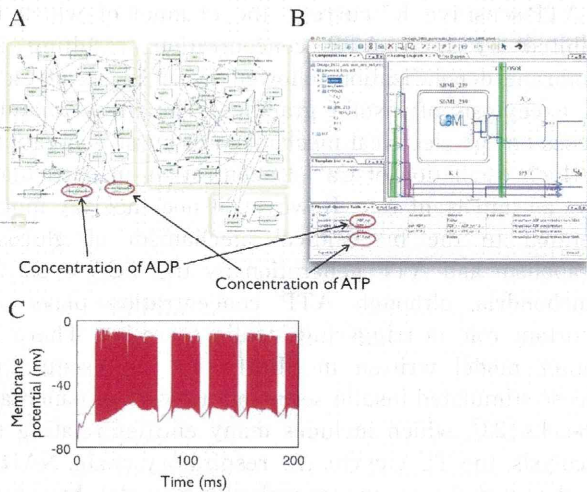

Fig. 6 SBML-PHML hybrid model of pancreatic β-cells. A. The network model on CellDesigner. B. Hybrid model on PhysioDesigner. In the hybrid model, ADP and ATP concentrations (indicated by red circles in A and B) defined in the SBML model are transferred to physical quantities in the PHML side with a "get" action. C. Simulation results of the hybrid model with Flint. The time course of the membrane potential showed a periodic burst.

subcellular biochemical phenomena such as signal transductions, while PHML was originally designed to describe modularity, hierarchical structures, and physiological system networks. The function of PhysioDesigner to embed SBML into another language without conversion is an unprecedented and effective method to achieve multilevel modeling of physiological systems.

Two methods can be considered to embed an SBML model into a PHML model. One is to convert the SMBL model into PHML, and import it as a module. The other is to retain the SBML model and define the relationship between the SBML species/parameters and the physical quantities. The former method was implemented, for example, for SBML and CellML exchange[25]. The latter method is adopted in our proposed modeling method, although the former is also technically feasible in our case.

The latter method has three advantages over the former method. First, targets modeled by SBML can be described by SBML most effectively, and many dedicated applications such as CellDesigner are available for working with SBML. With the aid of the Garuda platform, now PhysioDesigner can seamlessly consign an embedded SBML model in a module to CellDesigner for display and editing, and can receive the modified SBML model back. There is no need to reinvent the wheel, and to use such applications is the simplest and most direct way to work with SBML. However, direct modification of the embedded SBML model can lead to a collapse in the consistency of integration with PHML. Users must be careful to maintain the consistent bridges between the SBML and PHML models.

Second, since there is an entire SBML model in a PHML module, it is easy to know which version of the SBML model is used for the targeted phenomenon in the PHML model. If an updated SBML model exists, it is also simple to replace it.

Third, PhysioDesigner does not need to account for the SBML version to import a SBML model. When executing a simulation in Flint, Flint needs to extract equations and parameters from the SBML portion, hence it needs to parse the SBML model. But, this can be performed by the widely used libraries for SBML, which is much easier than maintaining our handcrafted converter from SBML to PHML.

On the other hand, the former method has an advantage in the process of integrating mathematical logic. The reason is as follows. In the case of the latter method, as explained in Section 4.1, users need to know Eq. 1 to obtain the expanded form (Eq. 4) to develop the SBML-PHML hybrid model. However, in some cases, it can be difficult to determine the original differential equation, because the equations are not explicitly described in SBML. If the SBML model is converted into PHML, integration in the mathematical logic may be simplified.

As described in Section 3.2, PhysioDesigner includes a dialog box to support the SBML model embedment process. However, users still need to maintain the consistency in the units of quantities and the time scales used in both SBML and PHML models. Support for this issue should be addressed in future development of PhysioDesigner. Physical quantities in PHML and species/parameters in SBML can be defined with unit information. As far as the units are defined and available in both models, PhysioDesigner has to, at least, raise a warning when it detects inconsistencies in the units.

SBML models are archived, for example, in the BioModels Database[13]. There is also a PHML model database at http://physiome.jp. PhysioDesigner, with the aid of the Garuda platform and CellDesigner, provides a wider activity arena for users by utilizing resources in databases for SBML and PHML together to build multilevel models. In PhysioDesigner, it is also possible to integrate the morphometric information as a skeletal structure to create a computable model with a template/instance framework[26] for large-scale modeling. Integration of SBML and morphology into a PHML model provides a novel way to create large-scale multilevel models.

One of the problems that could arise when the model size becomes huge is a shortage of computing power for simulations. To solve this problem, development is underway to render the simulator Flint executable on the K supercomputer and computing cloud. The version of Flint for cloud computing is called Flint K3, and the 1.0 alpha version is posted at http://flintk3.org. The enhancement of Flint will facilitate scaling-up of models in terms of computational power.

Acknowledgement

This work was supported in part by MEXT Grant-in-Aid

for Scientific Research on Innovative Areas "Integrative Multi-level Systems Biology".

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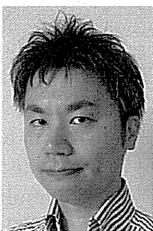
Masao OKITA



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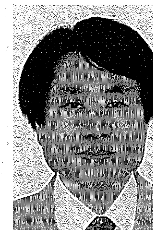
Ken-ichi HAGIHARA



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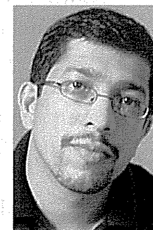

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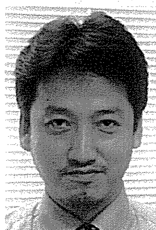

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Influenza Virus-Host Interactome Screen as a Platform for Antiviral Drug Development

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SUMMARY

Host factors required for viral replication are ideal drug targets because they are less likely than viral proteins to mutate under drug-mediated selective pressure. Although genome-wide screens have identified host proteins involved in influenza virus replication, limited mechanistic understanding of how these factors affect influenza has hindered potential drug development. We conducted a systematic analysis to identify and validate host factors that associate with influenza virus proteins and affect viral replication. After identifying over 1,000 host factors that coimmunoprecipitate with specific viral proteins, we generated a network of virus-host protein interactions based on the stage of the viral life cycle affected upon host factor downregulation. Using compounds that inhibit these host factors, we validated several proteins, notably Golgi-specific brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1) and JAK1, as potential antiviral drug targets. Thus, virus-host interactome screens are powerful strategies to identify targetable host factors and guide antiviral drug development.

INTRODUCTION

Viruses, which rely on host cellular functions to replicate, hijack the host cell machinery and rewire it for their own needs. A comprehensive understanding of host-virus interactions would greatly improve our understanding of the viral life cycle and be invaluable in identifying strategies to prevent or treat potentially deadly virus infections.

Influenza viruses cause annual epidemics and recurring pandemics, which have claimed millions of lives and had a considerable impact on public health and the global economy. Recent sporadic human infections with avian viruses of the H5N1 and H7N9 subtypes have raised concerns about the pandemic potential of these viruses (Gao et al., 2013; Li et al., 2014; Webster and Govorkova, 2006; Yen and Webster, 2009). Two antiviral drugs (that inhibit the ion channel [M2] or neuraminidase [NA] proteins) are available (Davies et al., 1964; Hayden, 2001), but the emergence of drug-resistant viruses has become a serious problem (Bright et al., 2005, 2006; Dawood et al., 2009; Nicoll et al., 2008). Therefore, there is an urgent need to identify targets for antiviral drugs.

In recent years, six genome-wide screens have identified a total of 1,449 human genes (including 110 human orthologs of *Drosophila* genes) with potential roles in the life cycle of influenza virus (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010; König et al., 2010; Shapira et al., 2009; Sui et al., 2009). Meta-analyses revealed limited overlap among these studies (de Chassey et al., 2012; Mehle and Doudna, 2010; Watanabe et al., 2010). This limited overlap may be caused by differences in the experimental conditions of the screens. Also, the experimental methods used in the screens might be suboptimal to investigate the whole life cycle of influenza viruses (e.g., using nonpermissive cells for influenza virus infection and/or nonauthentic influenza virus [i.e., recombinant viruses possessing reporter genes]). Moreover, the criteria used to determine the candidate host factors likely differed among the screens, and each screen might include a number of false positives. More importantly, most of these studies validated only subsets of potential host interaction

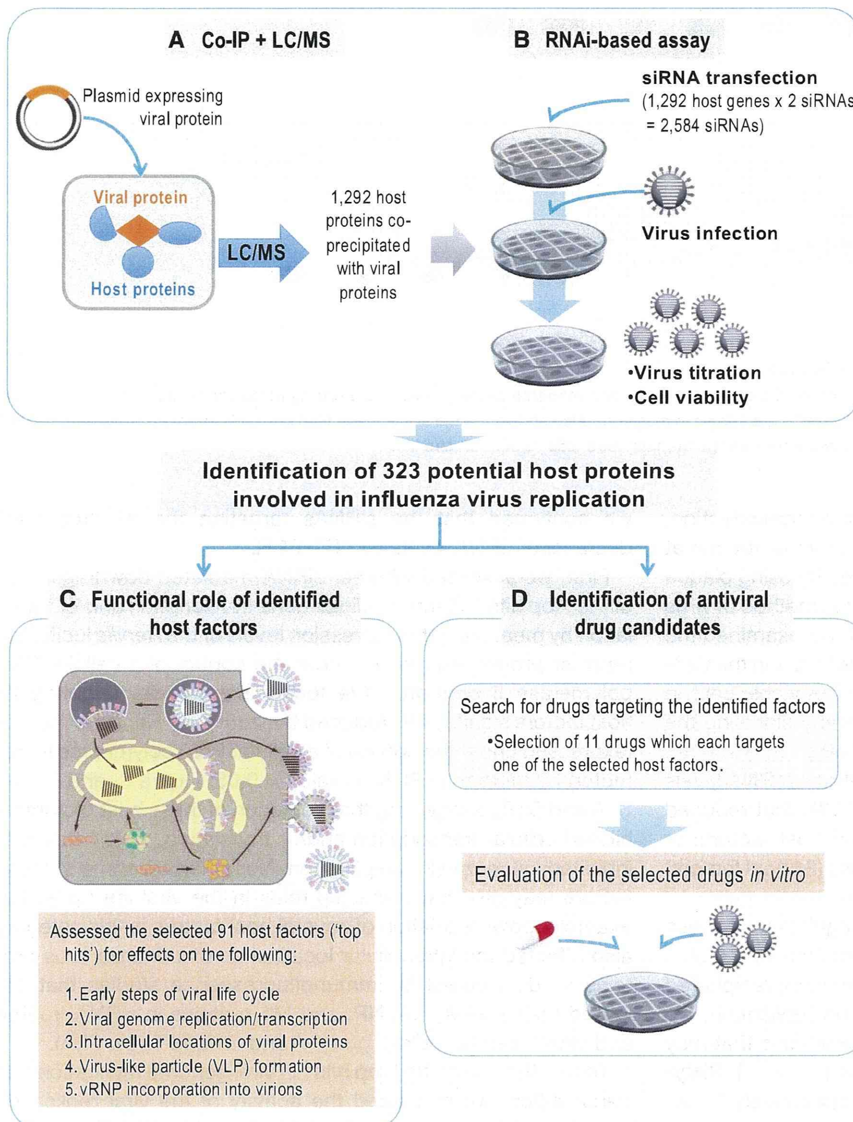

Figure 1. Overview of a Systematic Study to Elucidate the Physical and Functional Host-Viral Interactions in Influenza Virus Replication and to Identify Antiviral Drugs

(A and B) Schematic diagram of the identification of host proteins that coprecipitated with 11 influenza A viral proteins and affected viral replication. (A) Mass spectrometry analysis identified 1,292 host proteins that coimmunoprecipitated with one or more of the 11 FLAG-tagged influenza viral proteins. (B) To identify host factors that affect virus replication, cells were transfected with siRNAs targeted to each of the 1,292 candidate host genes and were then infected with influenza virus. Virus titers and cell viability were then determined. We identified 323 host genes whose mRNA levels were downregulated, while virus titers were reduced by more than two $\log_{10}$ units compared with a control (299 host factors) or increased by more than one $\log_{10}$ unit (24 host factors).

(C) To better understand the role of the identified host factors, we performed mechanistic studies assessing different steps in the viral life cycle for our "top hits," i.e., 91 host factors whose siRNA-mediated downregulation reduced viral replication in cultured cells by at least three $\log_{10}$ units while retaining > 80% cell viability.

(D) To identify antiviral drugs for influenza virus, we searched for drugs targeting the 299 host factors identified here and selected 11 drugs for in vitro testing. See also Figures S1–S4 and Tables S1, S2, and S3.

factors, and only a few of the validated candidates were assessed for their function(s) in the viral life cycle. We therefore used authentic influenza virus and a human cell line permissive for influenza virus replication to conduct a systematic analysis of influenza viral host interaction partners, which was followed by extensive validation studies and a systematic assessment of the functional roles of these host proteins in influenza virus replication. This information was then used to identify targets for antiviral drugs.

RESULTS AND DISCUSSION

Identification of Host Proteins that Coprecipitate with 11 Viral Proteins of Influenza A Virus and Are Involved in Viral Replication

We first attempted to establish a comprehensive map of viral-host protein interactions in human embryonic kidney (HEK) 293 cells, which support influenza virus replication (Hatta et al., 2007; Le Ru et al., 2010). Eleven FLAG-tagged viral proteins (i.e., PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2, and PB1-F2, which represent all of the viral proteins with the exception of the recently identified potential accessory factors) of an influenza A virus (A/WSN/33, H1N1 subtype; WSN) were individually expressed in HEK293 cells and then immunoprecipitated with an anti-FLAG antibody. Mass spectrometry analyses of the coprecipitated proteins identified 1,292 host proteins in total: 388, 322, 304, 351, 574, 675, 659, 531, 113, 42, and 81 host proteins coprecipitated with the viral PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2, and PB1-F2 proteins, respectively (Figure 1 and Table S1; note that the data for NS2 were reported previously [Gorai et al., 2012]).

The coprecipitated host proteins may be specific binding partners of influenza viral proteins with essential or nonessential functions in the viral life cycle. Alternatively, they may be nonspecific—that is, false positive—binding partners resulting from experimental artifacts such as the overexpression of viral proteins in our assay and/or the absence of other viral components. Therefore, to identify host factors that are specifically involved in virus replication, we transfected HEK293 cells with siRNAs targeted to each of the 1,292 candidate host genes (two siRNAs for each host gene were used, as shown in Table S2; AllStars Negative Control siRNA [QIAGEN] was used as a negative

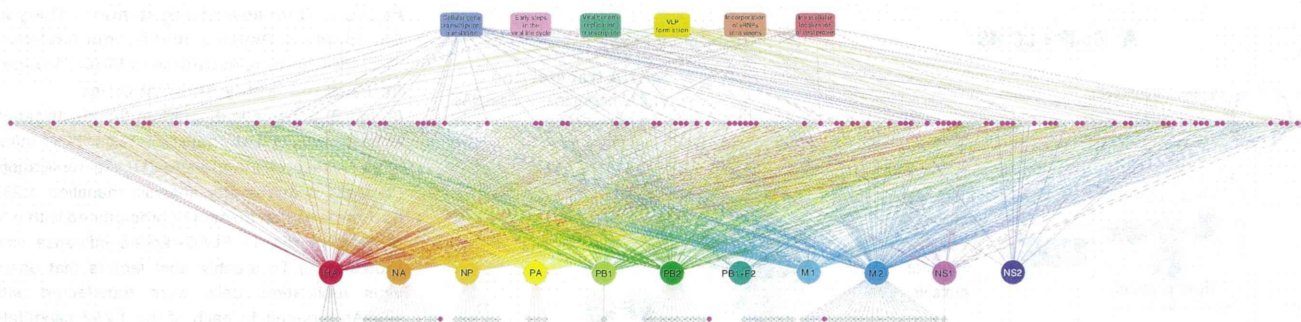

Figure 2. Network of Host-Influenza Viral Protein Interactions

Interactions among the viral proteins and the 323 host factors identified here (gray and magenta circles) were visualized by using Cytoscape (http://cytoscape.org/). "Top hits" (for a definition, see text and legend to Figure 1) are shown in magenta. Also shown are the steps in the viral life cycle affected by downregulation of the respective host factor. The network image is fully zoomable on the monitor. See also Tables S2 and S5.

control), infected cells with WSN virus at 24 hr posttransfection, and then harvested the culture supernatants for virus titration at 48 hr postinfection. Virus titers were determined by using plaque assays, which are a well-established and reliable method of virus titration in influenza virus research. In parallel, we examined the cell viability of siRNA-transfected HEK293 cells by using the Cell-Titer-Glo assay, which determines the number of viable cells in culture by quantifying the ATP levels and thereby signaling the presence of metabolically active cells (see also Experimental Procedures). We identified 323 host genes whose mRNA levels were downregulated, as confirmed by qRT-PCR, that reduced virus titers by more than two $\log_{10}$ units (299 host factors) or increased virus titers by more than one $\log_{10}$ unit (24 host factors) compared with the control siRNA (see Experimental Procedures, Figure S1, and Table S2). Moreover, downregulation of these host factors did not reduce cell viability by more than 40% (Table S2). For the set of 323 host factors that coimmunoprecipitated with viral proteins and affected influenza virus replication, we generated a network of virus-host protein interactions that may have critical roles in influenza virus replication (Figure 2). Sixty-three of these host factors had been identified previously (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010; König et al., 2010; Shapira et al., 2009; Sui et al., 2009) (Table S3 and Figure S2). Gene ontology and pathway analyses revealed that the host factors identified here are involved in various cellular functions, including many "housekeeping" processes such as transcription, translation, cell cycle, and mRNA splicing mechanisms (Supplemental Information, Figure S3, and Table S4).

The Roles of the Identified Host Factors in the Influenza Virus Life Cycle

Most of the previous genome-wide screens identified the affected viral life cycle step(s) for only a limited number of the host factors identified in their experiments (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010; König et al., 2010; Shapira et al., 2009; Sui et al., 2009). Because such information is essential for a better mechanistic understanding of the viral life cycle and to identify drug targets, we performed mechanistic studies for our "top hits," namely the 91 host factors whose siRNA-mediated downregulation reduced viral replication in cultured cells by at least three $\log_{10}$ units while retaining > 80% cell viability (Figures 1, 2, and 3 and Table S5). As described above, we confirmed that the siRNAs targeting the 91 "top hits" decreased mRNA levels by qRT-PCR.

First, we assessed whether siRNA-mediated downregulation of the "top hits" affected cellular gene transcription and/or translation by measuring the expression levels of the *Renilla* luciferase reporter protein expressed under the control of a cellular RNA polymerase II promoter. We found that siRNAs targeting 28 host factors significantly reduced the activity of *Renilla* luciferase (expressed under the control of a cellular RNA polymerase II promoter) by more than 80% (p value < 0.05; Figure 3, and Tables S5A and S5B), suggesting that depletion of these host factors inhibited cellular transcription and/or translation, which indirectly inhibited influenza virus replication. Nonetheless, some of these factors may also have specific roles in the viral life cycle. For example, downregulation of several host factors in this category also affected the intracellular localization of influenza virus proteins as determined by immunofluorescence studies that detected the viral HA, NA, NP, and M1 proteins in siRNA-treated and virus-infected cells (Figures 4 and S4 and Table S5A).

To test the role of the "top hits" in viral genome replication and transcription, we measured the activity of the viral replication complex (which comprises the PB2, PB1, PA, and NP proteins) based on its ability to replicate a virus-like RNA encoding the firefly luciferase reporter protein in a mini-replicon assay as described previously (Octaviani et al., 2010). We identified nine host factors (*BUB3*, *CCDC56*, *CLTC*, *CYC1*, *NIBP*, *ZC3H15*, *C14orf173*, *CTNNB1*, and *ANP32B*) that are critical for viral genome replication and transcription because their downregulation significantly decreased the relative viral RNA polymerase activity by more than 50% compared with a control (p value < 0.05; Tables S5A and S5B; see also Figure 3 for an overview). None of these factors affected host protein synthesis, suggesting a specific effect on viral replication. BUB3, a mitotic checkpoint protein, and CTNNB1, catenin (cadherin-associated protein) beta 1, were also detected in the genome-wide screens conducted by Brass et al. (Brass et al., 2009), Shapira et al. (Shapira et al., 2009), and Karlas et al. (Karlas et al., 2010) (Table S3A). CTNNB1 is part of a complex that forms adherens junctions and plays a role in cytoskeleton formation. Several of the factors identified here, including CTNNB1, share cellular interaction partners (Figure S5), suggesting that they may function in similar cellular processes. With the exception of BUB3 and CLTC, the host factors

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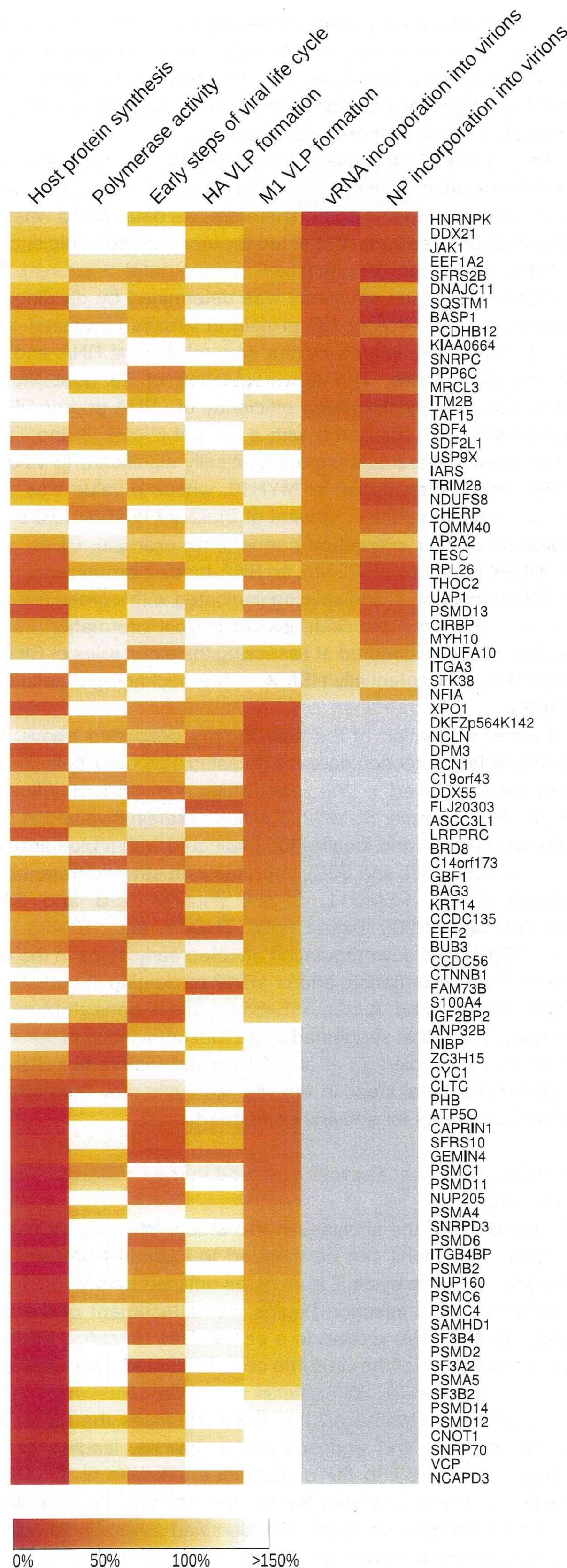

Figure 3. Effects of siRNA-Mediated Downregulation of the 91 "Top Hits" on the Influenza Virus Life Cycle

A summary of the effects of siRNA-mediated downregulation of the 91 "top hits" on influenza virus replication steps. The percentages indicate the relative efficiency compared with the negative control and correspond to the values presented in Table S5. For factors with a significant effect on the early steps in the viral life cycle, polymerase activity, or VLP formation, we did not test the efficiency of vRNA and NP virion incorporation; these factors are shown in gray in the respective columns.

identified in the polymerase activity assay did not coimmunoprecipitate with components of the viral replication complex (Table S1A), suggesting an indirect effect on viral replication. This finding may have important implications for drug development given that the inhibitory effects of drugs directed at these host factors may not be overcome easily by "escape" mutations in viral proteins.

To determine whether the "top hits" are involved in the early steps of the viral life cycle, we next infected siRNA-treated cells with a replication-incompetent PB2-knockout virus whose polymerase PB2-coding region was replaced with that of the *Renilla* luciferase reporter protein, as described previously (Ozawa et al., 2011) (see Experimental Procedures). Due to the lack of a functional PB2 gene, reporter expression is indicative of virus binding, internalization, and/or limited replication driven by the polymerase complex associated with the viral RNA segments of the infecting virions. Twenty-three host factors including several proteasome components affected these early steps in the viral life cycle. siRNAs targeting fourteen of these 23 host factors reduced cellular transcription/replication, whereas those targeting the remaining nine human genes (*BAG3*, *BRD8*, *CCDC135*, *DDX55*, *DPM3*, *EEF2*, *IGF2BP2*, *KRT14*, and *S100A4*) appeared to have influenza virus-specific effects (Figure 3 and Tables S5A and S5B). None of the siRNAs targeting these nine factors reduced viral replication as assessed in mini-replicon assays (see above; Tables S5A and S5B), suggesting that they have important roles in an earlier step in the viral life cycle such as virus binding, internalization, and/or transport of viral ribonucleoprotein (vRNP) complexes to the nucleus (see below).

Next, we attempted to determine whether the 91 "top hits" are critical for processes late in the viral life cycle, such as virion formation. To this end, we treated cells with siRNAs to the "top hits" and examined the effect on the formation of influenza virus-like particles (VLPs) derived from the M1, HA, and NA proteins (see Experimental Procedures). The efficiency of VLP production was calculated as the amount of M1 and HA in VLPs in the supernatant compared with the amount of M1 and HA in cell lysates. After removing the host genes that have general effects on cellular replication/transcription, we identified 15 host genes (*ASCC3L1*, *BRD8*, *C19orf43*, *DDX55*, *DKFZp564K142*, *DPM3*, *EEF2*, *FAM73B*, *FLJ20303*, *GBF1*, *NCLN*, *C14orf173*, *XPO1*, *LRPPRC*, and *RCN1*) whose siRNA-mediated downregulation decreased the average of VLP production by more than 50% compared with the control, although there were no statistically significant differences (Figure 3 and Tables S5A and S5C). None of these factors was identified in previous genome-wide screens (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010; König et al., 2010; Shapira et al., 2009; Sui et al., 2009). Four of these host factors (*BRD8*, *DDX55*, *DPM3*, and *EEF2*)

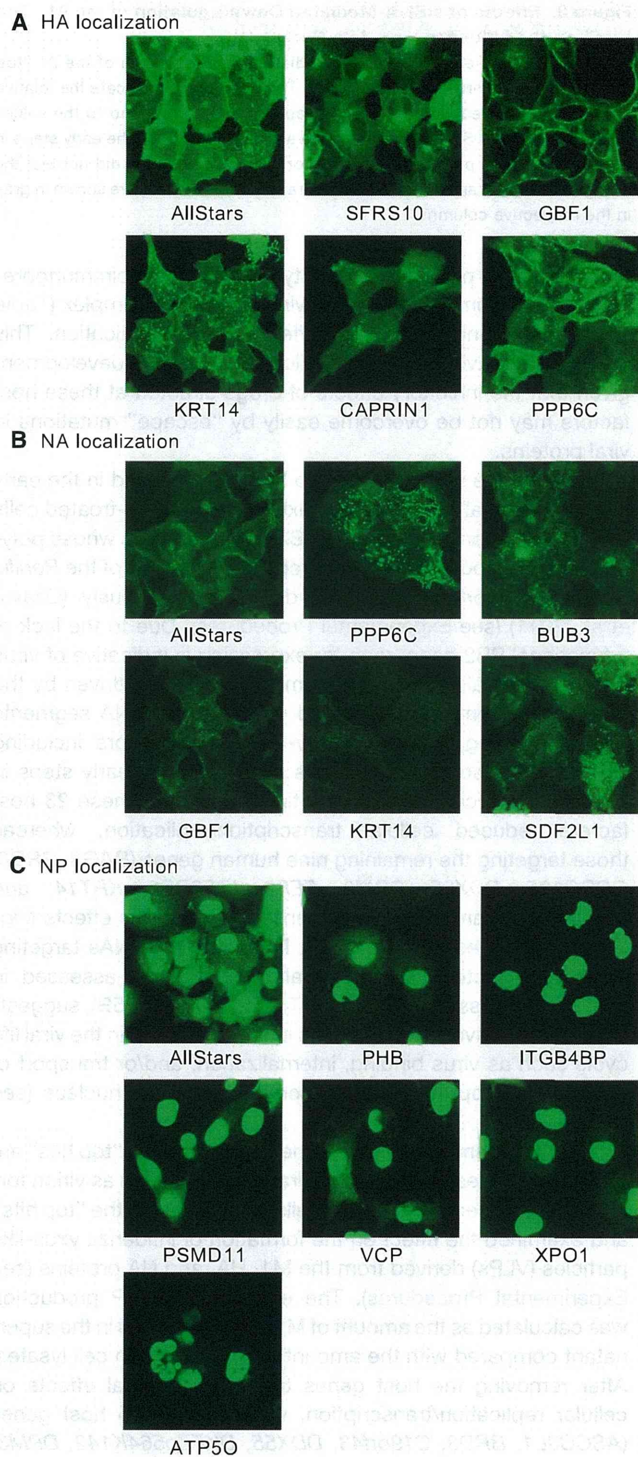

Figure 4. Effects of Selected siRNAs Targeting the 91 "Top Hits" on the Intracellular Localization of Viral Proteins in Infected Cells

(A–C) To examine whether the downregulation of the 91 "top hits" affects the intracellular localization of the viral proteins in virus-infected cells, siRNA-transfected HEK293 cells were infected with 200 pfu of WSN virus per well of a 24-well tissue culture plate, fixed at 12 hr postinfection, and then stained with an anti-HA, anti-NA, anti-NP, or anti-M1 antibody. The intracellular localization of HA (A), NA (B), and NP (C) is shown. None of the siRNAs affected M1 localization. See also Figure S4 and Table S5.

also affected the early steps in the viral life cycle and may thus play a role in the intracellular transport of viral proteins and/or vRNP complexes, functions that are essential during the early and late stages of infection. Hence, these host factors may be interesting targets for drug development.

Among the 91 "top hits," there were 35 host factors whose siRNA-mediated downregulation did not affect the virus life cycle steps assessed to this point. Therefore, we determined whether depletion of these factors affected the incorporation efficiency of vRNPs into progeny virions (see Experimental Procedures). The vRNP incorporation efficiency was determined by dividing the amount of viral RNA or NP protein in viruses harvested from the culture supernatants by the amount of viral RNA and NP in the cell lysates. The downregulation of 28 host factors decreased the incorporation efficiency of vRNA and/or NP by more than 50% compared with a control (Figure 3 and Table S5D); these differences were statistically significant (p value < 0.05), with the exception of MYH10, whose p value was 0.06 (Table S5D). In addition, several of these 28 host factors share common cellular interaction partners; for example, CIRBP and HNRNPK interact with RBMX, an RNA-binding protein with roles in RNA transcription and splicing (indicated with a green arrow in Figure S5E, and Table S5J). Based on this information, future studies could be directed at assessing the exact roles of CIRBP, HNRNPK, and potentially RBMX, in the packaging of influenza vRNPs.

Further evaluation of the host factors described above and their cellular interaction partners revealed a group of cellular factors likely involved in the intracellular transport of influenza vRNP. For example, SUMO2, a cellular protein involved in protein sumoylation and localization (indicated with a blue arrow in Figures S5B, S5D, and S5E), interacts with seven different host factors (BRD8, PSMD11, SF3B2 [Figures S5B and S5D], NSUN2, SNRNP200 [Figure S5D], THOC2, and TRIM28 [Figure S5E]) whose downregulation affected early steps in the viral life cycle, VLP formation, and/or vRNP packaging (Figures S5B, S5D, and S5E and Tables S5E–S5J). This is consistent with a previous study that suggested a role for sumoylation in the influenza viral life cycle (Wu et al., 2011). Host factors like SUMO2 that affect several steps in the viral life cycle may therefore be attractive targets for antiviral compounds.

Insights into Host Factors Involved in the Influenza Virus Life Cycle

On the basis of the analyses in this study, influenza virus-host protein interactions can be mapped to individual steps of the influenza virus life cycle (Figure 5), as outlined below.

Influenza virus infection begins with attachment of the viral hemagglutinin (HA) protein to a cell surface receptor, followed by internalization of the virus into cells, facilitated by endocytosis (Marsh and Helenius, 2006; Matlin et al., 1981; Rust et al., 2004; Sieczkarski and Whittaker, 2002). HA mediates the fusion between the viral and endosomal membranes, leading to the release of vRNPs into the cytoplasm (Stegmann et al., 1990). vRNP complexes are then transported through the cytoplasm to the nuclear core complex. We identified several host factors involved in these early steps in the viral life cycle (Figure 3 and Table S5), perhaps by facilitating intracellular transport of influenza vRNPs, as discussed earlier. For example, BRD8 (also

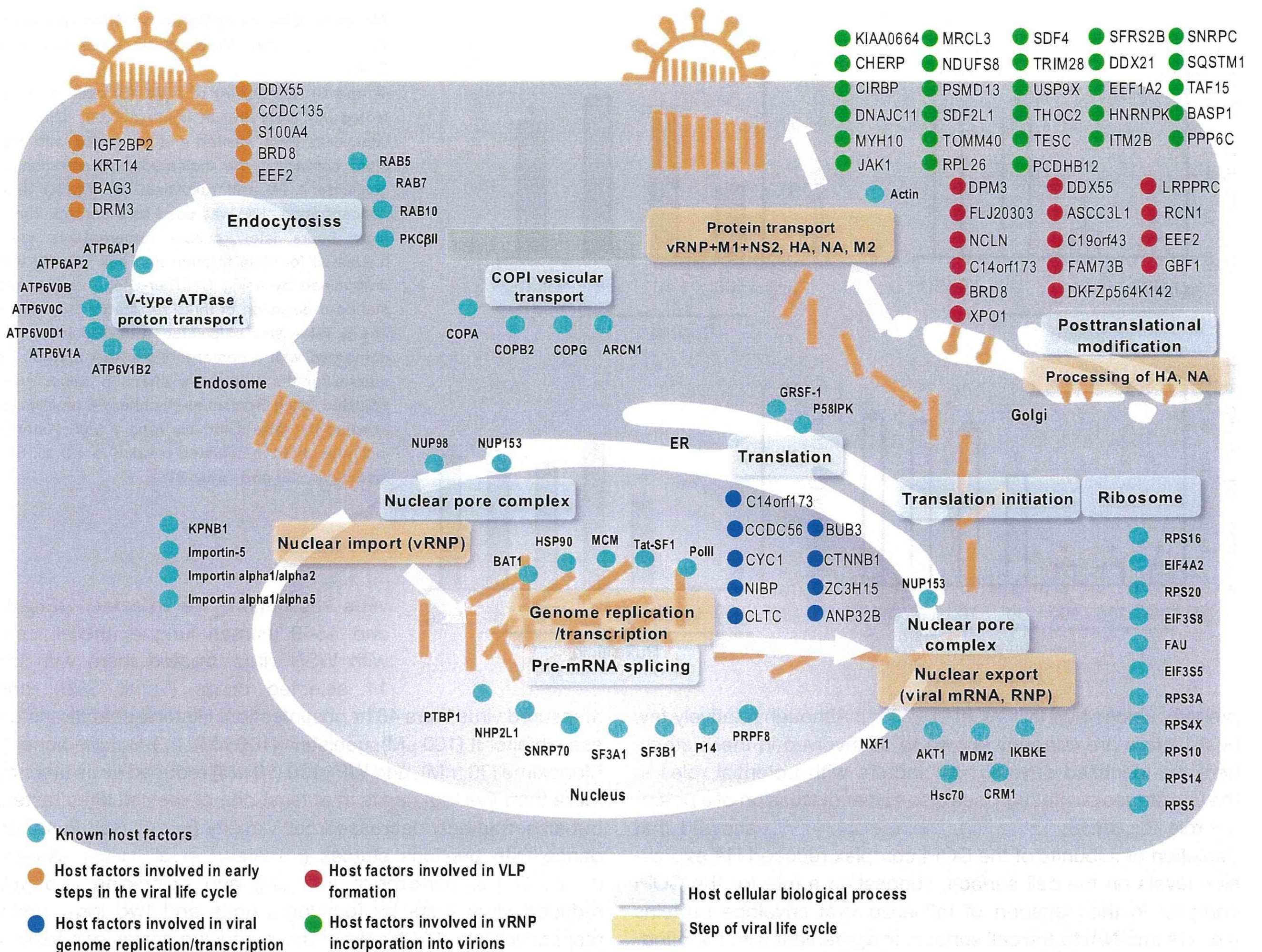

Figure 5. Putative Roles of Identified Host Factors in the Influenza Virus Life Cycle

Influenza virus is internalized by receptor-mediated endocytosis. The viral ribonucleoprotein (vRNP) complexes containing the eight viral genome RNAs (depicted by orange bars) are transported into the nucleus where replication and transcription of the viral genome take place. vRNPs formed with newly synthesized viral RNA, NP, and viral polymerase proteins are transported from the nucleus to the cytoplasm. HA and NA are processed posttranslationally during their transport from the ER to the Golgi apparatus. In the late stage of the viral life cycle, virion components are transported to the plasma membrane and progeny viruses then bud from the cells. The light orange boxes indicate individual steps of the influenza virus life cycle; the gray boxes indicate host cellular processes that are likely involved. Host factors identified in this study are grouped according to the viral life cycle steps they affected; light green circles indicate host factors identified in previous studies. Among the "known host factors," only *XPO1* (also known as *CRM1*) was identified in this study. See also Table S5.

known as SMAP or SMAP2) plays a role in intracellular vesicle trafficking (Tanabe et al., 2006) and binds to M1, the viral matrix protein, which is a major structural component of influenza virions. The interaction of M1 with BRD8 may affect intracellular vesicle trafficking and hence the transport of incoming and newly synthesized virus components to and from the plasma membrane.

The nuclear import of influenza vRNP is mediated by importins, which are part of the active import machinery of the host cell nuclear pore complex (NPC) (Deng et al., 2006; Gabriel et al., 2008; O'Neill et al., 1995; Resa-Infante et al., 2008; Tarendeau et al., 2007, 2008; Wang et al., 1997). Once the vRNPs are transported into the nucleus, the replication and transcription of influenza virus genomic RNA is facilitated by the viral polymerase subunits (PB1, PB2, and PA) and the nucleoprotein, NP (reviewed in Engelhardt and Fodor, 2006; Palese, 2007). Here, we identified several host factors that appear to be important for influenza viral RNA replication (summarized in Figure 3). Influenza virus also uses cellular machinery for nuclear export of vRNP complexes. Consistent with previous studies (Boulo et al., 2007; Elton et al., 2001; Neumann et al., 2000), we found that downregulation of *XPO1* (also known as *CRM1*) suppresses vRNP nuclear export (Figure 4C). Moreover, our 91 "top hits" include five other host factors whose suppression caused NP accumulation in the nucleus (Figure 4C), suggesting that these factors are likely involved in the nuclear export of influenza vRNPs.

In the late stage of the virus replication cycle, virion components such as vRNPs, M1, and the viral envelope proteins (HA, NA and M2) are transported to the plasma membrane where