LETTERS

Human host factors required for influenza virus replication

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Influenza A virus is an RNA virus that encodes up to 11 proteins and this small coding capacity demands that the virus use the host cellular machinery for many aspects of its life cycle[1]. Knowledge of these host cell requirements not only informs us of the molecular pathways exploited by the virus but also provides further targets that could be pursued for antiviral drug development. Here we use an integrative systems approach, based on genome-wide RNA interference screening, to identify 295 cellular cofactors required for early-stage influenza virus replication. Within this group, those involved in kinase-regulated signalling, ubiquitination and phosphatase activity are the most highly enriched, and 181 factors assemble into a highly significant host–pathogen interaction network. Moreover, 219 of the 295 factors were confirmed to be required for efficient wild-type influenza virus growth, and further analysis of a subset of genes showed 23 factors necessary for viral entry, including members of the vacuolar ATPase (vATPase) and COPI-protein families, fibroblast growth factor receptor (FGFR) proteins, and glycogen synthase kinase 3 (GSK3)-β. Furthermore, 10 proteins were confirmed to be involved in post-entry steps of influenza virus replication. These include nuclear import components, proteases, and the calcium/calmodulin-dependent protein kinase (CaM kinase) IIβ (CAMK2B). Notably, growth of swine-origin H1N1 influenza virus is also dependent on the identified host factors, and we show that small molecule inhibitors of several factors, including vATPase and CAMK2B, antagonize influenza virus replication.

Influenza viruses are a major cause of morbidity and mortality, and influenza A viruses in particular have the propensity to cause pandemic outbreaks such as occurred in 1918, 1957, 1968 and now in 2009 with the swine-origin H1N1 influenza virus[2]. Two of the viral proteins, neuraminidase (NA) and the M2 ion channel protein, are the targets for the US Food and Drug Administration (FDA)-approved influenza antiviral drugs; oseltamivir, zanamivir, amantadine and rimantadine[3]. Unfortunately, there is now widespread resistance to both of these drug classes[4]. Combined with the limited number of viral drug targets for influenza virus, this creates concern for the development of new influenza therapies.

An alternative therapeutic strategy that may greatly reduce the emergence of viral resistance is the pharmacological targeting of host factors required for viral replication. Genome-wide RNA interference (RNAi) screens have enabled the identification of host factors required by several RNA viruses[5–11], including an insect cell-based RNAi screen that implicated 110 *Drosophila* genes in influenza virus replication[12]. To more comprehensively characterize the host machinery used by influenza virus in mammalian cells, we have performed a genome-wide short-interfering RNA (siRNA) screen with human lung epithelial (A549) cells. To facilitate the readout for the high-throughput screen, the coding region for the influenza A/WSN/33 virus haemagglutinin (HA) protein was replaced with that of *Renilla* luciferase (Fig. 1a)[13]. As no HA is produced, this recombinant virus cannot complete its replication cycle. Thus, our RNAi screen focuses on the cellular requirements for viral entry, uncoating, nuclear import, and viral RNA transcription/translation, but it is not expected to identify factors involved in virus assembly, budding or release.

An arrayed siRNA library targeting more than 19,000 human genes was used to transfect human A549 cells (Fig. 1b and Supplementary Information). These cells were infected with the modified influenza virus (WSN-Ren), and luciferase readings were taken after 12, 24 and 36 h. Data from two independent screens were analysed using an integrative data analysis approach, which included redundant siRNA activity (RSA), as well as interactome and ontology-based analyses (see Supplementary Information)[6,14]. Using these methods, we were able to confirm 295 cellular genes for which at least two siRNAs reduced viral infection by 35% or more (~2 s.d. from mean of negative controls), without a concomitant induction of significant cellular toxicity (Supplementary Fig. 1 and Supplementary Table 1). Although some of these factors were previously known to be involved in influenza virus replication (confirming the robustness of our RNAi approach), most of the factors identified by this analysis represent host genes that have not previously been implicated in mediating influenza virus replication.

Analysis of overrepresented biological annotations identified more than 170 statistically enriched categories (Supplementary Table 2), which fell into 11 broadly related functional groups (Supplementary Fig. 2 and Supplementary Table 3). Signalling molecules, including those involved in the PI3K/AKT pathway, molecules that function to regulate cytoskeletal dynamics, and proteins involved in ubiquitination, phosphatase and protease activities were overrepresented among the 295 factors, underscoring the importance of these cellular functions during influenza virus infection (Table 1, see also Supplementary Tables 4 and 5). Consistent with these observations, we found that small molecule inhibition of two identified AKT pathway regulators,

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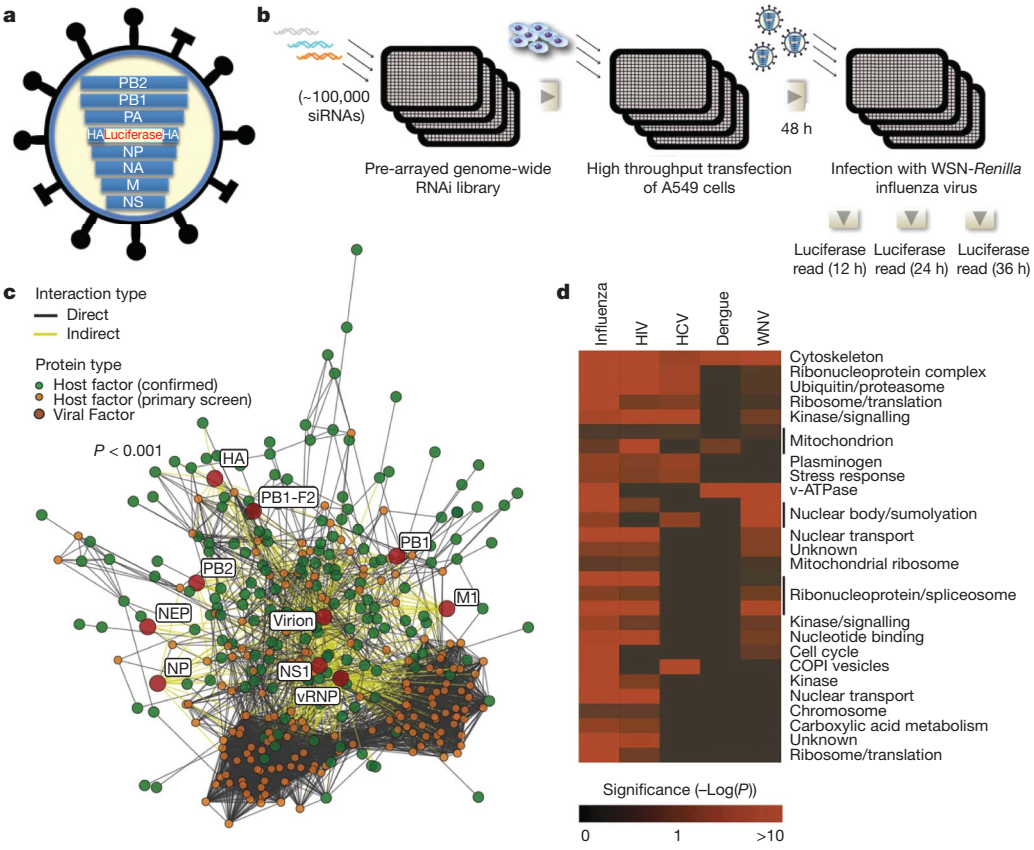

Figure 1 | A genome-wide RNAi screen for influenza virus host cellular factors. **a**, A schematic of the recombinant WSN-Ren virus showing the HA segment modified to express *Renilla* luciferase but maintaining the HA packaging sequences. **b**, An arrayed genome-wide RNAi library (100,000 siRNAs targeting more than 19,000 human genes) was transfected into A549 cells. Cells were subsequently infected with WSN-Ren and virus replication was monitored by measuring luciferase activities at the indicated times. **c**, A highly significant ($P < 0.001$, permutation test) host–pathogen interaction map for influenza virus containing 4,266 interactions between 181 confirmed influenza virus-host cellular factors (green circles), 10 influenza virus-encoded proteins or complexes (red circles), and a further 184 cellular proteins (orange circles). **d**, Predicted complexes from the human interactome used by influenza virus, HIV, HCV, dengue virus and West Nile virus (WNV) (based on results reported here and previous screens[5–8,10–12,28]). Intensity of red colour indicates the significance of enrichment (hypergeometric *P*-values) for proteins required by a virus within a complex.

mTOR (also known as FRAP1) and HSP90AA1, as well as microtubule assembly (TUBB), resulted in a dose-dependent inhibition of influenza virus replication (Supplementary Fig. 3)[15,16].

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 (Fig. 1c and Supplementary Figs 4 and 5). The integration of RNAi and interactome data sets produced a network containing 181 confirmed host cellular factors that mediate 4,266 interactions between viral or cellular proteins, and act as central rate-limiting 'hubs' in cellular pathways or processes required for influenza virus replication (Supplementary Fig. 6 and Supplementary Table 6). Although the coverage and quality of protein interaction databases available at present remains difficult to assess[17], the influenza interaction map was found to be highly significant ($P < 0.001$), indicating that this network topology is not randomly derived, and probably reflects a unique cellular sub-network.

Of the 295 identified host factors required for influenza virus replication, 53 were previously identified in RNAi screens for different RNA viruses (Supplementary Fig. 7 and Supplementary Table 7), including nine mammalian orthologues of host proteins required for influenza virus infection of *Drosophila* cells[12]. It is not at present clear whether this statistically significant ($P = 3.1 \times 10^{-9}$), but modest, overlap reflects false-negative activities in the current or aforementioned screen, or the differential host cell requirements between insect and mammalian cells for influenza virus replication. However, functional classification and protein interaction analysis of these shared factors showed that, collectively, these viruses rely on common host cellular mechanisms to promote discrete stages of their life cycles (Fig. 1d, Supplementary Fig. 8 and Supplementary Table 8).

To verify that the genes identified by the use of the reporter virus reflect the requirements in the context of a wild-type virus infection, 219 (of 295) identified genes were confirmed to inhibit multi-cycle replication of wild-type WSN virus with at least two siRNAs per gene. Furthermore, 76% of the remaining genes had one siRNA that inhibited wild-type influenza replication, indicating a high confirmation rate (Fig. 2a and Supplementary Table 9). For a subset of these genes, further assays were undertaken to confirm that depletion of these genes resulted in reduced viral gene expression (Fig. 2a and Supplementary Table 9), and also to ensure that inhibition of viral replication was not being triggered by a non-specific siRNA-mediated induction of an antiviral state (Supplementary Table 10).

Next, to identify potential factors specifically involved in virus entry steps, 45 of the top-scoring genes in the wild-type WSN assay were selected to be tested in a pseudotyped particle (PP) entry assay, designed to identify host factors that impede low-pH-dependent entry mediated specifically by influenza virus HA (WSN) and vesicular stomatitis virus (VSV)-G protein, while not affecting pH-independent entry promoted by the murine leukaemia virus (MMLV) envelope (Env)[18,19]. WSN-PP infection was reduced in the presence of siRNAs targeting 23 of these genes, including *CD81*, *FGFR4*, *GSK3B*, *MAP2K3* and the v-ATPase subunit *ATP6V0C* (Fig. 2a, b, Supplementary Table 11 and Supplementary Fig. 9). These genes were also required for efficient VSV-G-PP (but not MMLV-PP) infection, suggesting a role in low-pH-dependent virus entry. Notably, small molecule inhibitors of FGFR4, GSK3B and v-ATPase activities attenuated replication of WSN virus, further highlighting their importance in influenza virus infection (Supplementary Figs 3 and 10).

The COPI coat complex is made up of seven subunits, four of which (ARCN1, COPA, COPB2 and COPG) were among the confirmed factors in the protein-interaction network (Figs 1c and 2c). COPI association with endosomes is pH-dependent and coatomer complex is required for the formation of intermediate transport vesicles between the early and late endosomes[20,21]. Consistent with this role, depletion of both COPG and ARCN1 blocked WSN-PP infection (Fig. 2b). The requirement for ARCN1 during the influenza virus entry step was further demonstrated using a more direct virus-like particle (VLP) assay (Fig. 2d)[22], as well as immunolocalization studies (Fig. 2e).

Table 1 | Selected functional categories in early steps of influenza virus replication

Functional category	Genes	Cellular function	Proposed/known role in influenza virus replication	Replication block
IP3-PKC pathway	*ROCK1, CDC42BPA, KSR2, ARAF, PRKCI, CDC42BPB, CIT, AKAP13, RACGAP1, EIF2AK2, CDK4, ACVR2A, MAPK1, PRKCD, MAP2K2, GRK5, ARAF, HIPK1, PAK3, MAP2K3, PTPMT1, LIMK1, PIP5K1C, PAK2, GRK6, SGK1*	Signalling	Viral entry, promoting apoptosis; inhibitors of PKC block viral RNA replication and PKC activators enhance virus replication	Entry (*MAP2K3*)
COPI vesicles	*ARCN1, COPA, COPB2, COPG, USE1*	Early endosome maturation, retrograde golgi to ER transport	Entry (endosomal trafficking)	Entry (*ARCN1*)
Endosomal uptake, maturation, acidification and fusion	*ATP6V1A, ATP6V1B2, ATP6V0B, ATP6V0C, ATP6AP1, ATP6V0D1, ATP6AP2, RABEP1, PIP5K1C, VPS16, TRPV2, MARCH2, EPHB2*	vATPase complex: acidification of intracellular organelles, including endosomes	Low pH-dependent entry, ubiquitin/vacuolar protein sorting pathway required for entry	Entry (*ATP6V1A, ATP6V1B2, ATP6V0B, ATP6V0C, ATP6AP1*)
Actin organization and function	*GAK, APC2, CIT, PAK2, CDC42BPB, PAK3, CDC42BPA, SGCA, FSCN1, OXSR1, CD81, FGFR2, FGFR4, ITGA3, AKAP13, ACTC1, FGFR1, LIMK1, ROCK1, PIK3R4*	Actin organization and function	Intracellular transport, particle movement at cell periphery before virus fusion; actin also implicated in NP functions such as transcription, replication and genome-trafficking	Entry (*CD81, FGFR2 FGFR4, ITGA3, AKAP13*)
PI3K-AKT pathway	*AKT1, BCL3, FRAP1, GSK3B, HRAS, HSP90AA1, IKBKE, ITGA3, JAK2, MAP2K2, MAPK1, MDM2, PIK3R4, PIK3R4*	Signalling	Important for viral protein yields and nuclear export of vRNPs; involved in inhibition of apoptosis	Entry (*GSK3B*)
Endosomal recycling pathway	*RAB11B, RAB17*	Vesicle trafficking	Entry (endosomal trafficking)	Entry (*RAB11B*)
MAPK pathway	*MAP2K3, DUSP3, MAP3K12, MAPK1/ERK, MAP2K2/MEK, ARAF, CAD, CREB1, EPHB2, FGFR4, HRAS, JUN, NTRK2, PAK2, PRKACA, PRKCD, MAP2K5, MAP4K4, HIPK3, ATP6AP2, STK39, MINK1, PBK, TAOK1, ZNF436, CANT1, KSR2*	Signalling	Important for viral protein yields and nuclear export of vRNPs	Entry (*MAP2K3, DUSP3*)
Proteases	*CTSW, PCSK7, KLK9, ANPEP, PRSS35*	Post-translational processing	HA cleavage	Post-entry (*PRSS35*)
Calcium/calmodulin proteins	*CAMK2B, PRKACA, DAPK2, ADRA1B, CREB1, PRKAG2, DCLK2, GRK5, GRK6, KCNJ3, PKD1, PRKCD, STX5, CACGN4, AGTRAP*	Calcium regulation and signalling	Transcriptional regulation	Post-entry (*CAMK2B*)
Nuclear trafficking	*CSE1L, KPNB1, NUP214, NUP153, TNPO3*	Nuclear trafficking	Nuclear import of vRNA or viral/host proteins	Post-entry (*KPNB1, CSE1L*)
Trafficking	*STX10, STX5, GOPC, CLL1, NRBP1*	Membrane and receptor trafficking	Glycoprotein trafficking	ND
Sumoylation	*SUMO2, SUMO1, SAE1, SUMO4*	Post-translational modification	Unknown	ND
Microtubule organization and function	*MID1IP1, TUBB, PRKCI, PLK4, MARK2, DCLK1, NUDCD3, RACGAP1, MAP1LC3C*	Cytoskeletal organization	Microtubules implicated in intracellular viral transport	ND
Autophagy	*PRKAG2, MAP1LC3C, FRAP1, HRAS*	Stress response	Post-entry, blocks viral protein production	ND
Ubiquitination	*MDM2, UBQLN4, HECTD1, CBLL1, DTX2, EPS8L3, FBXO44*	Post-translational modification	Unknown	ND

References for the genes and proposed role in virus replication are provided in Supplementary Table 5. ND, no data.

To evaluate those factors that affect virus replication but not influenza virus entry, we monitored the localization of the influenza virus nucleoprotein (NP) in siRNA-depleted cells after infection with influenza A/WSN/33 virus (Fig. 3a and Supplementary Fig. 11; see Supplementary Information). In comparison to controls, cells depleted of CSE1L, PRSS35, F13A1, SF3A1, CAMK2B, KPNB1 and PPP1R14D show a significant decrease ($P < 0.01$) in nuclear to cytoplasmic ratios of NP protein at 180 min. With the exception of F13A1, depleting these factors did not inhibit entry by WSN pseudotyped virus or β-lactamase (Bla)-M1 VLPs (Fig. 3a), confirming their role in post-entry steps of influenza virus infection. Depletion of CSE1L, PRSS35 and F13A1 also led to a statistically significant ($P < 0.02$) reduction in nuclear to cytoplasmic NP ratios at 90 min post-infection, suggesting that they may have specific roles in early post-entry steps, such as viral uncoating or nuclear import of viral ribonucleoproteins (vRNPs; see also Supplementary Fig. 12). Consistent with a role in nuclear trafficking, imaging at higher resolution confirmed that RNAi-mediated inhibition of CSE1L, but not CAMK2B or KPNB1, results in a decrease in nuclear vRNPs typically seen 90 min after infection with influenza virus (Fig. 3b)[23]. Furthermore, CSE1L specifically inhibited influenza virus gene expression in a mini-genome replicon assay, suggesting that CSE1L activity is required for the nuclear import of vRNPs, as well as newly synthesized viral proteins (Fig. 3c and Supplementary Table 12).

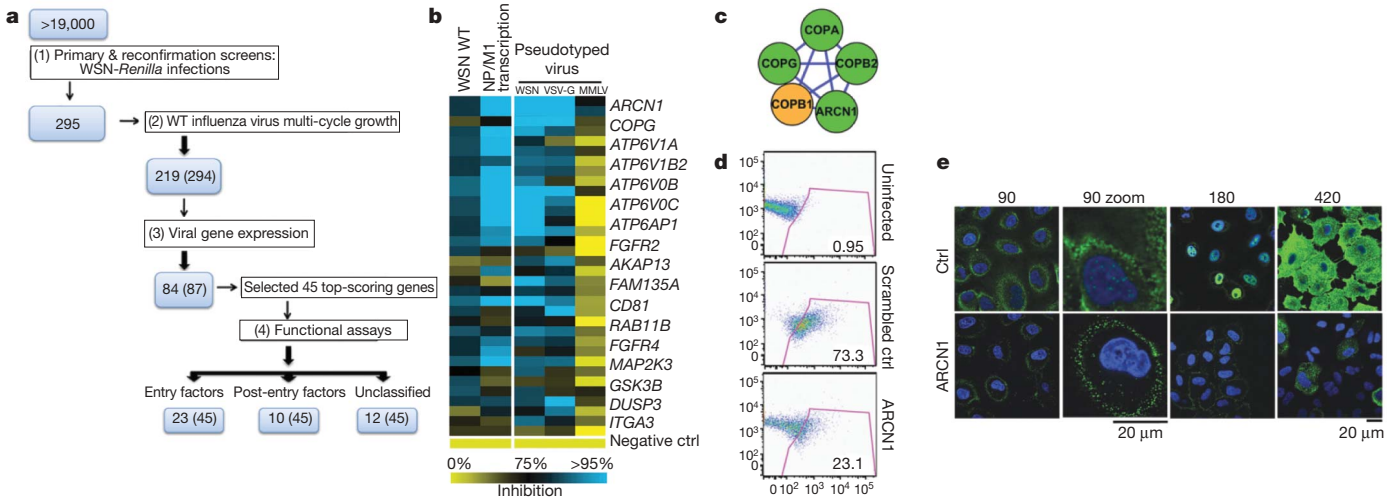

Figure 2 | Identification of host factors involved in influenza virus entry. **a**, Illustration of the screen progression from primary genome-wide analysis to the identification of factors involved in entry and post-entry steps in the virus life cycle. The number of confirmed genes and number of genes tested at each stage (in parentheses) are indicated. **b**, The relative effects of gene depletion (two siRNAs per gene) on infection of luciferase-encoding HIV particles pseudotyped with WSN, VSV or MMLV envelopes (right). Effects of RNAi on wild-type (WT) WSN virus replication and transcription of viral NP and M1 genes are also shown (left). Inhibition in each assay is shown as a continuum of blue (high inhibition) to yellow (low inhibition). Ctrl, control. **c**, The endosomal coat protein complex (COPI) was identified as 1 of 17 biochemical modules (MCODE clusters) likely to be important for influenza virus entry (see Fig. 1c and Supplementary Fig. 5)[29]. **d**, Infection of siRNA-transfected A549 cells with influenza virus VLPs carrying a β-lactamase (Bla)-M1 fusion protein. The percentages of cells containing detectable cytoplasmic β-lactamase activity are indicated. **e**, Cells depleted of ARCN1 and infected with wild-type WSN virus were fixed and stained for NP (green) and nuclei (blue) at the indicated times (in min) and analysed by confocal microscopy. The enlarged images at 90 min post-infection indicate the lack of incoming RNP complexes in the nucleus in cells depleted of ARCN1.

CAMK2B is a ubiquitously expressed calcium sensor that regulates diverse cellular functions, including actin cytoskeletal regulation and CREB-dependent transcription[24]. Our data implicate this kinase in the regulation of viral RNA transcription, as RNAi-knockdown of the kinase had a moderate effect on expression of an influenza mini-genome (Fig. 3c), but did not delay nuclear accumulation of

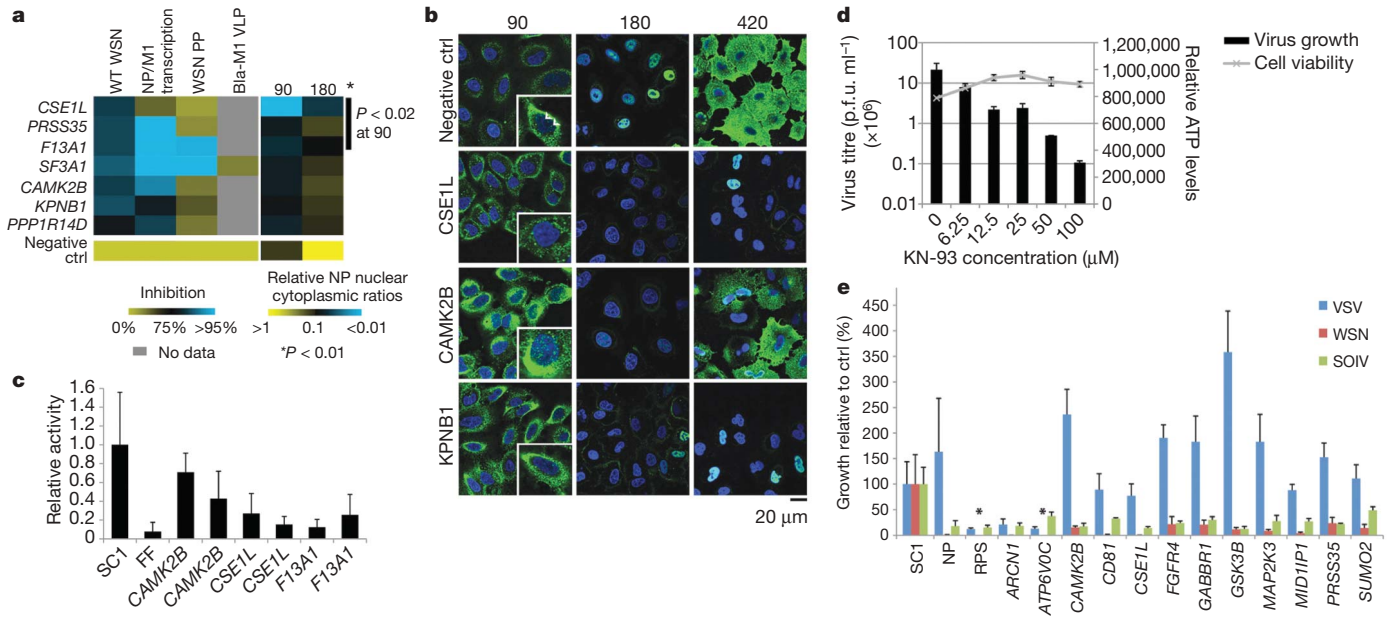

Figure 3 | Characterization of factors in post-entry replication events and conserved requirement by different influenza viruses. **a**, The effect of host factor depletion on the nuclear localization of viral NP protein at 90 and 180 min after A/WSN/33 virus infection is shown (right). Significant effects ($P < 0.01$, Welch t-test) are seen at 180 min with all genes, and with *CSE1L*, *PRSS35* and *F13A1* ($P < 0.02$) at 90 min. Levels of virus replication (WT WSN), viral gene (NP/M1) transcription and entry of WSN pseudotyped particles or Bla-M1 VLPs in cells lacking these factors are shown in the left panel. Values relative to negative controls are depicted in a continuum of blue (>95% inhibition) to yellow (little or no inhibition). **b**, Confocal imaging of influenza virus NP protein localization at the indicated times (in min) after A/WSN/33 virus infection in cells depleted of CSE1L, CAMK2B and KPNB1. Arrowheads in the 90-min inset indicate nuclear RNPs. **c**, The effects of host factor depletion on replication of an influenza virus mini-genome firefly reporter. The normalized fold reduction of firefly luciferase for each gene is shown relative to the scrambled siRNA control (SC1) ± s.d. Two different siRNAs were used to target each gene. All reductions are significant ($P < 0.05$, Student's t-test). FF, firefly luciferase siRNA. **d**, KN-93, a selective CAMK2B inhibitor, inhibits A/WSN/33 influenza viral replication in a dose-dependent manner in MDCK cells, without affecting cell viability (ATP levels). Mean titres ± s.d. of triplicate samples are shown. p.f.u., plaque-forming units. **e**, A549 cells were transfected with siRNAs targeting the indicated genes and subsequently infected with influenza A/WSN/33 virus, swine-origin influenza A/Netherlands/602/2009 (H1N1) virus (SOIV) or VSV. Virus growth is shown as the average percentage relative to the scrambled siRNA control (SC1) ± s.d. Asterisk denotes below level of detection (1×10^4 p.f.u. ml^{-1}). NP, siRNA for influenza A virus NP, RPS, siRNA for *RPS27A*.

vRNPs at 90 min post-infection (Fig. 3b). We also show that a specific inhibitor of CAMK2B, KN-93, inhibits influenza virus growth (Fig. 3d and Supplementary Fig. 13), suggesting that pharmacological targeting of this kinase may be an effective strategy for the development of host-factor-directed antivirals[25].

Finally, we assessed the requirements for 12 identified host cellular factors in the replication of a swine-origin influenza virus (SOIV) isolate from the 2009 pandemic (A/Netherlands/602/2009 (H1N1)) in comparison with influenza A/WSN/33 virus and VSV. Viral growth in siRNA-treated A549 cells showed that these proteins are all required for both SOIV and WSN replication but none of these factors, with the exception of the vATPase and COPI factors, are required for VSV replication (Fig. 3e, Supplementary Table 13 and Supplementary Fig. 14). These results indicate that factors identified here are probably important for the replication of several influenza virus strains.

The genome-wide analysis of influenza virus host factor requirements described here has revealed a large number of cellular proteins and biological pathways previously not known to be involved in the influenza virus life-cycle. These include identification of COPI complex, FGFR, GSK3B, CAMK2B, PRSS35 and others. As this study focused on host factors that regulate the early steps of influenza virus replication, additional analyses will probably help to determine the full complement of cellular proteins required during the complete replication cycle. Further understanding of the roles for these proteins in influenza virus infection will provide new insight into the host–pathogen interactions that orchestrate the viral replication cycle and new opportunities for the development of host-factor-directed antiviral therapies.

METHODS SUMMARY

***Renilla* luciferase influenza virus.** The coding region for the viral HA protein was replaced with that of *Renilla* luciferase and the packaging signals for the HA segment were incorporated, as previously described[13]. The recombinant WSN-Ren virus was generated by reverse genetics in the presence of complementing HA and amplified in HA-expressing MDCK cells[13].

Genome-wide RNAi screen. Genome-wide libraries comprising 98,737 synthetic siRNAs targeting 19,628 unique human genes were arrayed in 384-well plates (7 ng per siRNA) such that each well contained either two (47,560 wells) or one (3617 wells) unique and identifiable siRNA(s) per gene. Although use of low screening concentrations of RNAi may help to minimize off-target activities, this may also contribute to false-negative activities. The library matrix was introduced into A549 cells by a high-throughput transfection process[26,27], and after 48 h the cells were infected with WSN-Ren virus at a multiplicity of infection (M.O.I.) of 0.5. EnduRen Live Cell substrate (Promega) was added after 5 h and relative luminescence for each well was analysed on a plate reader (Viewlux) at 12, 24 and 36 h after infection. For the toxicity screen Cell-titer-glo (Promega) reagent was added 72 h after siRNA transfection. The screens were run minimally in duplicate and analysed using a scaling methodology that sets the positive control siRNA at an arbitrary value of 0.1, and the negative control siRNAs at 1. siRNAs targeting host factors were assigned a score based on the distribution of these values. For more details see Supplementary Information.

Inhibition of virus growth. siRNA-transfected A549 cells were infected with influenza A/WSN/33 virus or VSV (M.O.I. of 0.01) or swine origin influenza A/Netherlands/602/2009 virus (SOIV) (M.O.I. of 1) at 48 h after siRNA transfection. At 36 h after infection, supernatants were collected and virus titres were determined by plaque assay on MDCK cells (for A/WSN/33 and A/Netherlands/602/2009) or on Vero cells for VSV.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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