): p. 664-74.

114. Yang, Z., et al., *Simultaneous high-resolution analysis of vaccinia virus and host cell transcriptomes by deep RNA sequencing.* Proc Natl Acad Sci U S A. **107**(25): p. 11513-8.

115. Lefebvre, G., et al., *Analysis of HIV-1 expression level and sense of transcription by high-throughput sequencing of the infected cell.* J Virol. **85**(13): p. 6205-11.

116. Fields, S. and O. Song, *A novel genetic system to detect protein-protein interactions.* Nature, 1989. **340**(6230): p. 245-6.

117. Calderwood, M.A., et al., *Epstein-Barr virus and virus human protein interaction maps.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7606-11.

118. von Brunn, A., et al., *Analysis of intraviral protein-protein interactions of the SARS coronavirus ORFeome.* PLoS One, 2007. **2**(5): p. e459.

119. de Chassey, B., et al., *Hepatitis C virus infection protein network.* Mol Syst Biol, 2008. **4**: p. 230.

120. Andersen, L.E., Meliopoulos, V. A., Bakre, A., Coleman, J. K, Yan, X., Brooks, P., Crabtree, J., Tompkins, S. M., Tripp, R. A., *MicroRNA Regulation of Human Protein Kinase Genes Required for Influenza Virus Replication.* PloS Pathogens, submitted.

121. Meliopoulos, V.A., Andersen, L. E., Brooks, P., Yan, X., Bakre, A., Coleman, J. K, Brooks, P., Tompkins, S. M., Tripp, R. A., *MicroRNA Regulation of Human Protease Genes Essential for Influenza Replication.* PLoS One, in press.

CHAPTER 6

CONCLUSIONS

As noted in the Introduction, Influenza viruses have evolved to manipulate host barriers and are known to hijack host cell processes and parts to facilitate its replication. Influenza virus infection poses substantial burden to individuals around the world every year. Seasonal vaccination is the current most effective means to control the disease. Although antivirals do exist, they are largely ineffective due to the pressure put on the virus causing it to mutate. In theory, administering an antiviral drug targeting an essential host function for the virus would circumvent this dilemma, provided the antiviral target is not needed for host survival. However, understanding the viral and host interactions that are involved during viral replication is critically needed to properly implement vaccine design and disease intervention strategies, including antivirals.

The experiments completed in this study aim to identify human host kinase genes important for influenza virus replication and understand their regulation by miRNAs. The hypothesis addressed was that host genes involved in protein kinase signaling pathways are critical for influenza virus replication and together, with their miRNA regulators, provide novel therapeutic targets that may provide a disease intervention approach to reduce or prevent influenza virus replication. The specific aims that addressed the hypothesis were:

Specific Aim 1. To identify host genes in the human kinome required for influenza replication. The working hypothesis is that influenza virus requires host protein kinase gene expression to support virus replication, and these genes can be identified using siRNAs to selectively silence individual host kinase genes. The data show in chapter 3, that 22 out of 720 human protein kinase genes were modulated during A/WSN/33 influenza virus replication. In chapter 4, 17 of the 22 (NPR2, MAP3K1, DYRK3, EPHA6, TPK1, PDK2, EXOSC10, NEK8, PLK4, SGK3, NEK3, PANK4, ITPKB, CDC2L5, CALM2, PKN3, and HK2) were validated to be important for WSN replication by three different endpoints, infectious virus levels by $TCID_{50}$, influenza M gene levels, and influenza NP localization. Of these, 6 of the 17 kinases validated (CDC2L5, HK2, NEK3, PANK4, PLK4 and SGK3) were also important for A/New Caledonia influenza virus.

Specific Aim 2. To determine cellular pathways associated with kinase genes identified as important for influenza virus replication. The working hypothesis is that influenza virus co-opts kinase gene pathways to facilitate replication. The data show in chapter 4, that overlapping pathways include the factors FOXO, NFAT, and p53 damage pathways. As discussed in chapter 5, these were also common among other genome-screens for influenza virus.

Specific Aim 3. To understand host protein kinase gene regulation related to microRNAs (miRNA) expression. The working hypothesis is that host cell miRNA regulates host protein kinase genes providing an additional mechanism that affects influenza virus replication. The data show in chapter 5 that miR-148a and miR-181b target CDC2L5 and SGK3, respectively, and likely have a role in regulating influenza virus replication. Although not significant, miR-373 and miR-512-3p may act in a similar fashion by targeting NEK8 and miR-155 by targeting SGK3. However, this needs to be investigated further.

Together, these data advance our understanding of miRNA regulation of genes critical for virus replication and are important for development novel influenza intervention strategies. The importance of influenza virus to global human and animal health, coupled with the current limitations associated with vaccine production and anti-viral therapeutic pipelines, underscores the need for novel solutions to accelerate the development of virus intervention strategies. Genome-wide RNAi screening is an emerging technology with the power to detail host-virus interactions, furthering our understanding of virus pathogenesis and thereby driving the development of next-generation antivirals. The features of the virus-host interface are becoming clearer, and this study together with others has dissected some of the host components that affect influenza virus replication. Notably, there remains a gap in our understanding of the role of miRNA regulation of host genes, and how this interaction affects intracellular signaling pathways used during viral infection and replication; however, this study provides a framework for future studies. The findings reported here contribute toward a better understanding of host-pathogen interactions which may help in accelerating the rational design of therapeutics aimed to control influenza infection and disease pathogenesis.

APPENDICES

SUPPLEMENTAL DATA

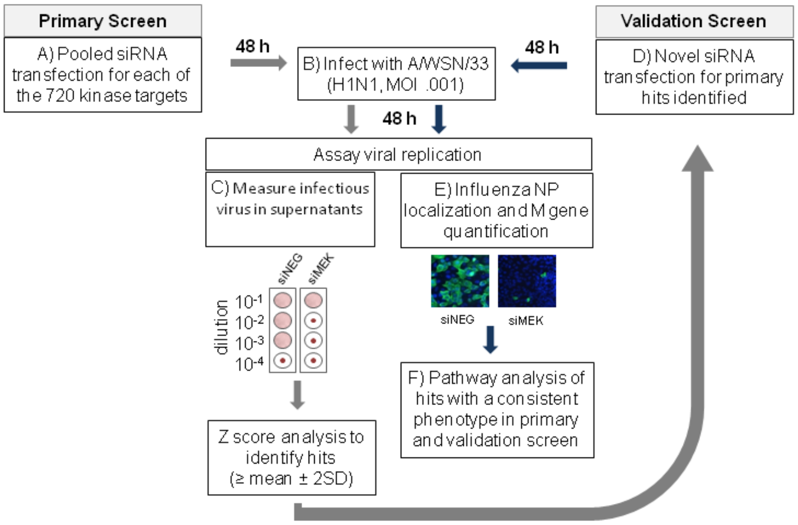

Figure S1. RNA interference screen strategy for identification of host factors affecting influenza infection. A) A549 cells were plated onto lyophilized siRNAs in 96-well flat-bottom plates and transiently transfected for 48h with 50 nM siRNA. B) At 48 hours post-transfection, cells were infected with influenza virus A/WSN/33 (MOI 0.001). C) 48 hours post-infection, viral replication was assayed by titration of A549 cell supernatant on MDCK cells. Each siRNA given a score based on the number of wells with detectable virus, and primary hits determined using Z score analysis. D) Those hits were then validated using a novel siRNA to repeat the screen and E) phenotype was confirmed by influenza NP localization as well as assaying influenza viral genome replication via quantitative real time PCR detecting influenza M gene. F) Last, validated gene hits were associated with the cellular pathways they affect or intersect. MDCK, Madin-Darby Canine Kidney; SD, standard deviation; siNEG, non-target negative control siRNA; siMEK, mitogen-activated protein kinase kinase 1 siRNA positive control.

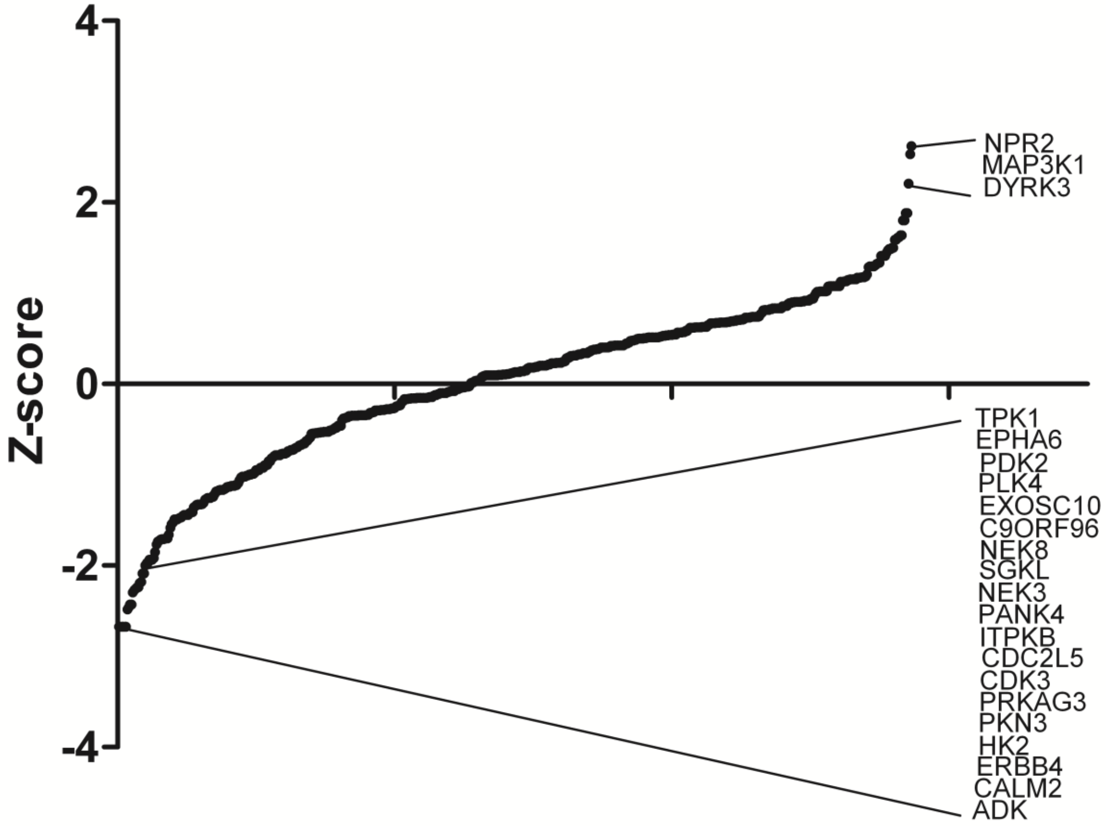

Figure S2. Primary screen identified 22 kinase factors associated with influenza infection. Calculated Z scores of the human kinase library identified primary hits (z scores ≥ 2 and ≤ -2) where silencing increased virus replication (positive Z score) and strongest hits that decreased virus replication (negative Z score). The position of each cellular kinase gene identified important for virus replication in the primary screen are indicated.

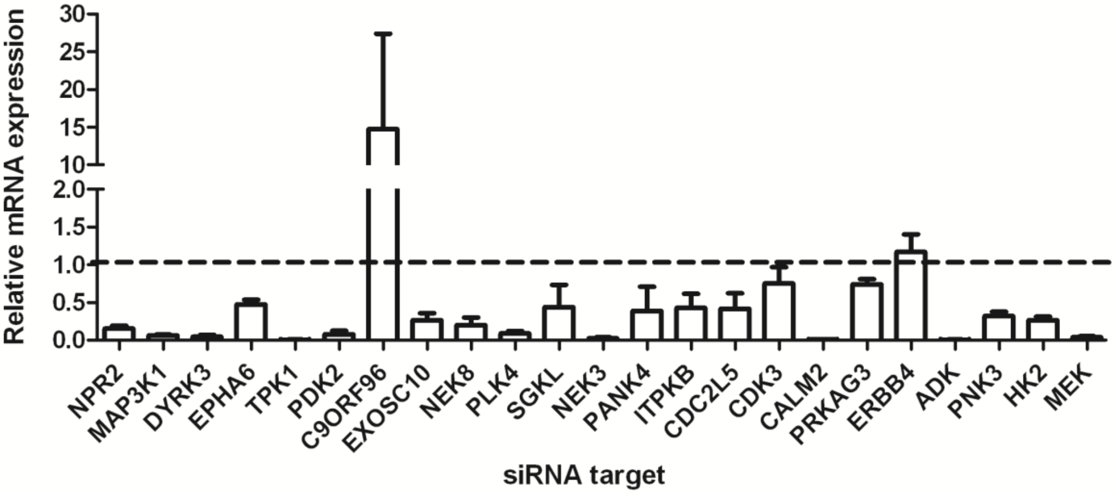

Figure S3. Quantitative PCR based validation of the knock down of genes targeted by siRNA. After 48 hours of silencing with siRNAs (50nM) in A549 cells, total RNA was extracted and used for quantification. The transcript copies divided by GAPDH of gene silenced cells normalized to the same values of non-target control siRNA transfected samples. CDK3, ERBB4, PRKAG3, and C9ORF96 were excluded from further studies because novel siRNAs were suboptimal at silencing. Values of control transfected cells are set as 0% silencing. The results are expressed as mean ± SEM from a representative experiment performed in triplicate.

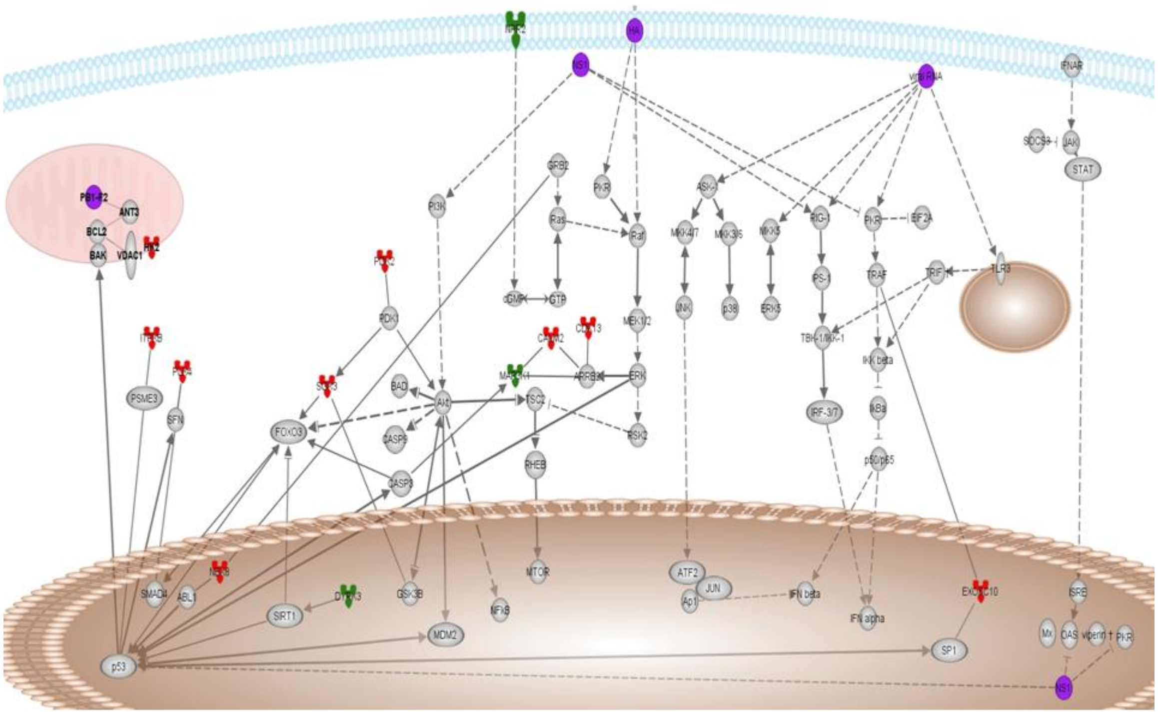

Figure S4. Cellular map of key biological interactions and relevance for influenza. To understand the network of host–pathogen interactions that govern the early steps of influenza virus replication, we used several protein interaction data sets to construct a host–pathogen interaction map depicting associations between the identified host factors, viral-encoded proteins, and other cellular proteins. Purple indicates influenza proteins, red indicates human kinase genes where silencing decreased influenza virus replication in the current screen, green indicates human kinase genes where silencing increased influenza virus replication in the current screen, and gray is other.

Table S1. Dharmacon siRNA sequences.

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_014911	AAK1	22848	
NM_001080395	AATK	9625	
NM_005157	ABL1	25	
NM_005158	ABL2	25	
NM_001105	ACVR1	27	
NM_004302	ACVR1B	90	
NM_001111033	ACVR1C	91	
NM_001616	ACVR2	130399	
NM_001106	ACVR2B	92	
NM_001077401	ACVRL1	93	
NM_020421	ADCK1	94	
NM_052853	ADCK2	57143	
NM_024876	ADCK4	57143	
NM_174922	ADCK5	90956	
NM_001123	ADK	79934	GAAUCUAUCUGCACCGUU
NM_031284	ADP-GK	203054	
NM_001619	ADRBK1	132	
NM_005160	ADRBK2	156	
NM_012301	AIP1	157	
NM_000476	AK1	9863	
NM_001625	AK2	9863	
NM_013410	AK3	204	
NM_016282	AK3L1	205	
NM_012093	AK5	50808	
NM_152327	AK7	26289	
NM_001014431	AKT1	208	
NM_001626	AKT2	208	
NM_005465	AKT3	10000	
NM_004304	ALK	238	
NM_018571	ALS2CR2	55437	
NM_139158	ALS2CR7	269	
NM_020547	AMHR2	54101	
NM_178510	ANKK1	54101	
NM_020639	ANKRD3	369	
NM_001654	ARAF1	9891	
NM_014840	ARK5	10926	
NM_006716	ASK	472	
NM_138292	ATM	545	
NM_001184	ATR	6790	
NM_198437	AURKA	9212	
NM_004217	AURKB	558	
NM_001015879	AURKC	558	
NM_001699	AXL	9223	
NM_004742	BAIAP1	10295	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_005881	BCKDK	10295	
NM_021574	BCR	613	
NM_001715	BLK	640	
NM_017593	BMP2K	640	
NM_004329	BMPR1A	657	
NM_001203	BMPR1B	658	
NM_001204	BMPR2	659	
NM_001721	BMX	660	
NM_004333	BRAF	673	
NM_001113182	BRD2	6046	
NM_007371	BRD3	8019	
NM_014299	BRD4	23476	
NM_001726	BRDT	676	
NM_000061	BTK	695	
NM_004336	BUB1	695	
NM_001211	BUB1B	699	
NM_153336	C10ORF89	699	
NM_174944	C14ORF20	701	
NM_022458	C7ORF2	118672	
NM_022755	C9ORF12	283629	
NM_153710	C9ORF96	64327	UAAAGGACGUGGUGCACAU
NM_006888	CALM1	64768	
NM_001743	CALM2	169436	GUUAACAGAUGAAGAAGUU
NM_005184	CALM3	805	
NM_003656	CAMK1	808	
NM_020397	CAMK1D	8536	
NM_020439	CAMK1G	57118	
NM_015981	CAMK2A	57172	
NM_172084	CAMK2B	57172	
NM_001221	CAMK2D	815	
NM_001222	CAMK2G	816	
NM_001744	CAMK4	817	
NM_018584	CAMKIINALPHA	814	
NM_172207	CAMKK1	84254	
NM_172215	CAMKK2	23729	
NM_013276	CARKL	8573	
NM_003688	CASK	23552	
NM_012119	CCRK	23552	
NM_030911	CDADC1	81602	
NM_001786	CDC2	81602	
NM_033487	CDC2L1	983	
NM_033529	CDC2L2	984	
NM_031267	CDC2L5	728642	GAAGAAAGUCGGCCGUAUA
NM_014826	CDC42BPA	8621	
NM_006035	CDC42BPB	8476	
NM_003503	CDC7	9578	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_052987	CDK10	9578	
NM_015076	CDK11	8558	
NM_001798	CDK2	23097	
NM_001258	CDK3	1017	GAAGAUCAGACUGGAUUUG
NM_000075	CDK4	1018	
NM_004935	CDK5	1019	
NM_003885	CDK5R1	1019	
NM_003936	CDK5R2	1020	
NM_001259	CDK6	8851	
NM_001799	CDK7	8941	
NM_001260	CDK8	1021	
NM_001261	CDK9	1022	
NM_004196	CDKL1	1024	
NM_003948	CDKL2	1024	
NM_016508	CDKL3	1025	
NM_001009565	CDKL4	8814	
NM_001037343	CDKL5	8999	
NM_000389	CDKN1A	51265	
NM_004064	CDKN1B	344387	
NM_000076	CDKN1C	6792	
NM_004936	CDKN2B	1026	
NM_001262	CDKN2C	1027	
NM_079421	CDKN2D	1028	
NM_022766	CERK	1030	
NM_001274	CHEK1	1031	
NM_145862	CHEK2	1032	
NM_212469	CHKA	64781	
NM_005198	CHKB	1111	
NM_001278	CHUK	11200	
NM_006383	CIB2	1119	
NM_007174	CIT	1120	
NM_001823	CKB	1147	
NM_001824	CKM	10518	
NM_020990	CKMT1B	11113	
NM_001099735	CKMT2	1152	
NM_001826	CKS1B	1158	
NM_001827	CKS2	1160	
NM_004071	CLK1	1164	
NM_003993	CLK2	1196	
NM_001292	CLK3	1198	
NM_020666	CLK4	57396	
NM_025233	COASY	80347	
NM_005713	COL4A3BP	10087	
NM_012071	COMMD3	23412	
NM_003909	CPNE3	8895	
NM_016441	CRIM1	51232	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_015083	CRK7	51755	
NM_005207	CRKL	1399	
NM_005211	CSF1R	1436	
NM_004383	CSK	1436	
NM_001892	CSNK1A1	1445	
NM_145203	CSNK1A1L	1452	
NM_001893	CSNK1D	122011	
NM_001894	CSNK1E	1453	
NM_022048	CSNK1G1	1454	
NM_001319	CSNK1G2	1454	
NM_001044722	CSNK1G3	1455	
NM_177560	CSNK2A1	1456	
NM_001896	CSNK2A2	1457	
NM_001320	CSNK2B	1459	
NM_004938	DAPK1	1460	
NM_014326	DAPK2	1612	
NM_001348	DAPK3	1612	
NM_004734	DCAMKL1	23604	
NM_000788	DCK	1613	
NM_013994	DDR1	9201	
NM_006182	DDR2	1633	
NM_201554	DGKA	780	
NM_004080	DGKB	4921	
NM_003648	DGKD	1606	
NM_001080745	DGKG	8527	
NM_152910	DGKH	1608	
NM_004717	DGKI	1608	
NM_001013742	DGKK	160851	
NM_001347	DGKQ	9162	
NM_080918	DGUOK	139189	
NM_001099436	DKFZP434C131	139189	
NM_001080826	DKFZP761P0423	1609	
NM_001098424	DLG1	1609	
NM_001364	DLG2	1716	
NM_020730	DLG3	25989	
NM_001365	DLG4	157285	
NM_001081562	DMPK	1739	
NM_012145	DTYMK	1740	
NM_022076	DUSP21	1741	
NM_199462	DUSTYPK	1742	
NM_130438	DYRK1A	1742	
NM_004714	DYRK1B	1760	
NM_003583	DYRK2	1841	
NM_003582	DYRK3	63904	GGAAUAGCCAAUAAGCUUA
NM_003845	DYRK4	25778	
NM_013302	EEF2K	1859	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_004952	EFNA3	9149	
NM_005227	EFNA4	8445	
NM_001962	EFNA5	8445	
NM_001406	EFNB3	8444	
NM_201283	EGFR	1944	
NM_004836	EIF2AK3	1944	
NM_001013703	EIF2AK4	1945	
NM_005232	EPHA1	1945	
NM_173641	EPHA10	1946	
NM_004431	EPHA2	1949	
NM_182644	EPHA3	1956	
NM_004438	EPHA4	9451	
NM_182472	EPHA5	440275	
NM_173655	EPHA6	2041	CGGAAUAUACUGGUCAAUA
NM_004440	EPHA7	1969	
NM_001006943	EPHA8	2042	
NM_004441	EPHB1	2043	
NM_017449	EPHB2	285220	
NM_004443	EPHB3	2045	
NM_004444	EPHB4	2046	
NM_004445	EPHB6	2047	
NM_004448	ERBB2	2048	
NM_001005915	ERBB3	2049	
NM_001042599	ERBB4	2050	GCAAGAAUUGACUCGAAUA
NM_139021	ERK8	2051	
NM_001433	ERN1	2064	
NM_033266	ERN2	2064	
NM_001039481	ETNK1	2065	
NM_002685	EXOSC10	2066	ACGAAAAGCUCUUGAAUUG
NM_006712	FASTK	2081	
NM_005246	FER	10595	
NM_002005	FES	5394	
NM_023108	FGFR1	10922	
NM_000141	FGFR2	10922	
NM_000142	FGFR3	2241	
NM_002011	FGFR4	2242	
NM_021923	FGFRL1	2260	
NM_001042729	FGR	2260	
NM_018208	FLJ10761	2263	
NM_023018	FLJ13052	2264	
NM_001018046	FLJ23074	53834	
NM_032237	FLJ23356	55224	
NM_144610	FLJ25006	55224	
NM_152534	FLJ32685	65220	
NM_152649	FLJ34389	80122	
NM_002019	FLT1	84197	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_004119	FLT3	84197	
NM_002020	FLT4	84197	
NM_022158	FN3K	124923	
NM_024619	FN3KRP	197259	
NM_004958	FRAP1	2321	
NM_000144	FRDA	2322	
NM_002031	FRK	2322	
NM_145059	FUK	2324	
NM_153048	FYN	64122	
NM_005255	GAK	79672	
NM_000154	GALK1	79672	
NM_002044	GALK2	2395	
NM_000162	GCK	2444	
NM_000167	GK	197258	
NM_033214	GK2	2534	
NM_005476	GNE	2580	
NM_005113	GOLGA5	2584	
NM_002929	GRK1	2585	
NM_001004057	GRK4	2645	
NM_005308	GRK5	2710	
NM_001004105	GRK6	2712	
NM_139209	GRK7	10020	
NM_031965	GSG2	6011	
NM_019884	GSK3A	2868	
NM_002093	GSK3B	2869	
NM_005316	GTF2H1	2870	
NM_004963	GUCY2C	131890	
NM_000180	GUCY2D	83903	
NM_001522	GUCY2F	2931	
NM_000858	GUK1	2931	
NM_052947	HAK	2932	
NM_002110	HCK	2965	
NM_152696	HIPK1	2984	
NM_022740	HIPK2	2986	
NM_001048200	HIPK3	2987	
NM_144685	HIPK4	115701	
NM_000188	HK1	204851	
NM_000189	HK2	28996	GAGUGGAGAUGCACAACAA
NM_002115	HK3	10114	
NM_014413	HRI	10114	
NM_017525	HSMDPKIN	147746	
NM_014365	HSPB8	3098	
NM_014586	HUNK	3099	
NM_004507	HUS1	3101	
NM_014920	ICK	27102	
NM_000875	IGF1R	55561	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_000876	IGF2R	55561	
NM_001006115	IHPK1	26353	
NM_016291	IHPK2	26353	
NM_054111	IHPK3	30811	
NM_003640	IKBKAP	3364	
NM_001556	IKBKB	22858	
NM_014002	IKBKE	3480	
NM_001099856	IKBKG	3480	
NM_001014794	ILK	3482	
AJ277481	ILK-2	9807	
NM_001079817	INSR	51447	
NM_014215	INSRR	117283	
NM_152230	IPMK	8518	
NM_001569	IRAK1	3551	
NM_001570	IRAK2	9641	
NM_007199	IRAK3	8517	
NM_016123	IRAK4	3611	
NM_005546	ITK	55522	
NM_014216	ITPK1	3643	
NM_002220	ITPKA	3645	
NM_002221	ITPKB	253430	GAUGAUCGACUUUGGGAAA
NM_025194	ITPKC	3656	
NM_002227	JAK1	11213	
NM_004972	JAK2	3702	
NM_000215	JAK3	3705	
NM_016281	JIK	3706	
NM_003947	KALRN	3706	
NM_172056	KCNH2	3707	
NM_144633	KCNH8	3716	
NM_002253	KDR	3717	
NM_006488	KHK	3718	
NM_025164	KIAA0999	3718	
NM_020791	KIAA1361	51347	
XM_290923	KIAA1639	8997	
NM_033403	KIAA1765	3757	
NM_032435	KIAA1804	131096	
NM_032430	KIAA1811	3795	
NM_024776	KIAA2002	23387	
NM_001093772	KIT	23387	
NM_014238	KSR	85443	
NM_173598	KSR2	84446	
NM_033276	KUB3	79834	
NM_001102406	LAK	8844	
NM_004690	LATS1	283455	
NM_014572	LATS2	91419	
NM_001042771	LCK	80216	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_002314	LIMK1	26524	
NM_001031801	LIMK2	26524	
NM_014916	LMTK2	3932	
NM_001080434	LMTK3	3984	
NM_001012418	LOC340156	3985	
XM_001134406	LOC390226	22853	
NM_138370	LOC91461	114783	
NM_024652	LRRK1	340156	
NM_198578	LRRK2	390226	
NM_206961	LTK	91461	
NM_001003786	LYK5	79705	
NM_001111097	LYN	120892	
NM_152900	MAGI-3	4058	
NM_005906	MAK	92335	
NM_002755	MAP2K1	4067	
NM_030662	MAP2K2	260425	
NM_002756	MAP2K3	4117	
NM_003010	MAP2K4	5604	
NM_002757	MAP2K5	5605	
NM_002758	MAP2K6	5606	
NM_145185	MAP2K7	6416	
NM_005921	MAP3K1	5607	GAUUAGAUGUCAAUACAGA
NM_002446	MAP3K10	5608	
NM_002419	MAP3K11	5609	
NM_006301	MAP3K12	4214	
NM_004721	MAP3K13	4294	
NM_003954	MAP3K14	4296	
NM_001001671	MAP3K15	7786	
NM_006609	MAP3K2	7786	
NM_002401	MAP3K3	9175	
NM_006724	MAP3K4	9020	
NM_005923	MAP3K5	389840	
NM_004672	MAP3K6	389840	
NM_145333	MAP3K7	10746	
NM_153497	MAP3K7IP1	10746	
NM_005204	MAP3K8	4215	
NM_033141	MAP3K9	4216	
NM_007181	MAP4K1	9064	
NM_004579	MAP4K2	6885	
NM_003618	MAP4K3	10454	
NM_004834	MAP4K4	1326	
NM_006575	MAP4K5	1326	
NM_138957	MAPK1	11184	
NM_002753	MAPK10	5871	
NM_002751	MAPK11	8491	
NM_002969	MAPK12	9448	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_002754	MAPK13	11183	
NM_139013	MAPK14	5594	
NM_001109891	MAPK3	5602	
NM_002747	MAPK4	5600	
NM_002748	MAPK6	6300	
NM_002749	MAPK7	5603	
NM_139047	MAPK8	1432	
NM_002752	MAPK9	5595	
NM_004759	MAPKAPK2	5595	
NM_004635	MAPKAPK3	5596	
NM_003668	MAPKAPK5	5597	
NM_018650	MARK1	5598	
NM_001039468	MARK2	5599	
NM_002376	MARK3	5601	
NM_031417	MARK4	5601	
NM_015112	MAST2	9261	
NM_015016	MAST3	7867	
NM_198828	MAST4	8550	
NM_032844	MASTL	4139	
NM_139354	MATK	2011	
NM_014791	MELK	4140	
NM_006343	MERTK	57787	
NM_000245	MET	23139	
NM_033115	MGC16169	23031	
NM_153361	MGC42105	23031	
NM_001040261	MGC45428	375449	
NM_032017	MGC4796	84930	
NM_024046	MGC8407	9833	
NM_020778	MIDORI	10461	
NM_015716	MINK	4233	
NM_198973	MKNK1	93627	
NM_017572	MKNK2	167359	
NM_182493	MLCK	166614	
NM_005372	MOS	83931	
NM_002436	MPP1	83931	
NM_005374	MPP2	79012	
NM_001932	MPP3	57538	
NM_002447	MST1R	50488	
NM_018238	MULK	8569	
NM_005592	MUSK	8569	
NM_000431	MVK	4342	
NM_053031	MYLK	4354	
NM_033118	MYLK2	4354	
NM_017433	MYO3A	4355	
NM_001083615	MYO3B	4355	
NM_018177	N4BP2	4356	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_017567	NAGK	4486	
NM_012224	NEK1	55750	
NM_145910	NEK11	4593	
NM_002497	NEK2	4598	
NM_152720	NEK3	4638	CCUGAAGACAUGAUACUUA
NM_003157	NEK4	53904	
NM_199289	NEK5	140469	
NM_014397	NEK6	55728	
NM_133494	NEK7	4750	
NM_178170	NEK8	4751	AGACAAAGCCCUUAUGAUC
NM_033116	NEK9	4752	
NM_016231	NLK	341676	
NM_000269	NME1	10783	
NM_001018139	NME2	10783	
NM_002513	NME3	140609	
NM_005009	NME4	284086	
NM_003551	NME5	91754	
NM_005793	NME6	91754	
NM_013330	NME7	51701	
NM_003995	NPR2	4830	UGAGAGAUGUUCAGUUCAA
NM_013392	NRBP	4831	
NM_178564	NRBP2	4832	
NM_198465	NRK	4833	
NM_001007792	NTRK1	10201	
NM_001018064	NTRK2	29922	
NM_002530	NTRK3	29959	
NM_022731	NUCKS	340371	
NM_012346	NUP62	203447	
NM_033516	NYD-SP25	4914	
NM_005109	OSR1	4915	
NM_014308	P101-PI3K	4916	
NM_020423	PACE-1	64710	
NM_020804	PACSIN1	23636	
NM_002576	PAK1	89882	
NM_002577	PAK2	89882	
NM_002578	PAK3	9943	
NM_001014834	PAK4	23533	
NM_020168	PAK6	23533	
NM_020341	PAK7	57147	
NM_138316	PANK1	29993	
NM_024960	PANK2	5058	
NM_024594	PANK3	5062	
NM_018216	PANK4	5063	GCGCUUCGCCAUCGACAUA
NM_005443	PAPSS1	10298	
NM_004670	PAPSS2	56924	
NM_015148	PASK	56924	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_002591	PCK1	57144	
NM_004563	PCK2	53354	
NM_006201	PCTK1	80025	
NM_002595	PCTK2	79646	
NM_002596	PCTK3	55229	
NM_006206	PDGFRA	9061	
NM_002609	PDGFRB	23178	
NM_006207	PDGFRL	5105	
NM_152835	PDIK1L	5106	
NM_002610	PDK1	5127	
NM_002611	PDK2	5128	CCACGUACCGCGUCAGCUA
NM_005391	PDK3	5156	
NM_002612	PDK4	5159	
NM_031268	PDPK1	149420	
NM_003681	PDXK	149420	
NM_002625	PFKFB1	5163	
NM_001018053	PFKFB2	5165	
NM_004566	PFKFB3	5166	
NM_004567	PFKFB4	5170	
NM_002626	PFKL	8566	
NM_000289	PFKM	5207	
NM_002627	PFKP	5208	
NM_012395	PFTK1	5209	
NM_000291	PGK1	5210	
NM_138733	PGK2	5211	
NM_002637	PHKA1	5213	
NM_000292	PHKA2	5230	
NM_000293	PHKB	5232	
NM_006213	PHKG1	5255	
NM_000294	PHKG2	5256	
NM_018323	PI4K2B	5260	
NM_018425	PI4KII	5261	
NM_002645	PIK3C2A	55300	
NM_002646	PIK3C2B	55361	
NM_004570	PIK3C2G	55361	
NM_002647	PIK3C3	5286	
NM_006218	PIK3CA	5287	
NM_006219	PIK3CB	5288	
NM_005026	PIK3CD	5288	
NM_002649	PIK3CG	5289	
NM_181504	PIK3R1	5291	
NM_005027	PIK3R2	5293	
NM_003629	PIK3R3	5295	
NM_014602	PIK3R4	5296	
NM_002650	PIK4CA	8503	
NM_002651	PIK4CB	30849	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_002648	PIM1	5297	
NM_006875	PIM2	5298	
NM_001001852	PIM3	5298	
NM_032409	PINK1	5292	
NM_003557	PIP5K1A	5292	
NM_003558	PIP5K1B	11040	
NM_012398	PIP5K1C	415116	
NM_005028	PIP5K2A	65018	
NM_003559	PIP5K2B	8394	
NM_024779	PIP5K2C	8395	
NM_152671	PIP5K3	23396	
NM_173492	PIP5KL1	5305	
NM_006823	PKIA	5305	
NM_032471	PKIB	8396	
NM_181871	PKLR	79837	
NM_002654	PKM2	200576	
NM_004203	PKMYT1	138429	
NM_013355	PKN3	5569	CUUCUGCGAUCCUGUCAUU
NM_005030	PLK1	5570	
NM_006622	PLK2	5313	
NM_004073	PLK3	5315	
NM_014264	PLK4	9088	GAAGAUAGCAAUUAUGUGU
NM_006556	PMVK	29941	
NM_001039582	PNCK	5347	
NM_007254	PNKP	10769	
NM_006251	PRKAA1	1263	
NM_006252	PRKAA2	10733	
NM_006253	PRKAB1	10733	
NM_005399	PRKAB2	10654	
NM_207518	PRKACA	139728	
NM_002731	PRKACB	11284	
NM_002732	PRKACG	11284	
NM_002733	PRKAG1	5563	
NM_024429	PRKAG2	5564	
NM_017431	PRKAG3	5565	GACCAGCAGCUCAGAAAGA
NM_002734	PRKAR1A	5566	
NM_002735	PRKAR1B	5567	
NM_004157	PRKAR2A	5568	
NM_002736	PRKAR2B	5571	
NM_002737	PRKCA	5571	
NM_002738	PRKCB1	51422	
NM_006254	PRKCD	53632	
NM_005400	PRKCE	5573	
NM_002739	PRKCG	5575	
NM_006255	PRKCH	5576	
NM_002740	PRKCI	5577	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_002741	PRKCL1	5578	
NM_006256	PRKCL2	5579	
NM_002742	PRKCM	5580	
NM_005813	PRKCN	5582	
NM_006257	PRKCQ	5583	
NM_002743	PRKCSH	5584	
NM_002744	PRKCZ	5586	
NM_001079882	PRKD2	5586	
NM_006904	PRKDC	5587	
NM_006258	PRKG1	5587	
NM_006259	PRKG2	23683	
NM_002759	PRKR	5588	
NM_018979	PRKWNK1	5589	
NM_006648	PRKWNK2	5590	
NM_001002838	PRKWNK3	25865	
NM_005044	PRKX	5591	
NM_002760	PRKY	5593	
NM_003913	PRPF4B	5593	
NM_002764	PRPS1	5610	
NM_175886	PRPS1L1	65125	
NM_002765	PRPS2	65268	
NM_006742	PSKH1	65267	
NM_033126	PSKH2	5613	
NM_005607	PTK2	5616	
NM_004103	PTK2B	8899	
NM_005975	PTK6	5631	
NM_152881	PTK7	221823	
NM_002822	PTK9	5634	
NM_007284	PTK9L	85481	
NM_017771	PXK	5747	
NM_001017423	PYCS	2185	
NM_002880	RAF1	5753	
NM_014226	RAGE	5754	
NM_022128	RBKS	11344	
NM_021975	RELA	54899	
NM_020630	RET	5832	
NM_018339	RFK	5894	
NM_006510	RFP	5891	
NM_031480	RIOK1	64080	
NM_018343	RIOK2	5970	
NM_003831	RIOK3	5970	
NM_003804	RIPK1	5979	
NM_003821	RIPK2	55312	
NM_006871	RIPK3	5987	
NM_021133	RNASEL	8780	
NM_005406	ROCK1	8737	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_004850	ROCK2	8737	
NM_001083592	ROR1	11035	
NM_004560	ROR2	6041	
NM_002944	ROS1	6093	
NM_001042452	RP6-213H19.1	9475	
NM_001006665	RPS6KA1	4919	
NM_001006932	RPS6KA2	4920	
NM_004586	RPS6KA3	6098	
NM_001006944	RPS6KA4	51765	
NM_182398	RPS6KA5	6195	
NM_014496	RPS6KA6	6196	
NM_003161	RPS6KB1	6197	
NM_003952	RPS6KB2	6197	
NM_012424	RPS6KC1	9252	
NM_031464	RPS6KL1	27330	
NM_002958	RYK	6198	
NM_014975	SAST	26750	
NM_001024401	SBK1	83694	
NM_001075099	SCAP1	6259	
NM_001048218	SCYL1	22983	
NM_005627	SGK	388228	
NM_016276	SGK2	8631	
NM_013257	SGKL	57410	GAAAGCUGCCCAAGUGUAA
NM_015191	SIK2	6446	
NM_014720	SLK	23678	
NM_015092	SMG1	23235	
NM_030952	SNARK	9748	
NM_173354	SNF1LK	23049	
NM_001100594	SNRK	81788	
NM_005876	SPEG	10290	
NM_021972	SPHK1	8877	
NM_020126	SPHK2	56848	
NM_005417	SRC	6725	
NM_080823	SRMS	6731	
NM_006947	SRP72	6732	
NM_003137	SRPK1	6733	
NM_182691	SRPK2	83983	
NM_032037	SSTK	6793	
NM_005990	STK10	6793	
NM_000455	STK11	6794	
NM_001008910	STK16	8576	
NM_004760	STK17A	9263	
NM_004226	STK17B	8859	
NM_004197	STK19	23617	
NM_053006	STK22B	81629	
NM_052841	STK22C	81629	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_032028	STK22D	83942	
NM_014370	STK23	83942	
NM_003576	STK24	26576	
NM_006374	STK25	9024	
NM_003957	STK29	6788	
NM_006281	STK3	56164	
NM_031414	STK31	202374	
NM_145001	STK32A	55351	
NM_018401	STK32B	282974	
NM_173575	STK32C	65975	
NM_030906	STK33	140901	
NM_080836	STK35	27148	
NM_015690	STK36	11329	
NM_007271	STK38	23012	
NM_015000	STK38L	27347	
NM_013233	STK39	6789	
NM_006282	STK4	55359	
NM_018423	STYK1	6850	
NM_003177	SYK	6872	
NM_004606	TAF1	138474	
NM_153809	TAF1L	138474	
NM_004783	TAO1	9344	
NM_013254	TBK1	29110	
NM_003215	TEC	7006	
NM_000459	TEK	7010	
NM_006285	TESK1	7016	
NM_007170	TESK2	10420	
NM_031272	TEX14	56155	
NM_004612	TGFBR1	7046	
NM_003242	TGFBR2	7048	
NM_003243	TGFBR3	7049	
NM_024838	THNSL1	79896	
NM_201629	TJP2	9414	
NM_004614	TK2	7084	
NM_012290	TLK1	9874	
NM_006852	TLK2	11011	
NM_015028	TNIK	23043	
NM_003985	TNK1	8711	
NM_005781	TNK2	10188	
NM_015978	TNNI3K	10188	
NM_018492	TOPK	55872	
NM_033550	TP53RK	112858	
NM_001042482	TPK1	27010	GCCAACCGCUUAUAUGAUA
NM_025195	TRIB1	10221	
NM_021643	TRIB2	28951	
NM_021158	TRIB3	57761	

Gene Accession	Gene Symbol	Gene ID	VALIDATION SCREEN
			Novel siRNA target
NM_007118	TRIO	54822	
NM_017662	TRPM6	60385	
NM_017672	TRPM7	84630	
NM_021733	TSKS	146057	
NM_032538	TTBK1	7272	
NM_173500	TTBK2	7297	
NM_003318	TTK	7301	
NM_003331	TYK2	7301	
NM_006293	TYRO3	83549	
NM_031432	UCK1	127933	
NM_175866	UHMK1	127933	
NM_003565	ULK1	8408	
NM_014683	ULK2	9706	
NM_017886	ULK4	54986	
NM_016308	UMP-CMPK	51727	
NM_012474	UMPK	7371	
NM_017859	URKL1	7443	
NM_003384	VRK1	7444	
NM_006296	VRK2	51231	
NM_001025778	VRK3	7465	
NM_003390	WEE1	65266	
NM_032387	WNK4	9942	
NM_005108	XYLB	7525	
NM_005433	YES1	7525	
NM_016653	ZAK	51776	
NM_001079	ZAP70	7535	

Table S2. Normalized primary screen scores, z-score analysis and cytotoxicity

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
1_A1	NEG	4	4	3	3														
1_B1	NEG	1	2	3	3														
1_C1	NEG	2	4	3	4														
1_D1	NEG	3	2	3	4														
1_E1	NEG	1	1	3	3														
1_F1	NEG	3	3	4	3														
1_G1	NEG	1	1	3	3														
1_H1	NEG	3	3	3	3				2.25	2.50	3.13	3.25				2.78			0
1_A12	MEK	0	0	2	2														
1_B12	MEK	0	0	2	1														
1_C12	MEK	1	2	0	1														
1_D12	MEK	2	2	1	1														
1_E12	MEK	1	1	1	1				0.36	0.40	0.38	0.37				0.38			19
1_F12	TOX	0	0	0	0														
1_G12	TOX	0	0	0	0														
1_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
1_A2	TTK	2	2	2	2				0.89	0.80	0.64	0.62				0.74	0.13	-0.35	32
1_B2	ACVRL1	2	2	3	3				0.89	0.80	0.96	0.92				0.89	0.07	0.12	26
1_C2	ALS2CR2	0	0	4	4				0.00	0.00	1.28	1.23				0.63	0.73	-0.68	23
1_D2	CDC2	3	1	3	3				1.33	0.40	0.96	0.92				0.90	0.38	0.16	12
1_E2	PRKAG3	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	4
1_F2	TEK	4	3	5	4				1.78	1.20	1.60	1.23				1.45	0.28	1.82	27
1_G2	ATM	2	1	3	2				0.89	0.40	0.96	0.62				0.72	0.26	-0.41	40
1_H2	RFP	1	1	0	0				0.44	0.40	0.00	0.00				0.21	0.24	-1.95	35
1_A3	SYK	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	38
1_B3	LYN	0	2	3	4				0.00	0.80	0.96	1.23				0.75	0.53	-0.32	31
1_C3	CARKL	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	30
1_D3	PIM2	1	2	2	4				0.44	0.80	0.64	1.23				0.78	0.33	-0.22	26
1_E3	GRK5	3	3	0	1				1.33	1.20	0.00	0.31				0.71	0.66	-0.43	31
1_F3	PANK1	4	4	3	2				1.78	1.60	0.96	0.62				1.24	0.54	1.17	31
1_G3	PHKA2	2	1	4	4				0.89	0.40	1.28	1.23				0.95	0.41	0.30	8
1_H3	PIP5KL1	2	2	3	3				0.89	0.80	0.96	0.92				0.89	0.07	0.12	34
1_A4	EPHA8	1	1	1	1				0.44	0.40	0.32	0.31				0.37	0.07	-1.47	16
1_B4	PAPSS1	2	1	3	3				0.89	0.40	0.96	0.92				0.79	0.26	-0.18	21
1_C4	ADK	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	12
1_D4	LRRK2	0	1	0	0				0.00	0.40	0.00	0.00				0.10	0.20	-2.29	31
1_E4	LIMK1	2	2	2	3				0.89	0.80	0.64	0.92				0.81	0.13	-0.12	35
1_F4	CSNK1G3	4	4	4	4				1.78	1.60	1.28	1.23				1.47	0.26	1.88	34
1_G4	MULK	0	0	2	2				0.00	0.00	0.64	0.62				0.31	0.36	-1.64	13
1_H4	NEK5	2	1	5	4				0.89	0.40	1.60	1.23				1.03	0.51	0.54	27
1_A5	DMPK	1	1	3	4				0.44	0.40	0.96	1.23				0.76	0.40	-0.28	48
1_B5	MAPK4	2	1	3	3				0.89	0.40	0.96	0.92				0.79	0.26	-0.18	20
1_C5	ABL2	2	3	2	1				0.89	1.20	0.64	0.31				0.76	0.38	-0.28	21
1_D5	HK2	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	20
1_E5	UMPK	2	2	3	3				0.89	0.80	0.96	0.92				0.89	0.07	0.12	21
1_F5	CKS2	2	3	3	3				0.89	1.20	0.96	0.92				0.99	0.14	0.43	23
1_G5	GRK6	3	4	4	4				1.33	1.60	1.28	1.23				1.36	0.16	1.55	31
1_H5	PLK2	1	2	2	1				0.44	0.80	0.64	0.31				0.55	0.22	-0.93	15
1_A6	COASY	2	2	1	1				0.89	0.80	0.32	0.31				0.58	0.31	-0.83	33
1_B6	MASTL	3	2	2	4				1.33	0.80	0.64	1.23				1.00	0.33	0.45	83
1_C6	PLK1	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	83
1_D6	FN3K	2	1	3	3				0.89	0.40	0.96	0.92				0.79	0.26	-0.18	21
1_E6	C7ORF2	2	2	3	4				0.89	0.80	0.96	1.23				0.97	0.19	0.36	18
1_F6	PHKG2	0	0	1	1				0.00	0.00	0.32	0.31				0.16	0.18	-2.11	36
1_G6	BMPR1B	2	3	3	2				0.89	1.20	0.96	0.62				0.92	0.24	0.19	40
1_H6	CSNK1G2	1	1	3	2				0.44	0.40	0.96	0.62				0.60	0.25	-0.75	43
1_A7	MYO3B	3	4	2	1				1.33	1.60	0.64	0.31				0.97	0.60	0.36	42
1_B7	TRIB1	2	2	3	3				0.89	0.80	0.96	0.92				0.89	0.07	0.12	28
1_C7	MINK	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	37
1_D7	CRIM1	2	2	3	3				0.89	0.80	0.96	0.92				0.89	0.07	0.12	46
1_E7	KSR	0	0	2	2				0.00	0.00	0.64	0.62				0.31	0.36	-1.64	11
1_F7	MAPK7	4	3	4	3				1.78	1.20	1.28	0.92				1.30	0.36	1.35	28
1_G7	STK33	2	2	4	4				0.89	0.80	1.28	1.23				1.05	0.24	0.60	33
1_H7	MARK2	2	3	2	3				0.89	1.20	0.64	0.92				0.91	0.23	0.18	25
1_A8	PRKWNK2	2	4	4	4				0.89	1.60	1.28	1.23				1.25	0.29	1.21	32
1_B8	BRD3	1	2	3	3				0.44	0.80	0.96	0.92				0.78	0.24	-0.21	26
1_C8	CDKL3	2	0	3	3				0.89	0.00	0.96	0.92				0.69	0.46	-0.48	28
1_D8	PIK4CA	1	2	0	1				0.44	0.80	0.00	0.31				0.39	0.33	-1.41	30

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
1_E8	MAP4K4	4	0	4	5				1.78	0.00	1.28	1.54				1.15	0.79	0.90	31
1_F8	KCNH8	1	3	3	3				0.44	1.20	0.96	0.92				0.88	0.32	0.09	42
1_G8	MIDORI	2	2	1	1				0.89	0.80	0.32	0.31				0.58	0.31	-0.83	32
1_H8	TNIK	1	3	0	0				0.44	1.20	0.00	0.00				0.41	0.57	-1.34	42
1_A9	PTK2	0	3	0	0				0.00	1.20	0.00	0.00				0.30	0.60	-1.68	27
1_B9	FLJ34389	0	1	1	1				0.00	0.40	0.32	0.31				0.26	0.18	-1.81	38
1_C9	MAP3K14	0	2	2	2				0.00	0.80	0.64	0.62				0.51	0.35	-1.03	38
1_D9	SSTK	2	1	0	0				0.89	0.40	0.00	0.00				0.32	0.42	-1.61	45
1_E9	PRKCN	1	3	3	2				0.44	1.20	0.96	0.62				0.80	0.34	-0.14	33
1_F9	CAMK2D	1	1	1	0				0.44	0.40	0.32	0.00				0.29	0.20	-1.71	23
1_G9	RNASEL	0	2	1	2				0.00	0.80	0.32	0.62				0.43	0.35	-1.27	46
1_H9	CALM3	2	0	3	3				0.89	0.00	0.96	0.92				0.69	0.46	-0.48	33
1_A10	FER	1	0	3	3				0.44	0.00	0.96	0.92				0.58	0.45	-0.82	32
1_B10	HK1	2	0	2	2				0.89	0.00	0.64	0.62				0.54	0.38	-0.96	40
1_C10	MAP3K7	1	0	2	4				0.44	0.00	0.64	1.23				0.58	0.51	-0.83	49
1_D10	TAO1	3	2	2	2				1.33	0.80	0.64	0.62				0.85	0.33	-0.02	24
1_E10	TTBK1	0	1	3	2				0.00	0.40	0.96	0.62				0.49	0.40	-1.09	41
1_F10	MAP4K5	1	0	2	2				0.44	0.00	0.64	0.62				0.42	0.30	-1.30	40
1_G10	STK16	2	1	3	2				0.89	0.40	0.96	0.62				0.72	0.26	-0.41	38
1_H10	PKM2	2	1	3	3				0.89	0.40	0.96	0.92				0.79	0.26	-0.18	36
1_A11	CAMK2B	1	1	3	3				0.44	0.40	0.96	0.92				0.68	0.30	-0.52	28
1_B11	THNSL1	1	1	2	2				0.44	0.40	0.64	0.62				0.52	0.12	-1.00	20
1_C11	CKB	1	1	2	2				0.44	0.40	0.64	0.62				0.52	0.12	-1.00	23
1_D11	NME3	0	1	0	0				0.00	0.40	0.00	0.00				0.10	0.20	-2.29	23
1_E11	ROCK1	1	2	2	1				0.44	0.80	0.64	0.31				0.55	0.22	-0.93	19
1_F11	PKN3	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	18
1_G11	GRK1	2	2	4	4				0.89	0.80	1.28	1.23				1.05	0.24	0.60	28
1_H11	CDKL5	3	4	2	2				1.33	1.60	0.64	0.62				1.05	0.50	0.59	11
2_A1	NEG	4	4	3	3	3													
2_B1	NEG	3	3	3	3	4													
2_C1	NEG	3	2	3	3	3													
2_D1	NEG	3	4	3	4	4													
2_E1	NEG	3	3	3	3														
2_F1	NEG	3	2	3	3														
2_G1	NEG	3	4	3	4														
2_H1	NEG	4	4	3	4				3.25	3.25	3.00	3.38	3.50			3.28			0
2_A12	MEK	1	1	2	1	3													
2_B12	MEK	1	3	0	0	0													
2_C12	MEK	1	2	0	2	2													
2_D12	MEK	0	0	1	1														
2_E12	MEK	2	2	2	2				0.31	0.49	0.33	0.36	0.48			0.39			0
2_F12	TOX	0	0	0	0	0													
2_G12	TOX	0	0	0	0														
2_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00	0.00			0.00			100
2_A2	PIK3CA	1	2	2	2				0.31	0.62	0.67	0.59				0.55	0.16	-0.93	0
2_B2	FLJ32685	3	3	4	5				0.92	0.92	1.33	1.48				1.17	0.29	0.95	0
2_C2	PAPSS2	3	4	4	4				0.92	1.23	1.33	1.19				1.17	0.17	0.96	0
2_D2	KIAA1811	2	3	4	4				0.62	0.92	1.33	1.19				1.01	0.32	0.49	3
2_E2	RIOK2	1	2	2	3				0.31	0.62	0.67	0.89				0.62	0.24	-0.71	0
2_F2	STK25	4	4	5	5				1.23	1.23	1.67	1.48				1.40	0.21	1.67	1
2_G2	PRKCL1	2	2	3	2				0.62	0.62	1.00	0.59				0.71	0.20	-0.45	2
2_H2	TRIO	3	3	4	4				0.92	0.92	1.33	1.19				1.09	0.20	0.73	3
2_A3	KIAA1804	3	4	0	0				0.92	1.23	0.00	0.00				0.54	0.63	-0.95	6
2_B3	ITPKC	4	4	3	3				1.23	1.23	1.00	0.89				1.09	0.17	0.72	0
2_C3	RPS6KB2	3	2	3	2				0.92	0.62	1.00	0.59				0.78	0.21	-0.21	0
2_D3	PDK2	0	1	1	0	1			0.00	0.31	0.33	0.00	0.29			0.19	0.17	-2.03	1
2_E3	FRAP1	4	3	4	4				1.23	0.92	1.33	1.19				1.17	0.17	0.96	0
2_F3	CDC7	3	3	3	4				0.92	0.92	1.00	1.19				1.01	0.12	0.47	0
2_G3	RIPK2	0	1	1	2				0.00	0.31	0.33	0.59				0.31	0.24	-1.65	0
2_H3	ETNK1	3	3	1	2				0.92	0.92	0.33	0.59				0.69	0.29	-0.48	4
2_A4	NAGK	4	4	3	3				1.23	1.23	1.00	0.89				1.09	0.17	0.72	0
2_B4	EPHA5	1	1	3	2				0.31	0.31	1.00	0.59				0.55	0.33	-0.91	0
2_C4	PFTK1	2	2	4	4				0.62	0.62	1.33	1.19				0.94	0.38	0.26	0
2_D4	MYLK2	1	1	0	1	0			0.31	0.31	0.00	0.30	0.00			0.18	0.17	-2.04	0
2_E4	PDGFRL	1	2	1	6				0.31	0.62	0.33	1.78				0.76	0.69	-0.29	1
2_F4	CAMK2G	4	4	5	5				1.23	1.23	1.67	1.48				1.40	0.21	1.67	1
2_G4	CKMT1B	1	1	1	2				0.31	0.31	0.33	0.59				0.39	0.14	-1.42	3
2_H4	ERK8	2	3	0	0				0.62	0.92	0.00	0.00				0.38	0.46	-1.42	0
2_A5	IRAK1	3	4	2	3				0.92	1.23	0.67	0.89				0.93	0.23	0.23	0
2_B5	MLCK	4	4	4	4				1.23	1.23	1.33	1.19				1.25	0.06	1.19	0
2_C5	NME5	4	4	4	4				1.23	1.23	1.33	1.19				1.25	0.06	1.19	0

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
2_D5	NEK11	2	1	2	3				0.62	0.31	0.67	0.89				0.62	0.24	-0.71	0
2_E5	AK7	1	1	4	8				0.31	0.31	1.33	2.37				1.08	0.99	0.69	0
2_F5	URKL1	2	3	3	4				0.62	0.92	1.00	1.19				0.93	0.24	0.24	0
2_G5	MKNK2	4	4	2	1				1.23	1.23	0.67	0.30				0.86	0.46	0.01	4
2_H5	ADP-GK	3	4	4	5				0.92	1.23	1.33	1.48				1.24	0.24	1.19	3
2_A6	EEF2K	4	3	0	0				1.23	0.92	0.00	0.00				0.54	0.63	-0.95	0
2_B6	PSKH1	0	0	3	2				0.00	0.00	1.00	0.59				0.40	0.49	-1.38	2
2_C6	SRC	2	1	0	0				0.62	0.31	0.00	0.00				0.23	0.29	-1.89	0
2_D6	KIAA1361	4	4	4	4				1.23	1.23	1.33	1.19				1.25	0.06	1.19	0
2_E6	EPHA10	2	1	3	8				0.62	0.31	1.00	2.37				1.07	0.91	0.67	2
2_F6	MAP3K9	4	4	5	4				1.23	1.23	1.67	1.19				1.33	0.23	1.45	0
2_G6	TNNI3K	4	4	3	3				1.23	1.23	1.00	0.89				1.09	0.17	0.72	1
2_H6	PIK3CG	3	3	0	0				0.92	0.92	0.00	0.00				0.46	0.53	-1.19	5
2_A7	ANKK1	2	3	3	3				0.62	0.92	1.00	0.89				0.86	0.17	0.01	0
2_B7	AURKC	3	3	1	1				0.92	0.92	0.33	0.30				0.62	0.35	-0.71	1
2_C7	NEK4	4	4	4	4				1.23	1.23	1.33	1.19				1.25	0.06	1.19	0
2_D7	SNF1LK	4	4	4	4				1.23	1.23	1.33	1.19				1.25	0.06	1.19	0
2_E7	STK17B	3	2	3	4				0.92	0.62	1.00	1.19				0.93	0.24	0.24	0
2_F7	GUCY2D	0	0	2	2				0.00	0.00	0.67	0.59				0.31	0.36	-1.63	3
2_G7	ALS2CR7	4	4	5	4				1.23	1.23	1.67	1.19				1.33	0.23	1.45	0
2_H7	FGFR3	3	3	1	2				0.92	0.92	0.33	0.59				0.69	0.29	-0.48	6
2_A8	MAP3K5	3	3	3	3				0.92	0.92	1.00	0.89				0.93	0.05	0.25	0
2_B8	PRKCE	1	3	1	1				0.31	0.92	0.33	0.30				0.47	0.31	-1.18	0
2_C8	CSNK1G1	3	4	4	4				0.92	1.23	1.33	1.19				1.17	0.17	0.96	0
2_D8	PTK9	4	4	3	3				1.23	1.23	1.00	0.89				1.09	0.17	0.72	0
2_E8	NPR2	4	2	8	8	6			1.23	0.62	2.67	2.37	1.71			1.72	0.83	2.64	1
2_F8	CAMKK2	3	0	2	3				0.92	0.00	0.67	0.89				0.62	0.43	-0.71	0
2_G8	HCK	4	4	4	6				1.23	1.23	1.33	1.78				1.39	0.26	1.64	2
2_H8	IRAK4	4	3	4	4				1.23	0.92	1.33	1.19				1.17	0.17	0.96	3
2_A9	PRKAA1	4	2	0	0				1.23	0.62	0.00	0.00				0.46	0.59	-1.19	0
2_B9	AK1	3	2	4	4				0.92	0.62	1.33	1.19				1.01	0.32	0.49	0
2_C9	RIOK1	3	3	0	0				0.92	0.92	0.00	0.00				0.46	0.53	-1.19	0
2_D9	LATS1	3	3	3	4				0.92	0.92	1.00	1.19				1.01	0.12	0.47	0
2_E9	GOLGA5	1	4	4	3				0.31	1.23	1.33	0.89				0.94	0.46	0.27	4
2_F9	KIAA1639	3	3	3	4				0.92	0.92	1.00	1.19				1.01	0.12	0.47	2
2_G9	CKS1B	4	4	3	5				1.23	1.23	1.00	1.48				1.24	0.20	1.17	15
2_H9	PCTK3	4	4	3	5				1.23	1.23	1.00	1.48				1.24	0.20	1.17	4
2_A10	RPS6KA4	3	4	2	4				0.92	1.23	0.67	1.19				1.00	0.26	0.45	0
2_B10	TRPM6	3	3	2	2				0.92	0.92	0.67	0.59				0.78	0.17	-0.23	0
2_C10	AKT1	3	2	4	3				0.92	0.62	1.33	0.89				0.94	0.30	0.27	0
2_D10	PFKP	4	2	4	3				1.23	0.62	1.33	0.89				1.02	0.33	0.50	6
2_E10	STK24	3	1	2	3				0.92	0.31	0.67	0.89				0.70	0.28	-0.47	7
2_F10	CAMKIINALPHA	3	2	3	3				0.92	0.62	1.00	0.89				0.86	0.17	0.01	8
2_G10	BMP2K	3	3	1	0				0.92	0.92	0.33	0.00				0.54	0.46	-0.93	6
2_H10	SGK2	4	4	3	4				1.23	1.23	1.00	1.19				1.16	0.11	0.94	3
2_A11	CALM1	3	4	5	4				0.92	1.23	1.67	1.19				1.25	0.31	1.21	0
2_B11	CLK1	2	1	1	1				0.62	0.31	0.33	0.30				0.39	0.15	-1.41	0
2_C11	SNRK	3	3	2	2				0.92	0.92	0.67	0.59				0.78	0.17	-0.23	0
2_D11	PRKG1	3	3	4	4				0.92	0.92	1.33	1.19				1.09	0.20	0.73	0
2_E11	DGKB	3	3	2	4				0.92	0.92	0.67	1.19				0.92	0.21	0.22	4
2_F11	KIT	4	3	5	4				1.23	0.92	1.67	1.19				1.25	0.31	1.21	1
2_G11	PHKB	3	3	5	4				0.92	0.92	1.67	1.19				1.17	0.35	0.98	0
2_H11	DYRK4	4	4	3	4				1.23	1.23	1.00	1.19				1.16	0.11	0.94	5
3_A1	NEG	4	4	3	3	2	2	3											
3_B1	NEG	3	3	3	3	3	3	4											
3_C1	NEG	3	3	4	3	4	3	3											
3_D1	NEG	3	3	3	3	1	1	4											
3_E1	NEG	4	4	3	3	4	4												
3_F1	NEG	3	2	3	3	3	4												
3_G1	NEG	3	3	4	4	2	2												
3_H1	NEG	3	3	4	3	4	3		3.25	3.13	3.38	3.13	2.88	2.75	3.50	3.14			0
3_A12	MEK	2	3	2	2	3	2	3											
3_B12	MEK	2	2	2	2	2	4	0											
3_C12	MEK	3	3	2	2	2	1	2											
3_D12	MEK	3	2	1	2	2	2												
3_E12	MEK	3	3	1	2	3	2		0.80	0.83	0.47	0.64	0.83	0.80	0.48	0.69			3
3_F12	TOX	0	0	0	0	0	0	0											
3_G12	TOX	0	0	0	0	0	0												
3_H12	TOX	0	0	0	0	0	0		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			100
3_A2	MAP3K2	3	3	4	3	4	4		0.92	0.92	1.23	0.92	1.39	1.45		1.14	0.25	0.88	0
3_B2	DGKK	4	4	4	4	3	3		1.23	1.28	1.19	1.28	1.04	1.09		1.19	0.10	1.01	0

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
3_C2	CSNK1E	1	0	2	2	3	4		0.31	0.00	0.59	0.64	1.04	1.45		0.67	0.52	-0.55	3
3_D2	JAK3	3	4	4	4	4	4		0.92	1.28	1.19	1.28	1.39	1.45		1.25	0.19	1.22	2
3_E2	FLT3	2	1	2	2	2	1		0.62	0.32	0.59	0.64	0.70	0.36		0.54	0.16	-0.96	1
3_F2	RPS6KA3	3	4	5	4	2	3		0.92	1.28	1.48	1.28	0.70	1.09		1.13	0.28	0.83	1
3_G2	MAP3K12	2	2	2	3	3	1		0.62	0.64	0.59	0.96	1.04	0.36		0.70	0.25	-0.46	3
3_H2	FN3KRP	3	4	4	3	4	4		0.92	1.28	1.19	0.96	1.39	1.45		1.20	0.22	1.05	21
3_A3	LATS2	4	4	2	1	4	4		1.23	1.28	0.59	0.32	1.39	1.45		1.04	0.47	0.59	5
3_B3	MPP2	3	4	2	1	3	4		0.92	1.28	0.59	0.32	1.04	1.45		0.94	0.42	0.25	0
3_C3	RELA	2	1	1	2	3	2		0.62	0.32	0.30	0.64	1.04	0.73		0.61	0.28	-0.75	7
3_D3	STK22D	4	4	4	3	4	4		1.23	1.28	1.19	0.96	1.39	1.45		1.25	0.17	1.21	2
3_E3	PIP5K2A	4	4	3	3	4	4		1.23	1.28	0.89	0.96	1.39	1.45		1.20	0.23	1.06	3
3_F3	EFNA4	4	3	3	4	3	3		1.23	0.96	0.89	1.28	1.04	1.09		1.08	0.15	0.70	5
3_G3	STK22C	4	3	2	3	3	3		1.23	0.96	0.59	0.96	1.04	1.09		0.98	0.21	0.39	8
3_H3	AKT2	2	2	1	2	2	2		0.62	0.64	0.30	0.64	0.70	0.73		0.60	0.16	-0.76	6
3_A4	P101-PI3K	3	3	2	2	1	2		0.92	0.96	0.59	0.64	0.35	0.73		0.70	0.23	-0.47	7
3_B4	BUB1	4	4	6	5	4	4		1.23	1.28	1.78	1.60	1.39	1.45		1.46	0.21	1.83	8
3_C4	CCRK	0	0	2	2	3	1	0	0.00	0.00	0.59	0.64	1.04	0.36	0.00	0.38	0.41	-1.44	3
3_D4	FGFR1	2	1	2	2	3	4		0.62	0.32	0.59	0.64	1.04	1.45		0.78	0.40	-0.23	1
3_E4	DAPK1	3	3	3	3	2	2		0.92	0.96	0.89	0.96	0.70	0.73		0.86	0.12	0.02	0
3_F4	ADCK1	3	2	1	1	1	2		0.92	0.64	0.30	0.32	0.35	0.73		0.54	0.26	-0.94	5
3_G4	PI4KII	4	4	2	2	4	4		1.23	1.28	0.59	0.64	1.39	1.45		1.10	0.38	0.75	11
3_H4	KIAA0999	3	3	2	2	3	4		0.92	0.96	0.59	0.64	1.04	1.45		0.94	0.31	0.25	1
3_A5	DGKG	3	4	3	4	4	4		0.92	1.28	0.89	1.28	1.39	1.45		1.20	0.24	1.07	0
3_B5	UHMK1	4	3	2	2	4	4		1.23	0.96	0.59	0.64	1.39	1.45		1.04	0.37	0.59	5
3_C5	MAPK9	3	3	5	2	2	4		0.92	0.96	1.48	0.64	0.70	1.45		1.03	0.36	0.53	0
3_D5	PNKP	0	0	3	2	3	0	0	0.00	0.00	0.89	0.64	1.04	0.00	0.00	0.37	0.47	-1.47	5
3_E5	FASTK	4	4	3	3	4	4		1.23	1.28	0.89	0.96	1.39	1.45		1.20	0.23	1.06	6
3_F5	MAP3K8	4	4	4	5	4	4		1.23	1.28	1.19	1.60	1.39	1.45		1.36	0.16	1.53	0
3_G5	TAF1L	2	1	3	3	3	2		0.62	0.32	0.89	0.96	1.04	0.73		0.76	0.27	-0.28	2
3_H5	PLK4	0	0	1	2	0	0	0	0.00	0.00	0.30	0.64	0.00	0.00	0.00	0.13	0.25	-2.18	14
3_A6	MAST3	3	4	2	2	4	4		0.92	1.28	0.59	0.64	1.39	1.45		1.05	0.38	0.59	12
3_B6	PRKCM	2	3	3	3	4	4		0.62	0.96	0.89	0.96	1.39	1.45		1.05	0.32	0.59	0
3_C6	TNK2	1	0	2	3	3	3		0.31	0.00	0.59	0.96	1.04	1.09		0.67	0.44	-0.57	0
3_D6	ERBB2	1	1	1	1	3	4		0.31	0.32	0.30	0.32	1.04	1.45		0.62	0.50	-0.70	0
3_E6	PRKG2	3	4	2	2	4	4		0.92	1.28	0.59	0.64	1.39	1.45		1.05	0.38	0.59	0
3_F6	CDK8	3	3	3	3	4	4		0.92	0.96	0.89	0.96	1.39	1.45		1.10	0.26	0.74	0
3_G6	CAMK1G	3	3	2	2	4	4		0.92	0.96	0.59	0.64	1.39	1.45		0.99	0.36	0.43	0
3_H6	HIPK3	3	4	0	0	1	1		0.92	1.28	0.00	0.00	0.35	0.36		0.49	0.52	-1.11	7
3_A7	EFNA3	4	4	2	2	4	4		1.23	1.28	0.59	0.64	1.39	1.45		1.10	0.38	0.75	10
3_B7	PIK4CB	4	4	3	2	3	4		1.23	1.28	0.89	0.64	1.04	1.45		1.09	0.29	0.72	0
3_C7	PDIK1L	4	4	8	0	4	4		1.23	1.28	2.37	0.00	1.39	1.45		1.29	0.76	1.32	1
3_D7	PRKAG1	3	3	2	2	4	4		0.92	0.96	0.59	0.64	1.39	1.45		0.99	0.36	0.43	5
3_E7	MPP1	3	3	3	3	4	2		0.92	0.96	0.89	0.96	1.39	0.73		0.98	0.22	0.37	0
3_F7	MKNK1	4	4	6	5	4	4	4	1.23	1.28	1.78	1.60	1.39	1.45	1.14	1.41	0.22	1.70	0
3_G7	HSMDPKIN	3	4	0	0	3	4		0.92	1.28	0.00	0.00	1.04	1.45		0.78	0.63	-0.21	0
3_H7	TYRO3	4	4	5	4	4	4		1.23	1.28	1.48	1.28	1.39	1.45		1.35	0.10	1.52	0
3_A8	BCKDK	4	4	4	4	4	4		1.23	1.28	1.19	1.28	1.39	1.45		1.30	0.10	1.37	3
3_B8	DLG4	4	4	4	5	4	4		1.23	1.28	1.19	1.60	1.39	1.45		1.36	0.16	1.53	0
3_C8	AXL	3	4	2	2	4	3		0.92	1.28	0.59	0.64	1.39	1.09		0.99	0.33	0.41	3
3_D8	FLJ10761	2	3	1	1	4	4		0.62	0.96	0.30	0.32	1.39	1.45		0.84	0.51	-0.04	0
3_E8	HSPB8	4	3	2	2	2	2		1.23	0.96	0.59	0.64	0.70	0.73		0.81	0.24	-0.14	0
3_F8	PIK3C2G	3	4	0	0	0	2		0.92	1.28	0.00	0.00	0.00	0.73		0.49	0.56	-1.11	0
3_G8	STK10	1	4	3	3	3	3		0.31	1.28	0.89	0.96	1.04	1.09		0.93	0.33	0.23	1
3_H8	MAPK3	3	4	3	3	4	3		0.92	1.28	0.89	0.96	1.39	1.09		1.09	0.21	0.72	8
3_A9	ITPKA	4	3	5	4	4	4		1.23	0.96	1.48	1.28	1.39	1.45		1.30	0.19	1.36	5
3_B9	BTK	3	4	3	3	4	4		0.92	1.28	0.89	0.96	1.39	1.45		1.15	0.25	0.90	0
3_C9	ANKRD3	4	3	3	3	4	0		1.23	0.96	0.89	0.96	1.39	0.00		0.91	0.48	0.16	0
3_D9	IGF1R	3	2	3	2	4	4		0.92	0.64	0.89	0.64	1.39	1.45		0.99	0.36	0.42	1
3_E9	CSF1R	3	3	6	4	3	2		0.92	0.96	1.78	1.28	1.04	0.73		1.12	0.37	0.81	0
3_F9	CDK4	2	2	3	3	4	4		0.62	0.64	0.89	0.96	1.39	1.45		0.99	0.36	0.42	3
3_G9	AIP1	4	1	3	2	3	4		1.23	0.32	0.89	0.64	1.04	1.45		0.93	0.41	0.23	0
3_H9	YES1	4	4	3	3	4	4		1.23	1.28	0.89	0.96	1.39	1.45		1.20	0.23	1.06	3
3_A10	NEK6	4	4	4	3	4	4		1.23	1.28	1.19	0.96	1.39	1.45		1.25	0.17	1.21	2
3_B10	MAP3K15	4	1	6	5	4	4		1.23	0.32	1.78	1.60	1.39	1.45		1.30	0.51	1.35	2
3_C10	RIPK1	4	4	5	4	4	0		1.23	1.28	1.48	1.28	1.39	0.00		1.11	0.55	0.79	5
3_D10	NEK9	4	3	3	3	4	4		1.23	0.96	0.89	0.96	1.39	1.45		1.15	0.24	0.90	7
3_E10	DYRK2	4	4	2	3	3	3		1.23	1.28	0.59	0.96	1.04	1.09		1.03	0.25	0.55	5
3_F10	PIM1	3	2	3	3	4	4		0.92	0.64	0.89	0.96	1.39	1.45		1.04	0.32	0.58	0
3_G10	ABL1	4	4	3	3	4	4		1.23	1.28	0.89	0.96	1.39	1.45		1.20	0.23	1.06	6
3_H10	NYD-SP25	4	4	3	3	4	4		1.23	1.28	0.89	0.96	1.39	1.45		1.20	0.23	1.06	9
3_A11	BLK	4	4	4	3	4	4		1.23	1.28	1.19	0.96	1.39	1.45		1.25	0.17	1.21	1

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
3_B11	GSK3A	3	2	4	4	3	4		0.92	0.64	1.19	1.28	1.04	1.45		1.09	0.29	0.72	5
3_C11	CDC42BPB	4	4	5	4	4	4		1.23	1.28	1.48	1.28	1.39	1.45		1.35	0.10	1.52	0
3_D11	FLJ23356	4	4	3	3	4	3		1.23	1.28	0.89	0.96	1.39	1.09		1.14	0.19	0.88	1
3_E11	PRKCL2	4	4	2	2	3	3		1.23	1.28	0.59	0.64	1.04	1.09		0.98	0.29	0.39	0
3_F11	DGKQ	4	4	3	4	4	4		1.23	1.28	0.89	1.28	1.39	1.45		1.25	0.20	1.22	0
3_G11	PAK6	2	3	2	3	4	2		0.62	0.96	0.59	0.96	1.39	0.73		0.87	0.30	0.07	0
3_H11	CDADC1	3	4	3	3	4	3		0.92	1.28	0.89	0.96	1.39	1.09		1.09	0.21	0.72	10
4_A1	NEG	4	3	4	4														
4_B1	NEG	4	4	4	4														
4_C1	NEG	4	4	4	5														
4_D1	NEG	3	3	3	4														
4_E1	NEG	3	3	4	5														
4_F1	NEG	4	4	4	4														
4_G1	NEG	4	4	4	4														
4_H1	NEG	4	4	4	4				3.75	3.63	3.88	4.25				3.88			0
4_A12	MEK	2	3	2	2														
4_B12	MEK	2	4	2	1														
4_C12	MEK	3	3	2	0														
4_D12	MEK	2	2	2	2														
4_E12	MEK	2	2	3	2				0.59	0.77	0.57	0.33				0.56			12
4_F12	TOX	0	0	0	0														
4_G12	TOX	0	0	0	0														
4_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
4_A2	STK31	4	4	3	4				1.07	1.10	0.77	0.94				0.97	0.15	0.36	4
4_B2	CDKN2C	2	4	3	3				0.53	1.10	0.77	0.71				0.78	0.24	-0.22	18
4_C2	CAMK1	3	3	4	4				0.80	0.83	1.03	0.94				0.90	0.11	0.15	11
4_D2	EPHB6	3	3	4	4				0.80	0.83	1.03	0.94				0.90	0.11	0.15	11
4_E2	MAST4	4	4	5	5				1.07	1.10	1.29	1.18				1.16	0.10	0.93	11
4_F2	TGFBR1	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	3
4_G2	PAK1	4	3	3	4				1.07	0.83	0.77	0.94				0.90	0.13	0.15	0
4_H2	ACVR1	3	3	4	3				0.80	0.83	1.03	0.71				0.84	0.14	-0.03	22
4_A3	AKT3	4	4	3	3				1.07	1.10	0.77	0.71				0.91	0.20	0.18	14
4_B3	MAP2K3	2	4	2	2				0.53	1.10	0.52	0.47				0.66	0.30	-0.60	8
4_C3	MARK3	3	3	4	3				0.80	0.83	1.03	0.71				0.84	0.14	-0.03	11
4_D3	MARK1	4	3	1	2				1.07	0.83	0.26	0.47				0.66	0.36	-0.60	15
4_E3	CKMT2	3	2	2	3				0.80	0.55	0.52	0.71				0.64	0.13	-0.64	0
4_F3	CDK5R2	4	3	3	2				1.07	0.83	0.77	0.47				0.78	0.25	-0.21	24
4_G3	RYK	4	4	5	6				1.07	1.10	1.29	1.41				1.22	0.16	1.11	12
4_H3	PRKACB	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	5
4_A4	NLK	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	0
4_B4	LCK	3	2	3	2				0.80	0.55	0.77	0.47				0.65	0.16	-0.62	13
4_C4	MGC42105	3	4	4	3				0.80	1.10	1.03	0.71				0.91	0.19	0.18	3
4_D4	PRKAR2A	2	3	3	4				0.53	0.83	0.77	0.94				0.77	0.17	-0.25	20
4_E4	PIK3R3	4	4	5	4				1.07	1.10	1.29	0.94				1.10	0.14	0.75	8
4_F4	LOC91461	4	4	5	3				1.07	1.10	1.29	0.71				1.04	0.24	0.58	26
4_G4	PDXK	4	4	2	2				1.07	1.10	0.52	0.47				0.79	0.34	-0.19	21
4_H4	STK39	4	4	4	3				1.07	1.10	1.03	0.71				0.98	0.18	0.38	12
4_A5	PFKFB3	3	4	4	4				0.80	1.10	1.03	0.94				0.97	0.13	0.36	0
4_B5	CIB2	3	4	3	4				0.80	1.10	0.77	0.94				0.90	0.15	0.16	9
4_C5	CHEK1	4	4	0	1				1.07	1.10	0.00	0.24				0.60	0.57	-0.76	22
4_D5	PINK1	3	3	3	3				0.80	0.83	0.77	0.71				0.78	0.05	-0.23	4
4_E5	ACVR2	4	4	3	3				1.07	1.10	0.77	0.71				0.91	0.20	0.18	10
4_F5	DKFZP761P0423	4	4	3	2				1.07	1.10	0.77	0.47				0.85	0.29	0.00	23
4_G5	NEK8	1	1	0	0				0.27	0.28	0.00	0.00				0.14	0.16	-2.18	16
4_H5	MATK	3	3	5	5				0.80	0.83	1.29	1.18				1.02	0.25	0.52	44
4_A6	TESK2	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	14
4_B6	AAK1	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	1
4_C6	SCAP1	4	4	2	3				1.07	1.10	0.52	0.71				0.85	0.28	-0.01	0
4_D6	MUSK	4	3	2	3				1.07	0.83	0.52	0.71				0.78	0.23	-0.22	5
4_E6	PRKWNK3	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	8
4_F6	DUSTYPK	4	4	5	2				1.07	1.10	1.29	0.47				0.98	0.36	0.40	24
4_G6	GK	2	1	4	2				0.53	0.28	1.03	0.47				0.58	0.32	-0.83	22
4_H6	RPS6KB1	3	2	2	3				0.80	0.55	0.52	0.71				0.64	0.13	-0.64	21
4_A7	EPHA7	4	3	3	4				1.07	0.83	0.77	0.94				0.90	0.13	0.15	12
4_B7	PIK3R2	2	3	2	1				0.53	0.83	0.52	0.24				0.53	0.24	-0.99	22
4_C7	CDC2L2	3	4	5	3				0.80	1.10	1.29	0.71				0.97	0.27	0.37	9
4_D7	ULK2	3	3	5	4				0.80	0.83	1.29	0.94				0.96	0.23	0.34	14
4_E7	PRKCZ	3	2	3	3				0.80	0.55	0.77	0.71				0.71	0.11	-0.44	18
4_F7	FLJ23356	4	4	5	5				1.07	1.10	1.29	1.18				1.16	0.10	0.93	13
4_G7	KSR2	4	4	4	3				1.07	1.10	1.03	0.71				0.98	0.18	0.38	21
4_H7	RFK	4	4	5	5				1.07	1.10	1.29	1.18				1.16	0.10	0.93	32

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
4_A8	STK35	2	3	4	4				0.53	0.83	1.03	0.94				0.83	0.22	-0.06	11
4_B8	IRAK2	3	3	5	5				0.80	0.83	1.29	1.18				1.02	0.25	0.52	1
4_C8	STK3	3	4	5	4				0.80	1.10	1.29	0.94				1.03	0.21	0.55	10
4_D8	MGC8407	4	3	0	0				1.07	0.83	0.00	0.00				0.47	0.56	-1.15	4
4_E8	ITK	4	2	4	4				1.07	0.55	1.03	0.94				0.90	0.24	0.14	10
4_F8	PDK3	3	4	2	3				0.80	1.10	0.52	0.71				0.78	0.25	-0.22	2
4_G8	PRKAR1A	3	4	3	5				0.80	1.10	0.77	1.18				0.96	0.21	0.34	9
4_H8	TTBK2	4	4	5	5				1.07	1.10	1.29	1.18				1.16	0.10	0.93	38
4_A9	STK11	4	2	3	3				1.07	0.55	0.77	0.71				0.77	0.22	-0.24	26
4_B9	ACVR1C	4	4	4	5				1.07	1.10	1.03	1.18				1.09	0.06	0.74	18
4_C9	FLT4	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	11
4_D9	AURKA	2	4	3	2				0.53	1.10	0.77	0.47				0.72	0.29	-0.40	10
4_E9	CAMKK1	4	4	2	4				1.07	1.10	0.52	0.94				0.91	0.27	0.17	7
4_F9	DGKA	4	3	2	3				1.07	0.83	0.52	0.71				0.78	0.23	-0.22	8
4_G9	NUP62	3	4	4	4				0.80	1.10	1.03	0.94				0.97	0.13	0.36	21
4_H9	CDC42BPA	2	4	2	2				0.53	1.10	0.52	0.47				0.66	0.30	-0.60	40
4_A10	JAK2	4	4	4	5				1.07	1.10	1.03	1.18				1.09	0.06	0.74	8
4_B10	ACVR1B	3	4	4	5				0.80	1.10	1.03	1.18				1.03	0.16	0.53	3
4_C10	CDC2L1	4	4	3	3				1.07	1.10	0.77	0.71				0.91	0.20	0.18	13
4_D10	PAK4	3	3	3	4				0.80	0.83	0.77	0.94				0.84	0.07	-0.05	21
4_E10	STK36	2	2	5	4				0.53	0.55	1.29	0.94				0.83	0.36	-0.07	5
4_F10	C9ORF12	0	4	3	4				0.00	1.10	0.77	0.94				0.70	0.49	-0.45	18
4_G10	ROR2	4	4	4	4				1.07	1.10	1.03	0.94				1.04	0.07	0.56	13
4_H10	GSG2	2	3	3	2				0.53	0.83	0.77	0.47				0.65	0.18	-0.61	11
4_A11	BRD2	4	4	3	3				1.07	1.10	0.77	0.71				0.91	0.20	0.18	22
4_B11	CPNE3	3	3	4	4				0.80	0.83	1.03	0.94				0.90	0.11	0.15	9
4_C11	MAP2K7	3	3	0	1				0.80	0.83	0.00	0.24				0.47	0.41	-1.18	3
4_D11	MAPK1	4	4	3	3				1.07	1.10	0.77	0.71				0.91	0.20	0.18	4
4_E11	MAPK8	4	4	3	2				1.07	1.10	0.77	0.47				0.85	0.29	0.00	6
4_F11	PTK6	0	0	2	2				0.00	0.00	0.52	0.47				0.25	0.29	-1.84	12
4_G11	PIP5K2C	4	3	5	6				1.07	0.83	1.29	1.41				1.15	0.26	0.90	14
4_H11	HIPK1	4	4	3	3				1.07	1.10	0.77	0.71				0.91	0.20	0.18	20
5_A1	NEG	3	3	2	1														
5_B1	NEG	2	2	1	1														
5_C1	NEG	2	3	1	1														
5_D1	NEG	4	3	2	3														
5_E1	NEG	3	2	2	2														
5_F1	NEG	3	2	2	3														
5_G1	NEG	1	1	2	2														
5_H1	NEG	3	3	1	1				2.63	2.38	1.63	1.75				2.09			0
5_A12	MEK	2	3	0	1														
5_B12	MEK	1	1	1	1														
5_C12	MEK	1	1	1	1														
5_D12	MEK	2	3	0	2														
5_E12	MEK	0	1	1	0				0.46	0.76	0.37	0.57				0.54			0
5_F12	TOX	0	0	0	0														
5_G12	TOX	0	0	0	0														
5_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
5_A2	MAP3K11	1	1	0	0				0.38	0.42	0.00	0.00				0.20	0.23	-1.98	18
5_B2	CDK5	2	2	0	0				0.76	0.84	0.00	0.00				0.40	0.46	-1.37	19
5_C2	ITPK1	2	3	0	0				0.76	1.26	0.00	0.00				0.51	0.62	-1.05	23
5_D2	BCR	3	2	1	1				1.14	0.84	0.62	0.57				0.79	0.26	-0.18	28
5_E2	ADCK2	1	2	2	2				0.38	0.84	1.23	1.14				0.90	0.38	0.14	27
5_F2	SPHK1	2	2	2	2				0.76	0.84	1.23	1.14				0.99	0.23	0.43	36
5_G2	PRKY	2	3	2	2				0.76	1.26	1.23	1.14				1.10	0.23	0.75	34
5_H2	MERTK	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	28
5_A3	AURKB	2	1	0	0				0.76	0.42	0.00	0.00				0.30	0.37	-1.69	10
5_B3	ADCK4	1	1	0	0				0.38	0.42	0.00	0.00				0.20	0.23	-1.98	0
5_C3	ILK-2	2	2	1	2				0.76	0.84	0.62	1.14				0.84	0.22	-0.04	37
5_D3	DCK	3	2	3	3				1.14	0.84	1.85	1.71				1.39	0.47	1.62	16
5_E3	PI4K2B	3	3	1	1				1.14	1.26	0.62	0.57				0.90	0.36	0.14	41
5_F3	ASK	4	3	2	2				1.52	1.26	1.23	1.14				1.29	0.16	1.33	17
5_G3	MAP3K1	4	4	3	3				1.52	1.68	1.85	1.71				1.69	0.13	2.55	44
5_H3	CERK	3	4	2	2				1.14	1.68	1.23	1.14				1.30	0.26	1.36	21
5_A4	LIMK2	3	2	1	1				1.14	0.84	0.62	0.57				0.79	0.26	-0.18	14
5_B4	HRI	2	2	1	1				0.76	0.84	0.62	0.57				0.70	0.13	-0.47	14
5_C4	EPHA2	3	2	2	3				1.14	0.84	1.23	1.71				1.23	0.36	1.16	29
5_D4	MGC16169	1	1	1	0				0.38	0.42	0.62	0.00				0.35	0.26	-1.51	17
5_E4	TRIB3	1	2	2	3				0.38	0.84	1.23	1.71				1.04	0.57	0.58	0
5_F4	PDGFRB	3	2	2	2				1.14	0.84	1.23	1.14				1.09	0.17	0.72	15
5_G4	ZAP70	2	2	2	1				0.76	0.84	1.23	0.57				0.85	0.28	0.00	3

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
5_H4	NUCKS	3	4	2	2				1.14	1.68	1.23	1.14				1.30	0.26	1.36	23
5_A5	GRK4	2	2	2	3				0.76	0.84	1.23	1.71				1.14	0.44	0.87	0
5_B5	MAPKAPK3	3	2	0	0				1.14	0.84	0.00	0.00				0.50	0.59	-1.08	3
5_C5	PHKG1	2	2	2	2				0.76	0.84	1.23	1.14				0.99	0.23	0.43	8
5_D5	STK22D	3	3	2	3				1.14	1.26	1.23	1.71				1.34	0.26	1.48	0
5_E5	FLJ23074	2	2	0	0				0.76	0.84	0.00	0.00				0.40	0.46	-1.37	0
5_F5	FGR	4	4	1	1				1.52	1.68	0.62	0.57				1.10	0.59	0.75	9
5_G5	STK23	4	2	3	3				1.52	0.84	1.85	1.71				1.48	0.45	1.91	5
5_H5	CIT	3	2	0	0				1.14	0.84	0.00	0.00				0.50	0.59	-1.08	28
5_A6	LOC340156	1	2	2	2				0.38	0.84	1.23	1.14				0.90	0.38	0.14	15
5_B6	ACVR2B	3	3	3	2				1.14	1.26	1.85	1.14				1.35	0.34	1.51	26
5_C6	FUK	4	4	1	1				1.52	1.68	0.62	0.57				1.10	0.59	0.75	24
5_D6	SRP72	3	3	2	3				1.14	1.26	1.23	1.71				1.34	0.26	1.48	47
5_E6	HIPK2	2	1	2	2				0.76	0.42	1.23	1.14				0.89	0.37	0.11	42
5_F6	ROR1	4	4	3	2				1.52	1.68	1.85	1.14				1.55	0.30	2.12	32
5_G6	PTK7	2	4	2	3				0.76	1.68	1.23	1.71				1.35	0.45	1.51	1
5_H6	CDKL4	3	3	3	3				1.14	1.26	1.85	1.71				1.49	0.34	1.94	37
5_A7	PRKX	2	1	3	2				0.76	0.42	1.85	1.14				1.04	0.61	0.58	0
5_B7	EGFR	1	1	0	0				0.38	0.42	0.00	0.00				0.20	0.23	-1.98	25
5_C7	AK3	4	3	1	1				1.52	1.26	0.62	0.57				0.99	0.47	0.43	21
5_D7	DGKI	2	2	2	1				0.76	0.84	1.23	0.57				0.85	0.28	0.00	11
5_E7	PRPS1	2	2	2	2				0.76	0.84	1.23	1.14				0.99	0.23	0.43	16
5_F7	CRK7	2	2	1	2				0.76	0.84	0.62	1.14				0.84	0.22	-0.04	28
5_G7	RPS6KL1	4	3	2	2				1.52	1.26	1.23	1.14				1.29	0.16	1.33	16
5_H7	KIAA2002	2	1	1	1				0.76	0.42	0.62	0.57				0.59	0.14	-0.79	29
5_A8	GNE	3	1	0	0				1.14	0.42	0.00	0.00				0.39	0.54	-1.40	13
5_B8	ROS1	2	1	1	1				0.76	0.42	0.62	0.57				0.59	0.14	-0.79	11
5_C8	CSNK2A2	1	3	0	0				0.38	1.26	0.00	0.00				0.41	0.60	-1.34	18
5_D8	CSK	4	2	1	1				1.52	0.84	0.62	0.57				0.89	0.44	0.11	20
5_E8	CSNK1D	2	2	3	2				0.76	0.84	1.85	1.14				1.15	0.49	0.90	0
5_F8	CDKN1B	3	2	2	2				1.14	0.84	1.23	1.14				1.09	0.17	0.72	17
5_G8	RPS6KA6	2	3	3	2				0.76	1.26	1.85	1.14				1.25	0.45	1.22	20
5_H8	OSR1	2	1	2	1				0.76	0.42	1.23	0.57				0.75	0.35	-0.32	20
5_A9	PRKAR1B	0	3	0	1				0.00	1.26	0.00	0.57				0.46	0.60	-1.20	0
5_B9	HK3	1	3	1	2				0.38	1.26	0.62	1.14				0.85	0.42	-0.01	21
5_C9	VRK2	4	2	2	3				1.52	0.84	1.23	1.71				1.33	0.38	1.45	0
5_D9	IKBKAP	3	2	2	2				1.14	0.84	1.23	1.14				1.09	0.17	0.72	8
5_E9	DYRK3	4	3	3	3				1.52	1.26	1.85	1.71				1.59	0.25	2.23	5
5_F9	PACSIN1	1	3	2	1				0.38	1.26	1.23	0.57				0.86	0.45	0.03	18
5_G9	SRPK1	1	3	0	0				0.38	1.26	0.00	0.00				0.41	0.60	-1.34	20
5_H9	RAGE	3	2	2	2				1.14	0.84	1.23	1.14				1.09	0.17	0.72	22
5_A10	MVK	1	0	1	1				0.38	0.00	0.62	0.57				0.39	0.28	-1.40	0
5_B10	DDR2	2	1	2	3				0.76	0.42	1.23	1.71				1.03	0.56	0.55	3
5_C10	KALRN	1	4	2	4				0.38	1.68	1.23	2.29				1.40	0.80	1.65	0
5_D10	ADRBK2	2	4	1	1				0.76	1.68	0.62	0.57				0.91	0.52	0.17	10
5_E10	TYK2	2	4	0	0				0.76	1.68	0.00	0.00				0.61	0.80	-0.73	3
5_F10	DAPK3	3	2	3	3				1.14	0.84	1.85	1.71				1.39	0.47	1.62	5
5_G10	PRKACA	4	2	3	3				1.52	0.84	1.85	1.71				1.48	0.45	1.91	0
5_H10	BMPR2	2	3	2	2				0.76	1.26	1.23	1.14				1.10	0.23	0.75	0
5_A11	PKIB	2	1	2	3				0.76	0.42	1.23	1.71				1.03	0.56	0.55	14
5_B11	DAPK2	3	3	2	2				1.14	1.26	1.23	1.14				1.19	0.06	1.04	0
5_C11	ARAF1	3	2	3	2				1.14	0.84	1.85	1.14				1.24	0.43	1.19	0
5_D11	ICK	2	2	0	0				0.76	0.84	0.00	0.00				0.40	0.46	-1.37	0
5_E11	MYO3A	4	3	0	0				1.52	1.26	0.00	0.00				0.70	0.81	-0.47	20
5_F11	PIK3R1	4	3	2	2				1.52	1.26	1.23	1.14				1.29	0.16	1.33	7
5_G11	PTK2B	4	3	2	2				1.52	1.26	1.23	1.14				1.29	0.16	1.33	16
5_H11	NRBP2	2	1	1	1				0.76	0.42	0.62	0.57				0.59	0.14	-0.79	18
6_A1	NEG	4	4	4	4														
6_B1	NEG	4	4	3	4														
6_C1	NEG	2	2	3	3														
6_D1	NEG	4	4	5	4														
6_E1	NEG	4	4	4	3														
6_F1	NEG	3	3	4	4														
6_G1	NEG	2	3	5	5														
6_H1	NEG	4	4	3	3				3.38	3.50	3.88	3.75				3.63			0
6_A12	MEK	3	3	3	3														
6_B12	MEK	3	2	3	3														
6_C12	MEK	3	3	2	2														
6_D12	MEK	0	0	3	3														
6_E12	MEK	2	2	2	2				0.65	0.57	0.67	0.69				0.65			8
6_F12	TOX	0	0	0	0														

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
6_G12	TOX	0	0	0	0														
6_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
6_A2	MAP2K4	1	1	2	2				0.30	0.29	0.52	0.53				0.41	0.14	-1.35	33
6_B2	CDK7	3	4	2	3				0.89	1.14	0.52	0.80				0.84	0.26	-0.05	9
6_C2	STK17A	2	3	3	4				0.59	0.86	0.77	1.07				0.82	0.20	-0.09	0
6_D2	MGC4796	4	3	4	5				1.19	0.86	1.03	1.33				1.10	0.20	0.76	8
6_E2	SRPK2	4	4	4	5				1.19	1.14	1.03	1.33				1.17	0.12	0.98	0
6_F2	CDKN1A	2	2	4	3				0.59	0.57	1.03	0.80				0.75	0.22	-0.31	20
6_G2	MAST2	4	4	4	4				1.19	1.14	1.03	1.07				1.11	0.07	0.77	2
6_H2	TNK1	1	1	2	2				0.30	0.29	0.52	0.53				0.41	0.14	-1.35	8
6_A3	FLJ13052	3	2	4	3				0.89	0.57	1.03	0.80				0.82	0.19	-0.09	10
6_B3	LRRK1	4	3	4	4				1.19	0.86	1.03	1.07				1.04	0.14	0.56	0
6_C3	TEX14	4	4	4	4				1.19	1.14	1.03	1.07				1.11	0.07	0.77	6
6_D3	PKIA	2	3	4	3				0.59	0.86	1.03	0.80				0.82	0.18	-0.10	1
6_E3	CLK2	3	3	1	0				0.89	0.86	0.26	0.00				0.50	0.44	-1.07	0
6_F3	PANK2	4	4	4	5				1.19	1.14	1.03	1.33				1.17	0.12	0.98	11
6_G3	CDK5R1	4	4	3	4				1.19	1.14	0.77	1.07				1.04	0.19	0.58	9
6_H3	STK29	3	3	2	3				0.89	0.86	0.52	0.80				0.77	0.17	-0.26	11
6_A4	TLK2	4	4	6	4				1.19	1.14	1.55	1.07				1.24	0.21	1.17	0
6_B4	FLJ25006	4	3	3	3				1.19	0.86	0.77	0.80				0.90	0.19	0.16	4
6_C4	MAPKAPK2	3	3	5	4				0.89	0.86	1.29	1.07				1.03	0.20	0.53	25
6_D4	RBKS	2	3	4	4				0.59	0.86	1.03	1.07				0.89	0.22	0.11	5
6_E4	TGFBR2	4	4	4	4				1.19	1.14	1.03	1.07				1.11	0.07	0.77	9
6_F4	EPHA3	2	2	4	4				0.59	0.57	1.03	1.07				0.82	0.27	-0.11	0
6_G4	WEE1	4	4	4	4				1.19	1.14	1.03	1.07				1.11	0.07	0.77	6
6_H4	NTRK3	4	4	3	3				1.19	1.14	0.77	0.80				0.98	0.22	0.37	0
6_A5	TJP2	4	4	6	5				1.19	1.14	1.55	1.33				1.30	0.18	1.37	18
6_B5	MAPK14	4	4	5	5				1.19	1.14	1.29	1.33				1.24	0.09	1.17	14
6_C5	LTK	4	4	5	5				1.19	1.14	1.29	1.33				1.24	0.09	1.17	4
6_D5	PHKA1	2	2	5	4				0.59	0.57	1.29	1.07				0.88	0.36	0.08	5
6_E5	STK22B	3	4	4	4				0.89	1.14	1.03	1.07				1.03	0.11	0.55	0
6_F5	PYCS	2	2	3	0				0.59	0.57	0.77	0.00				0.48	0.34	-1.12	0
6_G5	CDK6	3	3	1	2				0.89	0.86	0.26	0.53				0.63	0.30	-0.66	0
6_H5	LMTK2	3	3	2	3				0.89	0.86	0.52	0.80				0.77	0.17	-0.26	11
6_A6	ADCK5	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	38
6_B6	TBK1	3	4	5	5				0.89	1.14	1.29	1.33				1.16	0.20	0.95	2
6_C6	CASK	3	3	2	2				0.89	0.86	0.52	0.53				0.70	0.20	-0.47	10
6_D6	ERN1	4	4	0	0				1.19	1.14	0.00	0.00				0.58	0.67	-0.82	33
6_E6	GAK	3	3	0	0				0.89	0.86	0.00	0.00				0.44	0.50	-1.26	0
6_F6	IHPK1	3	3	3	3				0.89	0.86	0.77	0.80				0.83	0.05	-0.07	0
6_G6	CDKN2D	3	3	4	4				0.89	0.86	1.03	1.07				0.96	0.10	0.33	3
6_H6	DTYMK	4	4	3	2				1.19	1.14	0.77	0.53				0.91	0.31	0.17	0
6_A7	MAPK11	3	3	5	5				0.89	0.86	1.29	1.33				1.09	0.25	0.73	4
6_B7	ERN2	3	2	4	4				0.89	0.57	1.03	1.07				0.89	0.23	0.11	21
6_C7	DYRK1B	4	3	3	3				1.19	0.86	0.77	0.80				0.90	0.19	0.16	18
6_D7	STYK1	4	3	3	4				1.19	0.86	0.77	1.07				0.97	0.19	0.36	6
6_E7	RPS6KA2	0	0	2	1				0.00	0.00	0.52	0.27				0.20	0.25	-2.00	0
6_F7	BRD4	2	2	4	4				0.59	0.57	1.03	1.07				0.82	0.27	-0.11	0
6_G7	PRKCQ	3	3	4	3				0.89	0.86	1.03	0.80				0.89	0.10	0.13	9
6_H7	PIK3R4	4	4	1	1				1.19	1.14	0.26	0.27				0.71	0.52	-0.42	2
6_A8	PGK2	3	3	6	5				0.89	0.86	1.55	1.33				1.16	0.34	0.93	14
6_B8	PRKCI	4	2	4	4				1.19	0.57	1.03	1.07				0.96	0.27	0.34	22
6_C8	SBK1	4	3	3	3				1.19	0.86	0.77	0.80				0.90	0.19	0.16	21
6_D8	ARK5	2	3	2	2				0.59	0.86	0.52	0.53				0.62	0.16	-0.69	37
6_E8	MAPK6	3	0	4	4				0.89	0.00	1.03	1.07				0.75	0.50	-0.32	0
6_F8	TEC	2	2	4	4				0.59	0.57	1.03	1.07				0.82	0.27	-0.11	0
6_G8	PRKAG2	4	3	5	5				1.19	0.86	1.29	1.33				1.17	0.22	0.96	36
6_H8	GCK	4	4	2	2				1.19	1.14	0.52	0.53				0.84	0.37	-0.02	11
6_A9	BUB1B	2	4	5	5				0.59	1.14	1.29	1.33				1.09	0.34	0.72	12
6_B9	IKBKE	2	4	3	3				0.59	1.14	0.77	0.80				0.83	0.23	-0.08	24
6_C9	PCTK1	1	4	4	5				0.30	1.14	1.03	1.33				0.95	0.45	0.30	37
6_D9	PIP5K1B	4	2	4	4				1.19	0.57	1.03	1.07				0.96	0.27	0.34	22
6_E9	GRK7	4	2	3	3				1.19	0.57	0.77	0.80				0.83	0.26	-0.06	11
6_F9	FRDA	4	2	2	3				1.19	0.57	0.52	0.80				0.77	0.30	-0.26	9
6_G9	MAP3K3	3	4	1	1				0.89	1.14	0.26	0.27				0.64	0.45	-0.65	9
6_H9	EPHB2	4	4	2	2				1.19	1.14	0.52	0.53				0.84	0.37	-0.02	21
6_A10	MAP2K5	2	3	3	3				0.59	0.86	0.77	0.80				0.76	0.11	-0.29	11
6_B10	CSNK1A1L	4	2	4	5				1.19	0.57	1.03	1.33				1.03	0.33	0.54	0
6_C10	MAP3K4	3	2	4	5				0.89	0.57	1.03	1.33				0.96	0.32	0.32	33
6_D10	DGUOK	2	4	3	3				0.59	1.14	0.77	0.80				0.83	0.23	-0.08	0
6_E10	ADRBK1	3	4	5	5				0.89	1.14	1.29	1.33				1.16	0.20	0.95	0

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
6_F10	PRPS2	3	4	3	3				0.89	1.14	0.77	0.80				0.90	0.17	0.15	18
6_G10	FGFRL1	3	4	4	4				0.89	1.14	1.03	1.07				1.03	0.11	0.55	9
6_H10	SGKL	0	0	1	1				0.00	0.00	0.26	0.27				0.13	0.15	-2.19	2
6_A11	LAK	2	1	0	0				0.59	0.29	0.00	0.00				0.22	0.28	-1.92	14
6_B11	PIP5K1A	2	3	3	4				0.59	0.86	0.77	1.07				0.82	0.20	-0.09	0
6_C11	STK38L	3	2	3	3				0.89	0.57	0.77	0.80				0.76	0.13	-0.28	20
6_D11	PANK3	2	3	5	4				0.59	0.86	1.29	1.07				0.95	0.30	0.30	0
6_E11	CDK10	2	2	3	3				0.59	0.57	0.77	0.80				0.68	0.12	-0.51	47
6_F11	AK5	3	3	4	4				0.89	0.86	1.03	1.07				0.96	0.10	0.33	36
6_G11	NME1	4	4	4	3				1.19	1.14	1.03	0.80				1.04	0.17	0.57	6
6_H11	DLG3	3	3	2	2				0.89	0.86	0.52	0.53				0.70	0.20	-0.47	3
7_A1	NEG	4	4	4	3														
7_B1	NEG	4	4	4	4														
7_C1	NEG	3	3	4	4														
7_D1	NEG	3	2	5	5														
7_E1	NEG	4	4	5	5														
7_F1	NEG	4	4	5	5														
7_G1	NEG	3	3	5	5														
7_H1	NEG	4	4	5	5				3.63	3.50	4.63	4.50				4.06			0
7_A12	MEK	2	2	3	3														
7_B12	MEK	2	3	3	4														
7_C12	MEK	3	4	3	3														
7_D12	MEK	1	1	3	4														
7_E12	MEK	4	3	3	4				0.66	0.74	0.65	0.80				0.71			0
7_F12	TOX	0	0	0	0														
7_G12	TOX	0	0	0	0														
7_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
7_A2	RET	2	2	3	4				0.55	0.57	0.65	0.89				0.67	0.15	-0.57	15
7_B2	CDC2L5	1	0	0	0				0.28	0.00	0.00	0.00				0.07	0.14	-2.38	2
7_C2	KCNH2	3	3	5	6				0.83	0.86	1.08	1.33				1.02	0.23	0.52	5
7_D2	PRKCD	0	1	3	3				0.00	0.29	0.65	0.67				0.40	0.32	-1.37	0
7_E2	STK32B	4	4	4	5				1.10	1.14	0.86	1.11				1.06	0.13	0.62	0
7_F2	PKMYT1	4	4	5	5				1.10	1.14	1.08	1.11				1.11	0.03	0.78	2
7_G2	SLK	4	4	5	5				1.10	1.14	1.08	1.11				1.11	0.03	0.78	0
7_H2	PRKD2	1	1	4	4				0.28	0.29	0.86	0.89				0.58	0.34	-0.83	0
7_A3	MAP2K6	3	2	4	5				0.83	0.57	0.86	1.11				0.84	0.22	-0.03	0
7_B3	ALK	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	24
7_C3	CDKL1	4	4	0	0				1.10	1.14	0.00	0.00				0.56	0.65	-0.88	0
7_D3	ATR	4	3	4	3				1.10	0.86	0.86	0.67				0.87	0.18	0.06	20
7_E3	MAP3K7IP1	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	0
7_F3	PACE-1	2	3	5	4				0.55	0.86	1.08	0.89				0.84	0.22	-0.02	4
7_G3	CDKN1C	2	2	4	4				0.55	0.57	0.86	0.89				0.72	0.18	-0.40	0
7_H3	CAMKK1	4	3	5	5				1.10	0.86	1.08	1.11				1.04	0.12	0.57	0
7_A4	RPS6KC1	4	4	5	5				1.10	1.14	1.08	1.11				1.11	0.03	0.78	14
7_B4	SPEG	4	4	3	4				1.10	1.14	0.65	0.89				0.95	0.23	0.28	5
7_C4	NTRK2	1	1	3	5				0.28	0.29	0.65	1.11				0.58	0.39	-0.83	8
7_D4	CAMK2A	4	4	4	6				1.10	1.14	0.86	1.33				1.11	0.19	0.79	3
7_E4	PRKR	3	3	5	6				0.83	0.86	1.08	1.33				1.02	0.23	0.52	0
7_F4	IKBKB	3	3	3	3				0.83	0.86	0.65	0.67				0.75	0.11	-0.31	5
7_G4	PIP5K1C	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	0
7_H4	LYK5	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	0
7_A5	NME7	4	4	6	5				1.10	1.14	1.30	1.11				1.16	0.09	0.95	1
7_B5	TP53RK	3	3	4	5				0.83	0.86	0.86	1.11				0.92	0.13	0.19	8
7_C5	AATK	4	3	4	6				1.10	0.86	0.86	1.33				1.04	0.23	0.57	0
7_D5	KDR	3	3	4	4				0.83	0.86	0.86	0.89				0.86	0.03	0.02	0
7_E5	RIPK3	0	0	3	4				0.00	0.00	0.65	0.89				0.38	0.45	-1.42	0
7_F5	MAP3K13	2	3	3	5				0.55	0.86	0.65	1.11				0.79	0.25	-0.18	0
7_G5	PFKL	2	3	6	7				0.55	0.86	1.30	1.56				1.07	0.45	0.65	5
7_H5	COMMD3	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	0
7_A6	MAP3K10	4	4	6	5				1.10	1.14	1.30	1.11				1.16	0.09	0.95	14
7_B6	EFNA5	3	2	3	5				0.83	0.57	0.65	1.11				0.79	0.24	-0.19	0
7_C6	PRKCG	2	2	5	6				0.55	0.57	1.08	1.33				0.88	0.39	0.10	21
7_D6	TAF1	3	3	4	4				0.83	0.86	0.86	0.89				0.86	0.03	0.02	0
7_E6	ULK4	4	4	6	5				1.10	1.14	1.30	1.11				1.16	0.09	0.95	0
7_F6	CAMK1D	4	4	4	5				1.10	1.14	0.86	1.11				1.06	0.13	0.62	0
7_G6	PCK1	4	4	4	3				1.10	1.14	0.86	0.67				0.94	0.22	0.28	0
7_H6	TK2	2	2	4	4				0.55	0.57	0.86	0.89				0.72	0.18	-0.40	0
7_A7	UCK1	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	9
7_B7	MAPK13	3	3	4	5				0.83	0.86	0.86	1.11				0.92	0.13	0.19	3
7_C7	STK38	3	2	1	2				0.83	0.57	0.22	0.44				0.51	0.26	-1.03	0
7_D7	CDK11	4	4	6	6				1.10	1.14	1.30	1.33				1.22	0.11	1.12	0

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
7_E7	SPHK2	4	3	4	4				1.10	0.86	0.86	0.89				0.93	0.12	0.23	0
7_F7	IRAK3	0	0	4	4				0.00	0.00	0.86	0.89				0.44	0.51	-1.26	0
7_G7	CDK9	4	3	4	5				1.10	0.86	0.86	1.11				0.98	0.14	0.40	0
7_H7	CSNK2A1	4	4	6	6				1.10	1.14	1.30	1.33				1.22	0.11	1.12	0
7_A8	FGFR2	3	2	5	4				0.83	0.57	1.08	0.89				0.84	0.21	-0.03	18
7_B8	TRIB2	4	4	0	0				1.10	1.14	0.00	0.00				0.56	0.65	-0.88	8
7_C8	PMVK	4	4	5	4				1.10	1.14	1.08	0.89				1.05	0.11	0.61	8
7_D8	SNARK	4	4	4	4				1.10	1.14	0.86	0.89				1.00	0.14	0.45	1
7_E8	TESK1	2	2	2	2				0.55	0.57	0.43	0.44				0.50	0.07	-1.07	14
7_F8	MOS	3	4	4	3				0.83	1.14	0.86	0.67				0.88	0.20	0.07	0
7_G8	FGFR4	4	4	4	3				1.10	1.14	0.86	0.67				0.94	0.22	0.28	3
7_H8	SRMS	0	0	4	4				0.00	0.00	0.86	0.89				0.44	0.51	-1.26	0
7_A9	ERBB3	4	4	5	4				1.10	1.14	1.08	0.89				1.05	0.11	0.61	39
7_B9	DGKH	2	4	5	5				0.55	1.14	1.08	1.11				0.97	0.28	0.36	13
7_C9	EPHA1	0	0	5	4				0.00	0.00	1.08	0.89				0.49	0.57	-1.09	0
7_D9	SCYL1	4	4	5	4				1.10	1.14	1.08	0.89				1.05	0.11	0.61	0
7_E9	CSNK2B	4	4	5	4				1.10	1.14	1.08	0.89				1.05	0.11	0.61	9
7_F9	PIP5K2B	4	3	4	4				1.10	0.86	0.86	0.89				0.93	0.12	0.23	0
7_G9	CRKL	4	4	6	4				1.10	1.14	1.30	0.89				1.11	0.17	0.78	1
7_H9	MAP4K2	2	3	4	4				0.55	0.86	0.86	0.89				0.79	0.16	-0.19	0
7_A10	NEK3	1	0	1	0				0.28	0.00	0.22	0.00				0.12	0.14	-2.22	14
7_B10	FRK	3	3	4	3				0.83	0.86	0.86	0.67				0.80	0.09	-0.15	7
7_C10	RAF1	3	3	4	5				0.83	0.86	0.86	1.11				0.92	0.13	0.19	4
7_D10	PRKAB2	3	3	1	3				0.83	0.86	0.22	0.67				0.64	0.30	-0.64	0
7_E10	ERBB4	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	10
7_F10	STK4	4	4	6	6				1.10	1.14	1.30	1.33				1.22	0.11	1.12	1
7_G10	RPS6KA5	4	4	4	3				1.10	1.14	0.86	0.67				0.94	0.22	0.28	5
7_H10	KIAA1811	4	4	5	5				1.10	1.14	1.08	1.11				1.11	0.03	0.78	0
7_A11	COL4A3BP	3	4	4	4				0.83	1.14	0.86	0.89				0.93	0.14	0.24	42
7_B11	PIK3CB	1	2	1	3				0.28	0.57	0.22	0.67				0.43	0.22	-1.28	0
7_C11	PRKCSH	4	4	5	4				1.10	1.14	1.08	0.89				1.05	0.11	0.61	0
7_D11	MGC4796	4	4	2	2				1.10	1.14	0.43	0.44				0.78	0.40	-0.22	0
7_E11	PFKFB4	4	4	0	0				1.10	1.14	0.00	0.00				0.56	0.65	-0.88	0
7_F11	BAIAP1	1	0	4	4				0.28	0.00	0.86	0.89				0.51	0.44	-1.05	0
7_G11	STK32C	4	4	5	6				1.10	1.14	1.08	1.33				1.17	0.11	0.95	0
7_H11	PDK1	4	3	0	0				1.10	0.86	0.00	0.00				0.49	0.57	-1.10	0
8_A1	NEG	4	4	4	4														
8_B1	NEG	4	4	3	4														
8_C1	NEG	4	4	4	3														
8_D1	NEG	4	4	4	4														
8_E1	NEG	4	4	5	4														
8_F1	NEG	4	4	4	4														
8_G1	NEG	4	4	4	4														
8_H1	NEG	4	4	4	4				4.00	4.00	4.00	3.88				3.97			0
8_A12	MEK	1	1	0	0														
8_B12	MEK	4	4	2	1														
8_C12	MEK	3	4	2	2														
8_D12	MEK	3	4	2	3														
8_E12	MEK	3	3	2	3				0.70	0.80	0.40	0.46				0.59			20
8_F12	TOX	0	0	0	0														
8_G12	TOX	0	0	0	0														
8_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
8_A2	MELK	4	4	5	5				1.00	1.00	1.25	1.29				1.14	0.16	0.86	0
8_B2	TGFBR3	4	4	2	3				1.00	1.00	0.50	0.77				0.82	0.24	-0.10	3
8_C2	CSNK1A1	3	3	4	3				0.75	0.75	1.00	0.77				0.82	0.12	-0.10	7
8_D2	JIK	4	4	2	2				1.00	1.00	0.50	0.52				0.75	0.28	-0.30	0
8_E2	PTK9L	4	4	5	4				1.00	1.00	1.25	1.03				1.07	0.12	0.66	5
8_F2	PIM3	4	4	4	6				1.00	1.00	1.00	1.55				1.14	0.27	0.87	0
8_G2	PXK	4	4	3	3				1.00	1.00	0.75	0.77				0.88	0.14	0.09	0
8_H2	SAST	4	3	3	2				1.00	0.75	0.75	0.52				0.75	0.20	-0.30	0
8_A3	PRKCH	1	0	2	2				0.25	0.00	0.50	0.52				0.32	0.24	-1.63	9
8_B3	UMP-CMPK	4	4	6	4				1.00	1.00	1.50	1.03				1.13	0.25	0.85	0
8_C3	NME2	3	3	2	3				0.75	0.75	0.50	0.77				0.69	0.13	-0.48	3
8_D3	PKLR	4	4	5	4				1.00	1.00	1.25	1.03				1.07	0.12	0.66	11
8_E3	PIK3C2A	2	3	6	4				0.50	0.75	1.50	1.03				0.95	0.43	0.28	90
8_F3	LMTK3	3	3	1	1				0.75	0.75	0.25	0.26				0.50	0.29	-1.07	0
8_G3	HUNK	3	2	2	3				0.75	0.50	0.50	0.77				0.63	0.15	-0.67	0
8_H3	NEK2	4	3	4	4				1.00	0.75	1.00	1.03				0.95	0.13	0.28	0
8_A4	PAK3	4	4	5	3				1.00	1.00	1.25	0.77				1.01	0.19	0.47	4
8_B4	CHKA	3	3	2	4				0.75	0.75	0.50	1.03				0.76	0.22	-0.29	0
8_C4	MAK	3	4	3	2				0.75	1.00	0.75	0.52				0.75	0.20	-0.30	26

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
8_D4	PAK7	4	4	5	6				1.00	1.00	1.25	1.55				1.20	0.26	1.06	2
8_E4	STK32A	4	4	4	4				1.00	1.00	1.00	1.03				1.01	0.02	0.47	7
8_F4	CHEK2	4	4	4	5				1.00	1.00	1.00	1.29				1.07	0.15	0.67	0
8_G4	MAP4K1	1	3	4	4				0.25	0.75	1.00	1.03				0.76	0.36	-0.29	5
8_H4	MAPKAPK5	4	4	3	3				1.00	1.00	0.75	0.77				0.88	0.14	0.09	8
8_A5	PFKFB1	3	3	2	3				0.75	0.75	0.50	0.77				0.69	0.13	-0.48	10
8_B5	WNK4	4	4	4	4				1.00	1.00	1.00	1.03				1.01	0.02	0.47	9
8_C5	PIK3CD	4	4	3	2				1.00	1.00	0.75	0.52				0.82	0.23	-0.11	19
8_D5	VRK1	4	4	5	5				1.00	1.00	1.25	1.29				1.14	0.16	0.86	6
8_E5	GTF2H1	1	2	1	3				0.25	0.50	0.25	0.77				0.44	0.25	-1.24	16
8_F5	JAK1	4	4	5	5				1.00	1.00	1.25	1.29				1.14	0.16	0.86	0
8_G5	PCTK2	3	3	3	3				0.75	0.75	0.75	0.77				0.76	0.01	-0.29	1
8_H5	NRK	4	4	4	5				1.00	1.00	1.00	1.29				1.07	0.15	0.67	11
8_A6	INSRR	3	3	2	2				0.75	0.75	0.50	0.52				0.63	0.14	-0.68	0
8_B6	RPS6KA1	3	3	3	3				0.75	0.75	0.75	0.77				0.76	0.01	-0.29	1
8_C6	MAP3K6	4	4	4	4				1.00	1.00	1.00	1.03				1.01	0.02	0.47	4
8_D6	EPHA6	0	0	1	2				0.00	0.00	0.25	0.52				0.19	0.25	-2.01	0
8_E6	TOPK	4	3	5	4				1.00	0.75	1.25	1.03				1.01	0.20	0.47	0
8_F6	EIF2AK3	3	4	3	3				0.75	1.00	0.75	0.77				0.82	0.12	-0.10	4
8_G6	INSR	2	2	2	2				0.50	0.50	0.50	0.52				0.50	0.01	-1.06	0
8_H6	PFKFB2	4	4	5	4				1.00	1.00	1.25	1.03				1.07	0.12	0.66	11
8_A7	DLG2	4	4	2	3				1.00	1.00	0.50	0.77				0.82	0.24	-0.10	1
8_B7	IHPK3	4	4	5	3				1.00	1.00	1.25	0.77				1.01	0.19	0.47	13
8_C7	BRDT	1	2	3	4				0.25	0.50	0.75	1.03				0.63	0.34	-0.67	7
8_D7	GUCY2C	4	4	4	4				1.00	1.00	1.00	1.03				1.01	0.02	0.47	24
8_E7	ZAK	4	4	2	4				1.00	1.00	0.50	1.03				0.88	0.26	0.09	13
8_F7	PIK3C3	3	1	2	2				0.75	0.25	0.50	0.52				0.50	0.20	-1.06	8
8_G7	HIPK4	2	3	0	0				0.50	0.75	0.00	0.00				0.31	0.38	-1.64	0
8_H7	MARK4	4	4	4	5				1.00	1.00	1.00	1.29				1.07	0.15	0.67	3
8_A8	PIP5K3	4	4	3	6				1.00	1.00	0.75	1.55				1.07	0.34	0.68	5
8_B8	BMX	4	4	5	8				1.00	1.00	1.25	2.06				1.33	0.50	1.45	11
8_C8	EIF2AK4	4	4	5	3				1.00	1.00	1.25	0.77				1.01	0.19	0.47	17
8_D8	PNCK	4	4	4	5				1.00	1.00	1.00	1.29				1.07	0.15	0.67	6
8_E8	PDK4	1	1	2	4				0.25	0.25	0.50	1.03				0.51	0.37	-1.05	18
8_F8	ROCK2	4	4	3	6				1.00	1.00	0.75	1.55				1.07	0.34	0.68	0
8_G8	SIK2	2	3	1	0				0.50	0.75	0.25	0.00				0.38	0.32	-1.45	0
8_H8	EPHB1	2	1	3	2				0.50	0.25	0.75	0.52				0.50	0.20	-1.06	9
8_A9	BMPR1A	2	3	3	4				0.50	0.75	0.75	1.03				0.76	0.22	-0.29	13
8_B9	CLK4	4	4	6	6				1.00	1.00	1.50	1.55				1.26	0.30	1.25	5
8_C9	NME6	3	4	4	6				0.75	1.00	1.00	1.55				1.07	0.34	0.68	28
8_D9	VRK3	2	3	4	3				0.50	0.75	1.00	0.77				0.76	0.20	-0.29	7
8_E9	CHUK	3	3	3	3				0.75	0.75	0.75	0.77				0.76	0.01	-0.29	0
8_F9	PIK3C2B	4	4	3	6				1.00	1.00	0.75	1.55				1.07	0.34	0.68	0
8_G9	MET	4	4	5	4				1.00	1.00	1.25	1.03				1.07	0.12	0.66	4
8_H9	MAGI-3	4	4	3	4				1.00	1.00	0.75	1.03				0.95	0.13	0.28	0
8_A10	PRPS1L1	3	3	1	0				0.75	0.75	0.25	0.00				0.44	0.38	-1.26	12
8_B10	PAK2	2	2	3	4				0.50	0.50	0.75	1.03				0.70	0.25	-0.48	20
8_C10	DYRK1A	4	4	5	5				1.00	1.00	1.25	1.29				1.14	0.16	0.86	13
8_D10	PDGFRA	3	3	2	4				0.75	0.75	0.50	1.03				0.76	0.22	-0.29	8
8_E10	TPK1	0	0	1	2				0.00	0.00	0.25	0.52				0.19	0.25	-2.01	0
8_F10	SGK	4	4	4	3				1.00	1.00	1.00	0.77				0.94	0.11	0.28	0
8_G10	FYN	4	4	4	5				1.00	1.00	1.00	1.29				1.07	0.15	0.67	7
8_H10	SSTK	4	4	3	2				1.00	1.00	0.75	0.52				0.82	0.23	-0.11	8
8_A11	IGF2R	3	3	3	2				0.75	0.75	0.75	0.52				0.69	0.12	-0.49	2
8_B11	LOC390226	4	4	3	5				1.00	1.00	0.75	1.29				1.01	0.22	0.48	10
8_C11	CAMK4	4	4	5	6				1.00	1.00	1.25	1.55				1.20	0.26	1.06	19
8_D11	DDR1	4	4	4	5				1.00	1.00	1.00	1.29				1.07	0.15	0.67	37
8_E11	C14ORF20	3	3	4	5				0.75	0.75	1.00	1.29				0.95	0.26	0.29	13
8_F11	CLK3	4	3	4	4				1.00	0.75	1.00	1.03				0.95	0.13	0.28	12
8_G11	DKFZP434C131	3	4	2	3				0.75	1.00	0.50	0.77				0.76	0.20	-0.29	23
8_H11	IKBKG	0	1	2	2				0.00	0.25	0.50	0.52				0.32	0.24	-1.63	18
9_A1	NEG	4	4	3	3														
9_B1	NEG	4	4	3	3														
9_C1	NEG	4	4	3	3														
9_D1	NEG	4	4	3	3														
9_E1	NEG	4	4	3	3														
9_F1	NEG	4	4	4	4														
9_G1	NEG	4	4	3	3														
9_H1	NEG	4	4	3	3				4.00	4.00	3.13	3.13				3.56			0
9_A12	MEK	2	2	1	2														
9_B12	MEK	3	2	1	2														

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
9_C12	MEK	1	1	0	0														
9_D12	MEK	3	2	0	0														
9_E12	MEK	0	0	1	1				0.45	0.35	0.19	0.32				0.33			24
9_F12	TOX	0	0	0	0														
9_G12	TOX	0	0	0	0														
9_H12	TOX	0	0	0	0				0.00	0.00	0.00	0.00				0.00			100
9_A2	N4BP2	3	4	3	2				0.75	1.00	0.96	0.64				0.84	0.17	-0.05	15
9_B2	PRKCA	4	4	4	5				1.00	1.00	1.28	1.60				1.22	0.29	1.12	10
9_C2	GUK1	4	3	2	3				1.00	0.75	0.64	0.96				0.84	0.17	-0.05	9
9_D2	PLK3	4	4	2	2				1.00	1.00	0.64	0.64				0.82	0.21	-0.10	0
9_E2	AMHR2	0	0	1	2				0.00	0.00	0.32	0.64				0.24	0.31	-1.86	11
9_F2	PASK	4	4	1	2				1.00	1.00	0.32	0.64				0.74	0.33	-0.34	1
9_G2	EPHA4	4	4	3	2				1.00	1.00	0.96	0.64				0.90	0.17	0.14	0
9_H2	NEK7	3	3	2	1				0.75	0.75	0.64	0.32				0.62	0.20	-0.72	1
9_A3	NEK1	4	4	3	4				1.00	1.00	0.96	1.28				1.06	0.15	0.63	13
9_B3	CDKL2	2	2	1	1				0.50	0.50	0.32	0.32				0.41	0.10	-1.34	12
9_C3	CKM	2	2	2	2				0.50	0.50	0.64	0.64				0.57	0.08	-0.86	12
9_D3	EFNB3	3	3	3	2				0.75	0.75	0.96	0.64				0.78	0.13	-0.24	8
9_E3	DUSP21	4	4	4	4				1.00	1.00	1.28	1.28				1.14	0.16	0.87	0
9_F3	HUS1	4	4	2	2				1.00	1.00	0.64	0.64				0.82	0.21	-0.10	2
9_G3	DCAMKL1	4	4	4	4				1.00	1.00	1.28	1.28				1.14	0.16	0.87	11
9_H3	PRKWNK1	4	4	5	4				1.00	1.00	1.60	1.28				1.22	0.29	1.12	1
9_A4	XYLB	4	4	2	2				1.00	1.00	0.64	0.64				0.82	0.21	-0.10	19
9_B4	FES	4	4	3	3				1.00	1.00	0.96	0.96				0.98	0.02	0.39	8
9_C4	MAP4K3	3	4	3	3				0.75	1.00	0.96	0.96				0.92	0.11	0.20	1
9_D4	EPHB3	3	3	2	2				0.75	0.75	0.64	0.64				0.70	0.06	-0.48	13
9_E4	CDKN2B	4	3	4	3				1.00	0.75	1.28	0.96				1.00	0.22	0.44	5
9_F4	TLK1	4	4	2	3				1.00	1.00	0.64	0.96				0.90	0.17	0.14	4
9_G4	GK2	3	4	2	3				0.75	1.00	0.64	0.96				0.84	0.17	-0.05	3
9_H4	TSKS	4	3	2	3				1.00	0.75	0.64	0.96				0.84	0.17	-0.05	4
9_A5	RP6-213H19.1	4	4	2	2				1.00	1.00	0.64	0.64				0.82	0.21	-0.10	8
9_B5	PRKAB1	3	3	3	3				0.75	0.75	0.96	0.96				0.86	0.12	0.01	27
9_C5	PRKDC	3	3	5	4				0.75	0.75	1.60	1.28				1.10	0.42	0.74	5
9_D5	PRKCB1	2	4	2	3				0.50	1.00	0.64	0.96				0.78	0.24	-0.24	0
9_E5	DLG1	4	4	4	4				1.00	1.00	1.28	1.28				1.14	0.16	0.87	6
9_F5	PFKM	3	3	3	4				0.75	0.75	0.96	1.28				0.94	0.25	0.25	7
9_G5	KUB3	2	2	2	3				0.50	0.50	0.64	0.96				0.65	0.22	-0.62	8
9_H5	STK19	4	4	4	5				1.00	1.00	1.28	1.60				1.22	0.29	1.12	0
9_A6	SMG1	3	3	3	3				0.75	0.75	0.96	0.96				0.86	0.12	0.01	25
9_B6	HAK	4	4	2	2				1.00	1.00	0.64	0.64				0.82	0.21	-0.10	3
9_C6	PRPF4B	3	3	4	3				0.75	0.75	1.28	0.96				0.94	0.25	0.25	0
9_D6	DGKD	4	4	4	5				1.00	1.00	1.28	1.60				1.22	0.29	1.12	3
9_E6	C9ORF96	0	1	0	1				0.00	0.25	0.00	0.32				0.14	0.17	-2.16	7
9_F6	PRKAA2	0	0	2	1				0.00	0.00	0.64	0.32				0.24	0.31	-1.86	5
9_G6	TRPM7	3	3	3	2				0.75	0.75	0.96	0.64				0.78	0.13	-0.24	9
9_H6	MAPK12	3	2	2	2				0.75	0.50	0.64	0.64				0.63	0.10	-0.67	1
9_A7	IHPK2	4	4	3	2				1.00	1.00	0.96	0.64				0.90	0.17	0.14	17
9_B7	CDK2	3	3	0	1				0.75	0.75	0.00	0.32				0.46	0.36	-1.21	11
9_C7	RIOK3	4	4	1	3				1.00	1.00	0.32	0.96				0.82	0.33	-0.10	0
9_D7	EPHB4	4	4	1	3				1.00	1.00	0.32	0.96				0.82	0.33	-0.10	9
9_E7	MAP2K1	0	0	2	1				0.00	0.00	0.64	0.32				0.24	0.31	-1.86	13
9_F7	ITPKB	0	0	0	1				0.00	0.00	0.00	0.32				0.08	0.16	-2.35	14
9_G7	MYLK	4	3	1	3				1.00	0.75	0.32	0.96				0.76	0.31	-0.29	17
9_H7	GALK1	4	4	4	3				1.00	1.00	1.28	0.96				1.06	0.15	0.63	5
9_A8	ILK	4	4	5	4				1.00	1.00	1.60	1.28				1.22	0.29	1.12	2
9_B8	MAP2K2	3	3	2	3				0.75	0.75	0.64	0.96				0.78	0.13	-0.24	6
9_C8	KIAA1765	4	4	2	3				1.00	1.00	0.64	0.96				0.90	0.17	0.14	11
9_D8	MGC45428	2	2	0	1				0.50	0.50	0.00	0.32				0.33	0.24	-1.59	0
9_E8	GALK2	3	3	2	2				0.75	0.75	0.64	0.64				0.70	0.06	-0.48	23
9_F8	MPP3	3	3	2	3				0.75	0.75	0.64	0.96				0.78	0.13	-0.24	28
9_G8	GUCY2F	4	2	2	1				1.00	0.50	0.64	0.32				0.62	0.29	-0.72	16
9_H8	IPMK	3	3	3	4				0.75	0.75	0.96	1.28				0.94	0.25	0.25	9
9_A9	PSKH2	4	4	2	2				1.00	1.00	0.64	0.64				0.82	0.21	-0.10	3
9_B9	MAPK10	2	2	2	2				0.50	0.50	0.64	0.64				0.57	0.08	-0.86	0
9_C9	EXOSC10	1	0	1	0				0.25	0.00	0.32	0.00				0.14	0.17	-2.16	10
9_D9	CHKB	4	3	2	2				1.00	0.75	0.64	0.64				0.76	0.17	-0.29	0
9_E9	NTRK1	3	3	1	1				0.75	0.75	0.32	0.32				0.54	0.25	-0.96	6
9_F9	CDK3	0	1	0	0				0.00	0.25	0.00	0.00				0.06	0.13	-2.40	16
9_G9	GSK3B	4	4	3	3				1.00	1.00	0.96	0.96				0.98	0.02	0.39	14
9_H9	PANK4	0	0	1	0				0.00	0.00	0.32	0.00				0.08	0.16	-2.35	11
9_A10	NRBP	4	4	4	4				1.00	1.00	1.28	1.28				1.14	0.16	0.87	7

LOCATION (Plate_Well)	siRNA TARGET	RAW SCORE							NORMALIZED SCORE							MEAN	SD	Z SCORE	CYTOX
		EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7	EXP1	EXP2	EXP3	EXP4	EXP5	EXP6	EXP7				
9_B10	CALM2	0	0	0	0				0.00	0.00	0.00	0.00				0.00	0.00	-2.59	1
9_C10	PGK1	2	2	2	2				0.50	0.50	0.64	0.64				0.57	0.08	-0.86	13
9_D10	PRKAR2B	3	3	2	1				0.75	0.75	0.64	0.32				0.62	0.20	-0.72	6
9_E10	NME4	2	2	1	2				0.50	0.50	0.32	0.64				0.49	0.13	-1.10	14
9_F10	PDPK1	4	4	2	3				1.00	1.00	0.64	0.96				0.90	0.17	0.14	17
9_G10	MST1R	3	4	3	3				0.75	1.00	0.96	0.96				0.92	0.11	0.20	9
9_H10	PRKACG	4	4	2	3				1.00	1.00	0.64	0.96				0.90	0.17	0.14	0
9_A11	ULK1	4	2	1	2				1.00	0.50	0.32	0.64				0.62	0.29	-0.72	10
9_B11	KHK	2	2	1	2				0.50	0.50	0.32	0.64				0.49	0.13	-1.10	0
9_C11	AK3L1	3	2	2	3				0.75	0.50	0.64	0.96				0.71	0.19	-0.43	49
9_D11	PCK2	2	2	0	0				0.50	0.50	0.00	0.00				0.25	0.29	-1.83	20
9_E11	BRAF	4	4	1	0				1.00	1.00	0.32	0.00				0.58	0.50	-0.83	18
9_F11	C10ORF89	3	4	1	2				0.75	1.00	0.32	0.64				0.68	0.28	-0.53	11
9_G11	AK2	3	2	2	3				0.75	0.50	0.64	0.96				0.71	0.19	-0.43	17
9_H11	FLT1	4	4	4	4				1.00	1.00	1.28	1.28				1.14	0.16	0.87	3

Table S3. Primer sequences

PRIMER	SEQUENCE
NPR2-F	AAC TTG GAG AAG CTG GTG GAG GAA
NPR2-R	CTG CTG ACA ATG CTG TGA AGC CAA
NEK3-F	TTG GTT CAG CAT GAA AGC AG
NEK3-R	TGC ATT AGA TCC CCT CCA TC
EPHA6-F	GGC AGG TGA ATT TGG AGA AG
EPHA6-R	ACC CCT TCT AGG CGA ATG AT
EXOSC10-F	AGA GAG AGC GAG CAA CAA GC
EXOSC10-R	TCC AGC AAA AGC CTT GAA GT
ITPKB-F	GAT CGA CTT TGG GAA AAC CA
ITPKB-R	ACG AGA AAG GAA GCA CAG GA
PLK4-F	GGT CAG CCA CTC CCA AAT AA
PLK4-R	GGC CTT TCT TCT GCA TCT TG
CALM2-F	TGG CTG ACC AAC TGA CTG AA
CALM2-R	TGT GCC ATT ACC ATC AGC AT
ITPK1-F	CTG GAG AAG AAC GGC TTG AC
ITPK1-R	CTG GAC CAC GGT GTA GGA CT
PDK2-F	ATG GCA GTC CTC CTC TCT GA
PDK2-R	CAC CCA CCC TCT TCC TAA CA
CDK3-F	ACG GTG CCG TTT CAA AAT AG
CDK3-R	CTC AAC TCT TCC CCC AAA CA
CDC2L5-F	CTC GAA TTC TGC CTC CTG AC
CDC2L5-R	TCT CTT TTG GGG TCA TCC TG
SGKL-F	AGC TGC CCA AGT GTA AGC AT
SGKL-R	CAG GAA TCT TCA GGG CCA TA
MAP3K1-F	TGA ACA GCT ATG AAC GAG GCC AGT
MAP3K1-R	TTT CCT GTT CAC CTA GGG CCA GTT
DYRK3-F	TTG GTG GTC CCA ATA ATG GAG GGT
DYRK3-R	TAG GGC CAC GTA CTG TCG AAG TTT
PANK4-F	TGC GCA CCA TCA CCT ATA GCA TCA
PANK4-R	AGG ATT GTC CTG CTC AGC TCC TTT
TPK1-F	TCC TAC CAG CCA TTG TAG GCC AAT
TPK1-R	TCA TAT AAG CGG TTG GCA CCT CCA
NEK8-F	ATG ACT TGC AGC TGT CTC CTG GAT
NEK8-R	AAG GGC AAA GAG GTG GTA GGA GAA
C9ORF96-F	AAT GCG CAC CTC ACA CTC CTC TTA
C9ORF96-R	AAT GGT CTG GAA GGA CTG CTC TGT
PRKAG3-F	ACA CAC AAA CGC CTG CTC AAG TTC
PRKAG3-R	GGA ATG TGC CGA TGC CCA AAT CTT
ERBB4-F	TCT GCA TGG GTT GGT CAG GAG AAT
ERBB4-R	GCA TGG GTG TTT CAA CCA TCT GCT
ADK-F	AGA GGC AGC GAA TCG TGA TCT TCA
ADK-R	ACC TCC AAC AAA TGC ATC TCC AGC
PKN3-F	ACG CCA TCA AAG CAC TGA AGA AGC
PKN3-R	ATC TGC ATC ATG AGG TCA CCA CCA
HK2-F	TGC AGC GCA TCA AGG AGA ACA AAG
HK2-R	ACG GTC TTA TGT AGA CGC TTG GCA
GAPDH-F	GGT ATC GTG GAA GGA CTC ATG AC
GAPDH-R	ATG CCA GTG AGC TTC CCG TTC AG
MAP2K1-F	GCC CTC CAA CAT CCT AGT CA
MAP2K1-R	GAC AGT CCC ATG CTC CAG AT

Table S4. Hit gene overlap with other published host/influenza screens

Kinase target	Brass, A.L. et al. Cell 139, 1243-1254 (2009).	Shapira, S.D. et al. Cell 139, 1255-1267 (2009).	Konig, R. et al. Nature 463, 813-817 (2010).	Karlas, A. et al. Nature 463, 818-822 (2010).	Hao, L. et al. Nature 454, 890-893 (2008).	Andersen, L.E. et al.	Total
AQR	1	1	1	1			4
ARCN1	1		1	1	1		4
ARTN	1	1		1	1		4
ATP6AP1	1		1		1		3
ATP6AP2	1		1	1			3
ATP6V0B			1	1	1		3
ATP6V0C			1	1	1		3
ATP6V0D1	1		1	1			3
ATP6V1A	1			1	1		3
ATP6V1B2	1	1		1			3
B2M			1	1	1		3
BUB3	1			1	1		3
BZRAP1		1			1		2
C14orf109	1				1		2
C6orf62		1		1			2
CALCOCO2			1	1			2
CALM2	1		1				2
CAMK2B			1	1			2
CD81			1	1			2
CDK4		1		1			2
CFLAR	1	1					2
CLIC4			1	1			2
CLK1	1	1					2
COPA	1	1					2
COPB1	1	1					2
COPB2		1				1	2
COPG		1	1				2
DCLK2			1	1			2
DLG5		1	1				2
DMAP1		1	1				2
EIF2AK2		1		1			2
EIF4A2			1	1			2
EPHB2	1			1			2
FAM38A			1	1			2
FAU		1	1				2
FGFR2				1	1		2
FLNC		1	1				2
HAND2	1				1		2
HK2		1	1				2
IFIT5	1	1					2
IFITM3				1	1		2
IKBKE		1	1				2
IL17RA	1	1					2
IRF2			1		1		2
ISG15		1				1	2
ITPKB	1	1					2
JUN	1	1					2
KPNB1		1	1				2
KRTCAP2		1	1				2
MAP2K3			1	1			2
MAP3K12		1		1			2
MAPK1		1				1	2
MFAP1			1	1			2
MYC			1	1			2
NEK8	1				1		2
NHP2L1		1	1				2
NUP153		1	1				2
NUP98		1	1				2
NXF1	1	1					2
OSMR		1		1			2
PANK4				1		1	2
PGD	1		1				2
PHF2			1		1		2
PIK3R4		1	1				2
PLK3	1	1			1		3
PLK4			1	1			2
PLXNA2		1	1				2

Kinase target	Brass, A.L. et al. Cell 139, 1243-1254 (2009).	Shapira, S.D. et al. Cell 139, 1255-1267 (2009).	Konig, R. et al. Nature 463, 813-817 (2010).	Karlas, A. et al. Nature 463, 818-822 (2010).	Hao, L. et al. Nature 454, 890-893 (2008).	Andersen, L.E. et al.	Total
PPAN			1	1			2
PPP1R14D			1			1	2
PRKACA		1			1		2
PRPF8	1	1					2
PSENEN			1	1			2
PSMD11		1	1				2
PTPRN			1	1			2
PTS		1			1		2
RAB5A			1	1			2
RACGAP1	1		1				2
RBCK1	1				1		2
RPL13A			1	1			2
RPS10		1	1				2
RPS14	1	1					2
RPS16				1	1		2
RPS20			1		1		2
RPS4X	1				1		2
RPS5				1	1		2
RUNX1	1			1			2
SF3A1			1	1			2
SF3B1	1			1			2
SLC1A3	1	1					2
STARD5	1	1					2
SUPT6H	1			1			2
TNFRSF18		1		1			2
TNK2			1	1			2
TRIM21		1		1			2
TRIM28	1		1				2
VCP		1			1		2
WDR18	1		1				2

Table S5. DAVID summary of pathway analysis and hit kinase interactions

Database	Gene	Known interaction
BBID	MAP3K1	MAPK signaling cascades, Cholesterol Stress Response, signal transduction, Apoptosis, Mitogen signaling in growth control, IkBa Kinase JNK MEKK1
	PDK2	Insulin signaling, Signaling glucose uptake, Glycogen synthase-synthesis, mRNA translation-protein synthesis
BIOCARTA	MAP3K1	The 1BB-dependent immune response, Angiotensin II mediated activation of JNK Pathway via Pyk2 dependent signaling, BCR Signaling Pathway, CD40L Signaling Pathway, Ceramide Signaling Pathway, EGF Signaling Pathway, Map Kinase Inactivation of SMRT Corepressor, FAS signaling pathway (CD95), Fc Epsilon Receptor I Signaling in Mast Cells, fMLP induced chemokine gene expression in HMC-1 cells, Inhibition of Cellular Proliferation by Gleevec, Human Cytomegalovirus and Map Kinase Pathways, HIV-I Nef: negative effector of Fas and TNF, Signal transduction through IL1R,keratinocytePathway:Keratinocyte Differentiation,malPathway:Role of MAL in Rho-Mediated Activation of SRF, MAPKinase Signaling Pathway, NF-kB Signaling Pathway, p38 MAPK Signaling Pathway , PDGF Signaling Pathway, Links between Pyk2 and Map Kinases, Rac 1 cell motility signaling pathway, TNF/Stress Related Signaling, T Cell Receptor Signaling Pathway, TNFR1 Signaling Pathway, TNFR2 Signaling Pathway, Toll-Like Receptor Pathway, Neuropeptides VIP and PACAP inhibit the apoptosis of activated T cells
	PDK2	Regulation of eIF4e and p70 S6 Kinase, Skeletal muscle hypertrophy is regulated via AKT/mTOR pathway, mTOR Signaling Pathway, PTEN dependent cell cycle arrest and apoptosis
KEGG PATHWAY	EPHA6	Axon guidance
	ADK	Purine metabolism
	CALM2	Calcium signaling pathway, Phosphatidylinositol signaling system, Oocyte meiosis, Vascular smooth muscle contraction, Long-term potentiation, Neurotrophin signaling pathway, Olfactory transduction, Insulin signaling pathway, GnRH signaling pathway, Melanogenesis, Alzheimer's disease, Glioma
	EXOSC10	RNA degradation
	HK2	Glycolysis/Gluconeogenesis, Fructose and mannose metabolism, Galactose metabolism, Starch and sucrose metabolism, Amino sugar and nucleotide sugar metabolism, Insulin signaling pathway, Type II diabetes mellitus
	ITPKB	Inositol phosphate metabolism, Calcium signaling pathway, Phosphatidylinositol signaling system
	MAP3K1	MAPK signaling pathway, Ubiquitin mediated proteolysis, RIG-I-like receptor signaling pathway, Neurotrophin signaling pathway, GnRH signaling pathway
	NPR2	Purine metabolism, Vascular smooth muscle contraction
	PANK4	Pantothenate and CoA biosynthesis
	TPK1	Thiamine metabolism
PANTHER PATHWAY	EPHA6	Angiogenesis
	CALM2	B cell activation, Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction, T cell activation
	HK2	Glycolysis, Fructose galactose metabolism, Pentose phosphate pathway
	MAP3K1	Angiogenesis, Apoptosis signaling pathway, EGF receptor signaling pathway, FGF signaling pathway, Integrin signaling pathway, Toll receptor signaling pathway, Ras Pathway
	NPR2	Endothelin signaling pathway
	PANK4	Coenzyme A biosynthesis
	PKN3	Alzheimer disease-amyloid secretase pathway, Endothelin signaling pathway, Muscarinic acetylcholine receptor 1 and 3 signaling pathway, PDGF signaling pathway
	PDK2	TCA cycle
	TPK1	Thiamine metabolism
REACTOME PATHWAY	ADK	Metabolism of nucleotides
	CALM2	Signaling by NGF, Metabolism of nitric oxide, Opioid Signaling, Metabolism of carbohydrates, Hemostasis
	HK2	Metabolism of carbohydrates
	MAP3K1	Signaling in Immune system
	PANK4	Metabolism of vitamins and cofactors
	PLK4	Cell Cycle, Mitotic
	PDK2	Integration of energy metabolism
	TPK1	Metabolism of vitamins and cofactors

Table S6. Pubmed identification number for literature review

ID	PUBMED_ID
ADK	10215863,11242543,12112843,12477932,12820662,15489334,17154503,18309031,185014,18600536,18600545,621091,8577746,8917457,9070863,9843365,
CALM2	10026195,10026200,10075657,10075700,10088721,10207003,10333484,10411641,10416864,10488094,10493800,10547355,10681496,10692436,10731425,10737800,10747990,10748158,10757985,10781589,10816571,10823944,10852921,10860555,10893241,10899953,11054265,11171103,11178895,11181995,11248124,11278607,11323678,11395482,11448995,11457836,11585916,11591728,11685248,11710561,11734550,11736632,11741295,11807546,11827172,11953448,11981030,11984006,12006621,12032157,12034722,12051765,12135876,12153558,12189141,12221128,12223552,12358748,12408866,12446675,12475216,12477932,12485993,12508121,12509414,12556500,12577052,12626507,12715898,12724311,12727204,12805215,12808128,12821674,12871148,12925680,1332771,14551202,14594800,14613930,14702039,1474585,14765114,15087444,15104175,15140941,15147735,1519061,1520270,15225611,15229223,15316014,15342556,1544444,15489334,15522886,15583004,15592455,15632291,15670850,15719022,15723066,15746150,15746192,15806159,15815621,15817490,15902271,16013055,16030015,16084495,16127172,16154564,16189514,16258073,16299511,16344560,16359877,16545345,1660262,16613843,16627785,16760425,17287340,17569884,17580302,17654480,17687619,17719545,17884685,1790714,17975119,18001530,18083107,18178620,18452398,18669648,18768750,18940010,19023099,19034380,19080622,19429631,2123288,2161834,2223880,2244899,2445749,2455687,2487274,2507540,3111527,3182832,3350007,3472906,6385987,6847627,7093203,7490111,7525583,7559563,7593266,7607248,7634090,7642608,7803388,7836475,7887964,7925473,7947686,8076819,8206911,8226798,8314583,8476923,8486648,8625412,8639568,8663089,8670801,8756646,8769426,8780394,8824319,8900147,8901523,9003189,9009191,9092566,9096366,9115241,9188442,9188503,9202019,9242710,9278050,9282777,9341188,9374536,9438860,9490638,9512352,9580552,9681195,9728925,9753452,9799490,9804419,9822657,9837900,9923700,9927666,9952395,
CDK13	11162436,11347906,12477932,12853948,14702039,15144186,15231748,15302935,15489334,15635413,16721827,16730941,16964243,17081983,1731328,17344846,17525332,18220336,18669648,18691976,8125298,9013549,9373149,9847074,
DYRK3	10779429,12356771,12477932,15489334,15592455,15607427,16344560,16710414,17344846,18083107,18854306,9748265,9845759,
EPHA6	10607706,10730216,11128993,12084815,12471243,12477932,12975309,14702039,14726470,14986683,15194108,15271258,16344560,16641997,17344846,17974005,
EXOSC10	10465791,11110791,11426320,11719186,11812149,11879549,12419256,12429849,12477932,12788944,1383382,14527413,14702039,14743216,14872500,15231747,15489334,1644924,16710414,17412707,17545563,
HK2	11068878,11688991,12477932,12663490,14672622,14747281,15489334,15710218,15967114,16189514,16396496,16551620,16713569,17785433,18271924,18535403,18772588,19033437,19049977,7489847,7518342,7590357,7622509,7883120,7883122,7883123,7999069,8250948,8307259,8567628,8721778,8786021,8834247,8889548,8911786,9056853,9278438,9447955,
ITPKB	11104677,11846419,12477932,12747803,1330886,14702039,15489334,16189514,16354157,1654894,16710414,16740130,18088087,18669648,2176078,8889548,9374536,
MAP3K1	10465784,10523642,10611349,10713157,10805784,11746500,11756439,11782455,11784851,11815602,11884395,11903060,11971970,11971971,12048245,12049732,12079429,12185592,12228228,12456688,12584189,12600818,12763138,12878610,14500727,14612408,14743216,15205333,15262978,15276183,15299005,15372022,15829968,15866172,16044153,16046415,16286467,16344560,16434970,16636664,16714289,16760432,17301822,17344846,17397260,17529967,17889508,17997823,18032450,18187866,18220336,18287093,18308848,18355772,18420486,18434448,18437204,18467339,18521924,18669648,18691976,18784253,18785201,18973230,18984593,19028704,19094228,19232126,19262425,8597633,9008162,9135144,9405400,9689078,9712898,9733714,9808624,9836645,
NEK3	10224116,12063396,12477932,14702039,15057823,15345747,15489334,15618286,16344560,17118778,17297458,17344846,17924679,7522034,8274451,8889548,
NEK8	11864968,12477932,15019993,15489334,15761153,15872312,16625196,17474147,17974005,18199800,
NPR2	10073597,10082481,11208455,11464256,11556325,12022755,12477932,12709393,1309330,1327579,14759258,14988324,15146390,15164053,15262909,15371450,15459247,15489334,15722353,15911070,15911610,17344846,17429599,17652215,18945719,19086053,19326473,1979052,2570358,7679284,8765168,9624142,9634515,
PANK4	11479594,12477932,14702039,15489334,16038259,16132722,16710414,16959974,17974005,2981478,8125298,9373149,
PDK2	10191262,10438924,10748134,11483605,11485553,11486000,11701428,11978179,12477932,12573248,12676647,12816949,14702039,15169745,15489334,15491150,15491151,15955060,16401071,16517984,16962287,17344846,17669420,18029348,18220414,18220415,7499431,
PKN3	10441506,11259428,11432776,12477932,15164053,15282551,15489334,17192257,17344846,18669648,18691976,
PLK4	10607900,11489907,11597015,12477932,1311287,15342556,15489334,15761153,15967108,16189514,16244668,16326102,17344846,17681131,18239451,18691976,19001868,8274451,
SGK3	10548550,10585774,11035810,11514587,11707620,11749387,11751930,11884598,12054501,12218062,12397388,12477932,12590200,12632189,12634932,14604990,15489334,15496163,15578212,15737648,16036218,16341674,16421571,16485113,16543730,16790420,16888620,16959974,17167223,17344846,8125298,9373149,
TPK1	10567383,11342111,11342117,12477932,12690205,12853948,12953792,1332781,14702039,15489334,15664409,16087053,16262001,16344560,17295612,3896163,6303095,9847074,