Exploration of binary virus-host interactions
using an infectious protein complementation assay

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Running title: virus-host interactomics in an infectious context
Abbreviations

GT    GO terms
iPCA  infectious Protein Complementation Assay
LCS   literature-curated set
m.o.i. multiplicity of infection
MS    mass spectrometry
NLR   Normalized Luminescence Ratio
NPC   nuclear pore complex
OM    other methods
pfu   particle forming unit
PPI   protein-protein interaction
RNP   ribonucleoproteins
RRS   Random Reference Set
Y2H   yeast two-hybrid
Abstract

A precise mapping of pathogen-host interactions is essential to achieve a comprehensive understanding of the processes of infection and pathogenesis. The most frequently used techniques for interactomics are the yeast two-hybrid binary methodologies, which does not recapitulate the pathogen life cycle, and the TAP-MS co-complex methodologies, which can not distinguish whether interactions are direct or indirect. New technologies are thus needed to improve the mapping of pathogen-host interactions. Here, we detected binary interactions between influenza A virus polymerase and host proteins during the course of an actual viral infection, using a new strategy based on trans-complementation of the Gluc1 and Gluc2 fragments of *Gaussia princeps* luciferase. Infectious recombinant influenza viruses that encode a Gluc1-tagged polymerase subunit were engineered to infect cultured cells transiently expressing a selected set of Gluc2-tagged cellular proteins involved in nucleocytoplasmic trafficking pathways. A random set and a literature-curated set of Gluc2-tagged cellular proteins were tested in parallel. Our assay allowed sensitive and accurate recovery of previously described interactions while it revealed 30% of positive, novel viral-host protein-protein interactions within the exploratory set. In addition to cellular proteins involved in nuclear import pathway, components of the nuclear pore complex such as NUP62 and mRNA export factors such as NXF1, RMB15B and DDX19B were identified for the first time as interactors of the viral polymerase. Gene silencing experiments further showed that NUP62 is required for efficient viral replication. Our findings give new insights on the subversion of the host nucleocytoplasmic trafficking pathways by influenza A viruses. They also demonstrate the potential of our infectious protein complementation assay for high-throughput exploration of influenza virus interactomics in infected cells. With more infectious reverse genetics system becoming available, this strategy should be widely applicable to numerous pathogens.
INTRODUCTION

Influenza pandemics can be devastating. Even during typical epidemic years, approximately 250,000-500,000 people worldwide die as a result of severe complications associated with influenza infections. The elucidation of how influenza viruses interact with the host cell machinery and hijack cellular pathways has become an active field of research and could path the way to new antiviral approaches targeting virus–host interactions (1, 2). Several RNAi-based genome-wide screens have been performed for the identification of host factors involved in influenza virus replication (3-9). Because the influenza virus polymerase is a key component in the viral life cycle and is a strong determinant of virulence and host-range (10, 11), other studies have attempted to identify cellular proteins that interact with the viral polymerase using yeast two-hybrid (9, 12) or proteomics approaches (13-15). These approaches have limitations, as they do not recapitulate the entire viral cycle or can not distinguish whether interactions are direct or indirect (16). New technologies are thus needed to improve mapping of virus–host interactions and to design more effective therapeutics.

The genome of influenza A viruses consists of eight molecules of single-stranded RNA of negative polarity. The viral RNAs (vRNAs) are associated with the nucleoprotein (NP) and with the three subunits of the polymerase complex (PB1, PB2 and PA) to form ribonucleoproteins (RNPs). Once in the infected cells, the RNPs are transported to the nucleus, where they undergo transcription and replication (17). Here we used a new strategy to detect binary interactions between the viral polymerase and host proteins during the course of an actual viral infection. Our infectious Protein Complementation Assay (iPCA) is based on complementation of two trans-complementing fragments of *Gaussia princeps* luciferase, Gluc1 and Gluc2 (18, 19). Interaction-mediated luciferase activity is measured in cultured cells transiently expressing a cellular protein fused to Gluc2 and infected with a recombinant influenza virus having Gluc1 fused to one of its polymerase subunit. These engineered infectious viruses allowed us to achieve sensitive and accurate detection of influenza virus-host protein-protein interactions throughout the viral cycle.
EXPERIMENTAL PROCEDURES

Cells and viruses

293T, A549 and BSR cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). MDCK cells were grown in modified Eagle’s medium (MEM) supplemented with 5% FCS.

Wild-type A/WSN/33 (WSN) influenza virus was produced by reverse genetics using a procedure adapted from Fodor et al. (20). Vesicular stomatitis virus (VSV, Indiana strain) was kindly provided by O. Delmas (Institut Pasteur, Paris, France). WSN and VSV viruses were amplified on MDCK and BSR cells, respectively, at a multiplicity of infection (m.o.i.) of 0.001 pfu/cell. They were titrated on MDCK and BSR cells, respectively, using a plaque assay adapted from Matrosovich et al. (21).

Plasmids

The eight pPol™ plasmids containing the sequences corresponding to the genomic segments of influenza virus A/WSN/33, and the four recombinant pcDNA3.1 plasmids for the expression of WSN-PB1, -PB2, -PA and -NP proteins (20), were kindly provided by G. Brownlee (Sir William Dunn School of Pathology, Oxford, UK).

The pPol™-SL-PB2-Gluc1 and pPol™-SL-PB2-Gluc2 plasmids were obtained by subcloning the Gluc1 and Gluc2 coding sequences, respectively, between the NotI and NheI sites of the pPol™-PB2-GFPS11 plasmid (22).

The pPol™-PB1-SL-Gluc1 and pPol™-PA-LL-Gluc1 plasmids were obtained by modification of the pPol™-WSN-PB1 and pPol™-WSN-PA using standard PCR and cloning procedures in order to fuse the following sequences to the 5' end of the PB1- or PA-ORF: a) a short sequence encoding the short peptidic linker AAAGGS (SL, for PB1) or the long peptidic linker AAAGGGGSGGGGS (LL, for PA); b) the Gluc1 coding sequence; c) a stop codon combined with an SpeI restriction site (TAAACTAGT);
and d) the 5’ terminal 88 nucleotides of the PB1 segment or the 5’ terminal 100 nucleotides of the PA segment.

To produce the pPoll-PB1-SL-Gluc2 and pPoll-PA-LL-Gluc2 plasmids, the Gluc2 coding sequence was amplified using the pSPICA-N2 plasmid (18) as a template. The resulting PCR product was digested with *NotI* and *NheI* and subcloned between the *NotI* and *SpeI* sites of pPoll-PB1-Gluc1 and pPoll-PA-Gluc1 plasmids, respectively.

The Gateway®-compatible donor plasmids containing cellular ORFs were obtained from the Human ORFeome resource (hORFeome v3.1). They were transferred into the Gateway®-compatible pSPICA-N2 destination vector (18), using the LR clonase (Invitrogen) according to the manufacturer specifications. The resulting plasmids allowed the expression of Gluc2-cellular-ORF fusion proteins.

The proteins included in the RRS were randomly selected within the RRS from Braun *et al* (23).

All constructs were verified by sequencing using a Big Dye terminator sequencing kit and an automated sequencer (Perkin Elmer). The sequences of the oligonucleotides used for amplification and sequencing can be provided upon request.

*Production of recombinant viruses by reverse genetics*

The method used for the production of recombinant influenza viruses by reverse genetics was adapted from previously described procedures (20). Briefly, the eight pPoll-WSN and four pcDNA3.1-WSN plasmids (0.5 µg of each) were co-transfected into a sub-confluent monolayer of co-cultivated 293T and MDCK cells (4 x 10⁵ and 3 x 10⁵ cells, respectively, seeded in a 35 mm dish), using 10 µL of FuGENE® HD transfection reagent (Roche). After 24 h of incubation at 35°C, the supernatant was removed, cells were washed twice with DMEM and incubated at 35°C in DMEM containing TPCK-trypsin at a final concentration of 1 µg/mL for 48 h. The efficiency of reverse genetics was evaluated by titrating the supernatant on MDCK cells in plaque assays. For subsequent amplification of the recombinant PB1-, PB2- and PA-Gluc1 or PB1-, PB2- and PA-Gluc2 viruses, MDCK cells were infected at a m.o.i. of 0.0001 and incubated for 3 days at 35°C in DMEM containing TPCK-trypsin at a
concentration of 1 µg/mL. Viral stocks were titrated by plaque assays on MDCK cells as described previously (21). Viral RNA was extracted and subjected to reverse-transcription and amplification using specific oligonucleotides. The products of amplification were purified using a QIAGenQuick gel extraction kit (Qiagen) and were sequenced using a Big Dye terminator sequencing kit and an automated sequencer (Perkin Elmer). The sequences of the oligonucleotides used for amplification and sequencing can be provided upon request.

**Antiviral activity assay**

MDCK cells were seeded at a concentration of 2 x 10^4 cells per well in 96-well white plates (Greiner Bio-One). After 24 h, cells were rinsed twice with DMEM and co-infected with a combination of P-Gluc1 and P’-Gluc2 viruses (0.01 pfu of each virus/cell in 50 µL). After viral adsorption for 1 h at 35°C, 50 µL of DMEM supplemented with 4% of FCS were added and cells were incubated at 35°C for 24 h. Cells were either left untreated, or incubated with ribavirin (Sigma) or nucleozin (Sigma) at a final concentration of 1-100 µM or 0.01-1 µM respectively for 24 h post-infection, or incubated with amantadine (Sigma) at a final concentration of 10 µM during the 30 min preceding infection, during viral adsorption, and for 24 h post-infection. At 24 h post-infection, cells were mildly rinsed in Ca^{2+}/Mg^{2+}-DPBS, and 40 µL of Renilla lysis buffer (Promega) were directly added to each well. After incubation for 1 h at room temperature, the *Gaussia princeps* luciferase enzymatic activity was measured using the Renilla luciferase assay reagent (Promega) and a Berthold Centro XS luminometer (*Renilla* luminescence counting program, integration time 10 seconds after injection of 50 µL of the reagent).

**Viral-host protein-protein interaction assay**

293T cells were seeded at a concentration of 3 x 10^4 cells per well in 96-well white plates (Greiner Bio-One). After 24 h, they were co-transfected in triplicates with 100 ng of the recombinant p-Gluc2-cellular-ORF constructs and 50 ng of the pCI plasmid (Promega), using polyethylenimine PEI
At 17 h post-transfection, cells were rinsed with DMEM and infected with 50 µL of a viral suspension containing $9 \times 10^5$ pfu of either the PB2-Gluc1, PB1-Gluc1 or PA-Gluc1 recombinant virus.

As a control, cells were either 1) co-transfected with 100 ng of the empty p-Gluc2 plasmid and 50 ng of pCI, and subsequently infected with $9 \times 10^5$ pfu of either the PB2-Gluc1, PB1-Gluc1 or PA-Gluc1 recombinant virus; or 2) co-transfected with 100 ng of the p-Gluc2-cellular-ORF constructs and 50 ng of the empty p-Gluc1 plasmid, and subsequently infected with $9 \times 10^5$ pfu of the wild-type WSN virus. After viral adsorption for 1 h at 35°C, 50 µL of DMEM supplemented with 4% FCS were added to each well, and cells were incubated at 35°C for 6 h. Cells were mildly rinsed in Ca$^{2+}$/Mg$^{2+}$-DPBS, and 40 µL of Renilla lysis buffer (Promega) were directly added to each well. After incubation for 1 h at room temperature, the *Gaussia princeps* luciferase enzymatic activity was measured using the Renilla luciferase assay reagent (Promega) and a Berthold Centro XS luminometer (*Renilla* luminescence counting program, integration time 2 seconds after injection of 50 µL of the reagent). For each p-Gluc2-cellular-ORF plasmid, three Normalized Luminescence Ratios (NLRs) were calculated as follows: the luminescence activity measured in cells transfected with the p-Gluc2-cellular-ORF plasmid and infected with the PB2-Gluc1, PB1-Gluc1 or PA-Gluc1 virus (Arbitrary Units, mean of triplicates), divided by the sum of the luminescence activities measured in both control samples as described above (Arbitrary Units, mean of triplicates).

**Enrichment analysis**

The integrated dataset of human protein interaction available in HIPPIE database was used to generate the subnetwork of PPI connected as first neighbors to proteins directly targeted by the influenza virus polymerase subunits (layer one). This subset of PPI connected to layer one was scored according to experimental evidence as described in HIPPIE (24). Interactions with scores $\geq 0.75$ considered as high-confidence interactions, were selected as layer two. The resulting subnetwork was analyzed with ClueGO (Cytoscape plugin, (25)) to extract the non-redundant biological information.
based on enrichment for biological process descriptors in gene ontology annotations associated with each polymerase subunit. Significance of each term was calculated with hypergeometric test and a Bonferroni P value correction according to ClueGO standard statistical options (25). GO term analysis was restricted to P value ≤ 0.01 and “global” network predefined selection criteria in order to get a more global functional enrichment information and obtain insights on main biological processes susceptible to be targeted by the influenza virus polymerase subunits.

siRNA transfection and infection assays

Small interfering RNAs (siRNAs) targeting CCT2, RANGAP1 and NUP62 were purchased from Dharmacon (ON-TARGETplus SMARTpool). Non-target siRNA (ON-TARGETplus Non-targeting Control pool, Dharmacon) was used as a negative control. A549 cells (2 x 10^4 cells) were transfected in suspension with 25 nM of siRNA using 0.2 µL of DharmaFECT1 transfection reagent (Dharmacon) and were plated in 96-well plates. The medium was replaced with fresh medium 24 h later. At 48 h post-transfection, cells were infected with influenza A/WSN/33 virus at a m.o.i. of 0.001 pfu/cell or with VSV at a m.o.i of 0.0001 pfu/cell. After viral adsorption for 1 h at 37°C, 50 µL of DMEM supplemented with 4% FCS were added per well and cells were incubated at 37°C for 24 h. Supernatants were collected and viral titers were determined by plaque assays on MDCK or BSR cells, as described above. Cell viability was determined by assaying total intracellular ATP with the CellTiter-Glo Luminescent Viability Assay kit (Promega) according to the manufacturer’s recommendations. Down-regulation of siRNA-targeted genes was evaluated at 48 h and 72 h post-transfection by RT-qPCR using Maxima First Strand cDNA Synthesis kit and Solaris qPCR Expression Assays and Master Mix (Thermo Scientific) according to the manufacturer’s recommendations.
RESULTS

Using a plasmid-based reverse genetics system (20, 26, 27), we produced a set of infectious recombinant A/WSN/33 (WSN) influenza viruses that express a PB1, PB2 or PA polymerase subunit fused at their C-terminal end to Gluc1 or Gluc2 complementation fragments of *Gaussia princeps* luciferase. Viability of the recombinant viruses depended on the peptidic linker separating the Gluc fragment from the polymerase subunits, and on sequence duplication ensuring the conservation of packaging signals at the 5’ end of the viral genomic segments (26) (Fig. 1). Despite moderate attenuation compared to the wild-type WSN virus, the viruses expressing a viral fusion protein (vP-Gluc1 or vP-Gluc2) were replication-competent, the vP-Gluc1 viruses showing higher titers than their vP-Gluc2 counterparts upon multi-cycle amplification on MDCK cells (2-10 x 10⁶ pfu/mL compared to 0.3-3 x 10⁶ pfu/mL).

We checked whether the viral fusion proteins were indeed expressed in vP-Gluc1 and vP-Gluc2 infected cells, and whether they could participate in the reconstitution of a functional *Gaussia princeps* luciferase. To this end, MDCK cells were infected with six distinct pairs corresponding to every possible combination of vP-Gluc1 and vP-Gluc2 viruses. High luciferase activities were detected in cell lysates at 24 h post-infection (Fig. 2A, black bars), which likely resulted from the natural assembly of viral polymerase heterotrimeric. A strong and dose-dependent reduction of luciferase activities was observed in the presence of ribavirin (Fig. 2A, white bars, and Figure 2B) and in the presence of nucleozin (Figure 2C), but not in the presence of amantadine (Fig. 2A, grey bars), consistent with the A/WSN/33 virus used in these experiments being sensitive to ribavirin and nucleozin but resistant to amantadine (28, 29). The observed EC₅₀ values for ribavirin and nucleozin were 9.7 µM and 0.13 µM, respectively, in accordance with literature data (30, 31). These data showed that the vP-Gluc recombinant viruses were well designed for the detection of protein-protein interactions involving the polymerase subunits during the course of infection. In addition, they indicated that the vP-Gluc viruses...
can be used to measure inhibition of viral replication in an assay which covers the entire viral cycle, unlike existing reporter-based methods (3, 32, 33), and therefore could be instrumental for the development of improved high-throughput screens for chemical antivirals or genome-wide siRNAs.

We next explored the potential of the vP-Gluc1 viruses for the detection of binary interactions between each subunit of influenza virus polymerase (PB1, PB2 and PA) and cellular factors. To validate the assay and to determine the cutoff for non-interacting protein pairs, we selected i) a literature-curated set (LCS) composed of 58 human proteins that were shown to interact with influenza virus polymerase by yeast two-hybrid (9, 12) (Y2H: 34 proteins), by TAP-MS (13-15) (MS: 15 proteins), or by other methods (OM: 9 proteins) (highlighted in orange in Table 1 and supplemental Table S1), and ii) an a priori “negative” set of 36 randomly picked human proteins or Random Reference Set (RRS) (Table 1). To identify novel host interactors of the viral polymerase subunits, we selected a set of proteins that had not been documented with respect to their interaction with influenza virus polymerase, specifically 31 proteins annotated to the GO terms “nucleocytoplasmic transport” (GO:0006913) or “nuclear pore” (GO:0005643) (GT, Table 1 and supplemental Table S1), plus 5 proteins that affected influenza virus replication in at least two independent RNAi screens (3) and were functionally related to intracellular transport (RNAi, Table 1 and supplemental Table S1). While this work was in progress, 10 proteins from this selection were identified as polymerase interactors (12, 13, 34) and were thus shifted into the LCS. The resulting exploratory set comprised 26 proteins (GT: 22 proteins; RNAi: 4 proteins) (highlighted in purple in Table 1).

Expression plasmids encoding the selected human proteins fused to the Gluc2 fragment at their N-terminal end were produced. The LCS, the RRS and the exploratory set were tested in triplicate for the production of luciferase activity upon transfection in 293T cells and subsequent infection with the recombinant influenza viruses expressing either PB1-Gluc1, or PB2-Gluc1, or PA-Gluc1 (Fig. 3A). For each human-viral protein pair, luciferase activity was expressed as a Normalized Luminescence Ratio (NLR) over control protein pairs (18) (Fig. 3A).
The NLR obtained for the RRS set are shown in Table 1. The theoretical root mean square deviation with 97.5% confidence interval obtained from Gaussian fits corresponded to a NLR of 3.2, 2.2 and 2.6 for the PB2, PB1 and PA-Gluc1 viruses respectively (Fig. 3B). The NLR cutoff for non-interacting protein pairs was set accordingly at 4.0, a stringent value reducing false positive background below 2.5%.

Comparison of the results obtained in two independent experiments with the LCS and the exploratory set showed strong reproducibility (Bartlett’s test for equal variances, $\chi^2(1) = 31.5642$, 25.9196 and 39.6757 for PB2-Gluc1, PB1-Gluc1 and PA-Gluc1 screens, respectively, Prob $> \chi^2 < 0.001$, the mean NLRs are shown in Table 1).

Within the LCS, 33 out of 58 proteins produced a NLR > 4.0 with at least one of the vP-Gluc1 viruses (Table 1, highlighted in green, and Fig. 3C), a recovery rate of 56.9%. The high recovery rate among proteins identified by MS (80%, 12 out of 14 proteins) or by OM (77.8%, 7 out of 9 proteins) (Fig. 4B) underlines the sensitivity of our assay. The lower recovery rate among proteins identified by Y2H is in agreement with the fact that some biophysical interactions which can be forced in the context of Y2H cannot be recovered in the context of human cell expression, and thus correspond to biological false positives (35). The proportion of false negatives might be further reduced by reversing Gluc1 and Gluc2 fusion with respect to viral and host protein, which may modulate the background signal, and by testing both N- and C-terminal fusions with host proteins, as N-terminal fusions may disrupt protein-protein interactions in some cases. Moreover, new edges were identified within the LCS, i.e. some of the proteins that had been shown to interact with one of the viral polymerase subunit in pair-wise screens were found to interact with another subunit as well (Fig. 4A, green edges connected to blue nodes) most likely indicating an interaction with a heterodimer or with the PB1-PB2-PA heterotrimer.

Supporting the accuracy of this interpretation is the fact that the importin 5 protein (IPO5), known to interact with the PB1-PA dimer but not with the PB2 protein (36), produced NLRs > 4 with the PB1- and PA-Gluc1 viruses but not with the PB2-Gluc1 virus (Table 1 and Fig. 4A).
Within the exploratory set, 8 out of 26 proteins produced a NLR > 4.0 with at least one of the vP-Gluc1 viruses (Table 1 and Fig. 3C) and were thus identified as novel interactors of the viral polymerase (Fig. 4A, green nodes). These include the DDX19A and DDX19B helicas, the karyopherin alpha 5 (KPNA5), the NUP62 nucleoporin and NUP62CL, a shorter protein that shows 79% similarity with the C-terminal domain of NUP62, the Ran GTPase activating protein 1 (RANGAP1), the putative RNA binding motif protein 15B (RBM15B) and the nuclear RNA export factor 1 (NXF1). The NUP62, NUP62CL and RANGAP1 proteins interacted with PB2 only, whereas the five other proteins interacted with all three polymerase subunits (Fig. 4A). The karyopherin alpha 5 and the Ran GTPase activating protein 1, a regulator of the Ran GTP/GDP cycle, are involved in the nuclear import pathway. The NUP62 and the DDX19B are components of the nuclear pore complex, and along with NXF1 and its cofactor RBM15B are involved in mRNA export from the nucleus.

In order to extract functional information from our interactomic dataset, we started by selecting the 41 host proteins that were found to interact with influenza virus polymerase subunits in infectious native conditions using the iPCA assay (Fig. 4A, blue and green nodes). Based on this selection identified herein as “layer one”, we generated a second layer of human proteins corresponding to their first neighbors in the HIPPIE high confidence score filtered network for human PPI (24) (see Experimental Procedures for scoring). To capture biological information associated to the subnetwork of the human interactome resulting from the merging of both interactomic layers, we used ClueGO, a Cytoscape plugin which extract and statistically analyze gene ontology annotations (25) (see Experimental Procedures for details on the enrichment analysis). The functionally grouped annotation network obtained with ClueGO (Fig. 4C) showed enrichment for processes related to mRNA and protein biosynthesis and metabolism, protein transport, and apoptosis, in agreement with the fact that these processes can be hijacked or modulated by influenza virus infection (3). In addition, this functional enrichment analysis suggested the interaction of the polymerase subunits with the chromatin compartment and with signal transduction pathways, in agreement with recent studies (9, 37-39).
Interestingly, some of the enriched processes were connected with only two or one of the viral polymerase subunits (Fig. 4C), thus highlighting their specificities.

We performed siRNA-mediated knockdown on two novel interactors of the viral polymerase identified by iPCA, RANGAP1 and NUP62, to investigate their role in influenza virus replication. A549 cells treated with siRNAs targeting RANGAP1, NUP62, or with a non-targeting control siRNA, were infected with the A/WSN/33 influenza virus at a m.o.i. of 0.001 or with a vesicular stomatitis virus (VSV) at a m.o.i. of 0.0001. CCT2 siRNA was used as a positive control as it has been shown to reduce influenza virus growth in A549 cells (40). At 24 h post-infection, supernatants were harvested and virus titers were determined by plaque assays (Fig. 5). siRNA-mediated knockdown of CCT2, RANGAP1 and NUP62 reduced the expression of target mRNAs by more than 80%, as evaluated by RT-qPCR and showed no toxicity (data not shown). Down-regulation of CCT2 and RANGAP1 expression inhibited influenza virus replication by 3.4 and 4.5 fold, respectively. In NUP62-silenced cells, a 22.3 fold reduction in influenza virus titers was observed (Fig. 5). In contrast, CCT2, RANGAP1 and NUP62 siRNAs did not affect VSV replication (data not shown). These data clearly demonstrate that NUP62 and RANGAP1 are involved in influenza virus replication.
DISCUSSION

Transcription and replication of the genome of influenza A viruses take place in the nucleus of the infected cells. To gain access to the nucleus, the viral ribonucleoproteins (vRNPs) and the newly synthetized polymerase subunits must interact with the host nuclear import machinery (11, 41). Import of macromolecules through the nuclear pore complex (NPC) is mediated by import receptors, such as importins/karyopherins that bind the cargo, interact with nucleoporins and facilitate translocation through the NPC (42). Within the LCS, we confirmed the interaction of one or several viral polymerase subunits with importins IPO5 (importin 5, previously known as RANBP5), KPNA1 (importin alpha 5), KPNA2 (importin alpha 1), KPNA3 (importin alpha 4), KPNA6 (importin alpha 7) and with the nucleoporin NUP50 that has been proposed to prevent the nuclear PB2 rebinding to importin alpha 5 (11, 34, 41). Within the exploratory set, we identified KPNA5 (importin alpha 6), RANGAP1, NUP62 and NUP62CL as additional partners. KPNA5 shares more than 90% similarity with KPNA1 and KPNA6 and human KPNA5 expression seems to be restricted to testis (43). KPNA5 could be a species-specific importin, as mouse KPNA5 has not been identified while bovine KPNA5 was found to be expressed ubiquitously (44). The relative importance of KPNA5 and its potential redundancy with KPNA1 and KPNA6 is thus likely to differ from species to species, in line with the fact that the differential use of importin alpha isoforms by influenza virus is a determinant of host range (11, 45).

Inside the nucleus, the small GTPase Ran in a GTP-bound form binds to importins, enables their dissociation from the cargo and their translocation to the cytoplasm. The cytoplasmic Ran GTPase activating protein RANGAP1 stimulates the GTPase activity of Ran, which results in the release of importins for another import cycle (42). An interaction between the viral polymerase and RANGAP1 could thus modulate the recycling of importins or other transport receptors. NUP62 is a nucleoporin located at the core of the NPC, and NUP62CL is a shorter protein that shows 79% similarity with the C-terminal domain of NUP62. Our findings add influenza virus polymerase to the list of other viral proteins known to interact with NUP62, such as HTLV Tax, HSV ICP27 or EBV BGLF4 proteins (46-
Several viruses interfere with nucleocytoplasmic transport via degradation and/or disruption of nucleoporins. In particular, NUP62 is cleaved by the enterovirus protease 2A, hyperphosphorylated by the cardiovirus L protein and displaced from the NPC upon adenovirus or HIV-1 infection (49). Our gene silencing experiments demonstrate that NUP62 is required for efficient influenza virus replication. It remains to be explored how is NUP62 involved in viral replication, whether the interaction between influenza virus polymerase and NUP62 enhances transport of vRNPs or viral mRNAs across the NPC and whether it could alter the nucleocytoplasmic trafficking of host factors or mRNAs.

NXF1, the major cellular transport receptor for export of cellular mRNAs, was identified in four independent RNAi screens as an important host factor for influenza virus replication (3, 5-7, 9). Indeed, a subset of influenza virus mRNAs is exported through the NXF1 pathway (50). However the mechanisms by which influenza virus mRNAs are exported to the cytoplasm are unclear. Whether or not the viral polymerase is associated with any or with a subset of viral mRNAs still remains controversial (51-53). Here we clearly demonstrate an association of influenza virus polymerase with NXF1 and with the NXF1-cofactor RMB15B, (54). Moreover, we show that influenza virus polymerase interacts with DDX19B (and DDX19A which shows 98% similarity with DDX19B), a DEAD-box helicase that localizes to the cytoplasmic side of the NPC by binding to NUP214 and plays an essential role in mRNA export by remodeling mRNP complexes during their passage through the NPC (55). The interaction of the viral polymerase with these mRNA export factors is likely to modulate the export of cellular and/or viral mRNAs. Taken altogether, our findings should contribute to a better understanding of the molecular mechanisms by which influenza viruses exploit the host nucleocytoplasmic trafficking pathways for their replication.

We report the first application of luciferase complementation to proteins expressed in the context of an infectious pathogen. Our iPCA assay allows the detection of binary protein-protein interactions in infectious, native conditions. We illustrate how this assay can be combined with an annotated human protein-protein interaction network to generate a high-confidence function-specific subnetwork used by the influenza virus. The fact that it covers the entire pathogen’s life cycle represents a clear advantage.
over the yeast two-hybrid screen. This feature is also very useful for the development of high-throughput screens for chemical antivirals or genome-wide siRNAs. The iPCA assay can readily be handled inside high-security laboratories. As a consequence it applies to highly pathogenic microorganisms, unlike mass-spectrometry which does not apply to infectious cell lysates that cannot be handled experimentally outside high-security laboratories. It showed an excellent sensitivity and accuracy when applied to influenza virus against random, literature-curated and exploratory sets of human proteins, as we were able to confirm most of the already documented influenza polymerase-host interactions and to identify novel interactions. These observations suggest that fusion to the small-sized GLuc1 or GLuc2 fragment is overall well tolerated and preserves protein-protein interactions. The iPCA assay or adaptations of it should prove useful in elucidating many aspects of influenza virus biology. With more infectious reverse genetics systems becoming available, this strategy should be widely applicable to other viral and non-viral pathogens.
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References


Figure Legends

**Fig. 1. The PB1, PB2 and PA viral RNA (vRNA) segments used in reverse genetics experiments.**

The vRNAs encoding a PB1-Gluc1/2 (A), PB2-Gluc1/2 (B), or PA-Gluc1/2 (C) fusion proteins are represented schematically. The PB1-, PB2- and PA open reading frames (light grey boxes) are fused to the sequences encoding the Gluc1 or Gluc2 (Gluc1/2) domain of *Gaussia princeps* luciferase (white boxes) and flanked by the 3’ and 5’ viral non-coding regions (NCR, thick lines). The Gluc1 and Gluc2 domains are separated from the viral proteins with a small (PB1 and PB2) or large (PA) linker (SL and LL black boxes, respectively). The duplicated PB1, PB2 and PA coding sequences are represented as hatched boxes. The length of the sequence kept identical to the wild-type at the 5’ extremity of the genomic segment is indicated by a double-headed arrow. Black arrowheads indicate stop codons.

**Fig. 2. Antiviral activity assay.** *A*, Luciferase activities measured in MDCK cells co-infected with six distinct indicated combinations of a P-Gluc1 and a P’-Gluc2 virus, in the absence of antiviral compound (black bars), in the presence of 100 μM ribavirin (white bars), or in the presence of 10 μM amantadine (grey bars). Data are expressed as the mean +/- SD of quadruplicate repeats. Dotted line: background corresponding to the sum of the luciferase activities obtained with the P-Gluc1 or the P’-Gluc2 viruses alone. *B*, Percentage of the luciferase activities measured in MDCK cells co-infected with the PB2-Gluc1 and PB1-Gluc2 viruses in the presence of increasing concentrations of ribavirin (upper panel) or nucleozin (lower panel). The data are expressed as the mean +/- SD of quadruplicate.

**Fig. 3. Identification of cellular interactors of influenza virus polymerase by iPCA.** *A*, Schematic of the experiment. 293T cells seeded in 96-well plates are transfected with an ordered set of 84 plasmids encoding human proteins fused to Gluc2 (Gluc2-human P), or with control plasmids. At 18 h post-transfection, 293T cells are infected at high multiplicity of infection (>1 pfu/cell) with an influenza
virus expressing a Gluc1-tagged polymerase (viral P-Gluc1) or with the wild-type control virus. At 6 h post-infection, cell lysates are prepared and Normalized Luminescence Ratios (NLRs) determined by dividing the luminescence activity measured in cells co-expressing a Gluc2-human P and a viral P-Gluc1 by the sum of the luminescence activities measured in the corresponding control samples. B, Gaussian curves fitted on log(NLR) values of the RRS and the literature-curated set (LCS) with the PB2-Gluc1, PB1-Gluc1 and PA-Gluc1 viruses. The positive threshold value of 4 is indicated. C, Heat map of the Normalized Luminescence Ratios. The 84 screened cellular proteins are arranged by hierarchical clustering according to their NLRs. The 58 proteins that belong to the LCS and the 26 proteins that belong to the exploratory set are indicated. The color scale indicates the range of the NLR measured upon screening with the PB2-Gluc1, PB1-Gluc1 or PA-Gluc1 recombinant influenza virus.

**Fig. 4. Analysis of cellular interactors of influenza virus polymerase.** A, Influenza virus polymerase interaction network. Viral polymerase subunits are represented by pink diamonds. Cellular interactors are represented by circles and edges are colored according to the recovery (blue), the non-recovery (grey) or the novelty (green) of the interaction. B, Histogram representation of the number of recovered/non-recovered interactors among the mass-spectrometry (MS), yeast two-hybrid (Y2H) and other methods (OM) reference subsets within the literature-curated set, and the number of newly identified interactors among the exploratory set. C, ClueGO visualization as a functionally grouped annotation network with edges connecting polymerase subunits to gene ontology biological process attributes. Edges width is proportional to the relative enrichment of the biological processes evaluated by protein count.

**Fig. 5. Influenza virus replication in siRNA-treated A549 cells.** A549 cells were transfected with siRNA targeting the cellular proteins CCT2, RANGAP1 or NUP62 or with non-target siRNA (NT). At 48 h post-transfection, cells were infected with influenza A/WSN/33 virus at a m.o.i. of 0.001.
Supernatants from infected cells were harvested at 24 h post-infection and viral titers were determined by plaque assay on MDCK cells. The data are expressed as the mean +/- SD of independent triplicate.
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<sup>a</sup>LC: Literature Curated; Y2H: Yeast Two-Hybrid; MS: Mass Spectrometry; OM: Other Methods; Exp: Experimental; GT: GO Terms; RRS: Random Reference Set

<sup>b</sup>Mean of two independent determinations
Figure 1
Figure 2

(A) Bar graph showing luciferase activity (AU) for different combinations of Gluc1 and Gluc2 with and without inhibitors. The bars represent the following conditions:
- Black: no inhibitor
- White: 100 μM ribavirin
- Gray: 10 μM amantadine

(B) Graph showing RLU (% of control) against the concentration of ribavirin (μM) for a specific condition.

(C) Graph showing RLU (% of control) against the concentration of nucleozin (μM) for a specific condition.
Literature-curated set

Random Reference Set

Literature-curated set

Exploratory set

Gluc2-human P

Gluc1-viral P

Plasmid transfection

Virus infection

Gluc2-human P

Gluc1-viral P

NLR =

Gluc2-human P

+ Gluc1-viral P

Analysis

T0h

T18h

T24h

Gluc2-human P

+ Gluc1

Gluc2

Frequency (%)

Figure 3
Figure 4
Figure 5