Generation and comprehensive analysis of a influenza virus polymerase cellular interaction network

Running title: Host cell partners of the influenza polymerase

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Abstract

The influenza virus transcribes and replicates its genome inside the nucleus of infected cells. Both activities are performed by the viral RNA-dependent RNA polymerase that is composed of three subunits: PA, PB1 and PB2 and recent studies have shown that it requires host-cell factors to transcribe and replicate the viral genome. To identify these cellular partners, we generated a comprehensive physical interaction map between each polymerase subunit and the host cellular proteome. 109 human interactors were identified by yeast two-hybrid screens, whereas 90 were retrieved by literature mining. We built the FluPol interactome network composed of the influenza polymerase (PA, PB1, PB2) and the nucleoprotein NP and 234 human proteins that are connected through 279 viral-cellular protein interactions. Analysis of this interactome map revealed enriched cellular functions associated with the influenza polymerase, including host factors involved in RNA Polymerase II-dependent transcription and mRNA processing. We confirmed that eight influenza polymerase interacting proteins are required for virus replication and transcriptional activity of the viral polymerase. These are involved in cellular transcription (C14orf166, COPS5, MNAT1, NMI, and POLR2A), translation (EIF3S6IP), nuclear transport (NUP54) and DNA repair (FANCG). Conversely, we identified PRKRA that acts as an inhibitor of the viral polymerase transcriptional activity, thus it is required for the cellular antiviral response.

Keywords: yeast two-hybrid; flu; viral host-cell interactome; viromics; systems virology; functional interaction
Introduction

Influenza A virus is responsible for annual epidemics of respiratory disease and reoccurring pandemics, and represents a worldwide important public health problem. Its genome is composed of eight single-stranded negative polarity RNA segments (vRNA). They are individually encapsidated by nucleoprotein (NP) and the RNA-dependent RNA polymerase (RdRP), forming a ribonucleoprotein complex (vRNP) (reviewed in 38). Each vRNA behaves as an independent template for transcription and replication that are both taking place in the nucleus of infected cells. The virus RNA polymerase is a heterotrimer composed by PA, PB1 and PB2. PB1 is the core subunit of the complex and contains the polymerase activity, while PB2 recognizes capped cellular mRNA (38) and PA possesses an endonuclease activity (12). After viral decapsidation, vRNPs are transported into the nucleus where they are engaged in primary transcription. This is initiated by cap snatching of cellular pre-mRNA: the PB2 subunit recognizes capped mRNAs while the PA subunit cleaves them 10-15 nt downstream of the cap, generating cap-containing primers for virus mRNA synthesis (28). Viral mRNAs are then translated by cytoplasmic ribosomes, allowing newly synthesized components of the viral polymerase and NP to accumulate in the nucleus. It has recently been proposed that the nuclear accumulation of newly synthesized viral proteins could be responsible for the switch from viral transcription to replication (56). For the genome replication, untranslated sequences at the 5’- and the 3’-end of each genomic vRNA segment act as promoter elements that are recognized by the viral polymerase. vRNA segments are copied into complementary positive-strand RNA (cRNA), which are encapsidated by NP and serve as templates for de novo vRNA synthesis (22, 38).

The influenza polymerase performs numerous functions during the virus life cycle, suggesting that many cellular factors interact with this complex and are required for the viral genome transcription and replication.
Only a few cellular partners have been previously described in literature, but recent studies identified several influenza polymerase interactors using proteomic approaches (23, 33). Moreover, a global survey of influenza virus host-cell partners was established using the yeast two-hybrid technology (51). A series of functional genome-wide siRNA screenings were also conducted for the identification of host factors involved in influenza replication (5, 18, 24, 27, 51, and reviewed in 58). We present here a more specific analysis that is focused on the RdRP cellular interactors. We reconstructed a global influenza polymerase physical interaction map by performing yeast two-hybrid screens with each subunit as bait, and by retrieving protein-protein interactions from literature. Through this interaction network, we identified cellular functions specifically targeted by the viral polymerase. We selected nine functionally relevant host-cell partners, which interactions with the viral proteins were validated in human cells. We show that these cellular factors affect both the viral polymerase activity and the virus replication using functional assays: eight of them are required for virus replication and polymerase activity. Conversely, PRKRA acts as an antiviral factor since virus replication and polymerase activity are enhanced when its expression is depleted.
Material and methods

Cloning of influenza ORFs

NP, PA, PB1 and PB2 ORFs from A/PR/8/34 (H1N1) and A/WSN/33 (H1N1) (kindly provided by G. Brownlee and V. Moulès, respectively), PA and PB1 from A/VietNam/1194/2004 (H5N1) (kindly provided by V. Moulès), PA from A/Turkey/651242/2006 (H5N1) (the H5N1 genomic RNA were kindly provided by V. Moulès) and PB2 from A/Victoria/3/75 (H3N2) (kindly provided by D. Hart) were amplified from plasmids encoding corresponding cDNA genomic segments or from genomic viral RNA by using ORF-specific Gateway primers (containing attB1.1 at 5’ end and attB2.1 at 3’ end and without ATG and stop codons 48). Four PB2-fragments isolated from the ESPRIT technology were screened: the long (aa 234-496) and the short cap-binding domain (aa 318-483, 17), the “627” domain (aa 538-693), encompassing the K627 residue (55) and the C-terminus domain (“DPDE”, aa 678-759, 54). We constructed the compensatory point mutations: K627E in “627 domain” (52), D701N (49), R702K (15) and a double point mutant D701N/R702K in the DPDE domain. After PCR, ORFs were cloned by in vitro recombination into donor vectors (pDONR207/223). All clones were sequence verified and stored into a repository, viralORFeotheque. We developed a database that provides an integrated set of bioinformatic tools to clone viral ORFs in the Gateway® system, viralORFeome (http://www.viralorfeome.com, 43).

Yeast two-hybrid (Y2H)

Viral ORFs were cloned by recombination into pGBK7-gw and transformed in AH109 (bait strain, Clontech). Y2H screens were performed by yeast mating (53). Briefly, the human cDNA libraries (spleen, fetal brain and respiratory epithelium libraries were from Invitrogen and customized CloneMiner for the latter) were transformed in Y187 (prey strain, Clontech)
and each bait strain was mated with the library prey strain. Diploids were plated on SD-W-L-H+3AT and positive clones were streaked twice on this selection medium. AD-cDNA were PCR amplified and sequenced. Interaction Sequence Tags were analysed through pISTil and deposited in the viralORFeome database (42). All partners of PB2 domains identified in Y2H screens were re-tested against each PB2 fragment in an Y2H array system (in an all-against-all matrix). First, AD-cDNA encoding for cellular interactors identified with PB2 domains were transformed in the yeast prey strain together with linearized pACT2-gw. Bait and prey strains were arrayed in a 96-well format using a robotic workstation (Tecan Freedom Evo), and mated in an all-against-all array on YPD plates. Diploids were selected on SD-W-L for 2 days, and then transferred on selection medium (SD-W-L-H + increasing concentrations of 3-AT). Interactions were scored as positive if observed at least twice in 3 independent arrays (53). Among the 79 partners identified with PB2 domains, 59 were positively scored, confirming the results obtained during the screen. The 20 remaining partners were discarded.

**Gene Ontology categories enrichment using BiNGO and Golorize on Cytoscape**

BiNGO (Biological Network Gene Ontology) is a Cytoscape plugin that assess which Gene Ontology (GO) categories are overrepresented in a network (32). It provides single p values, calculated with the hypergeometric test and takes into consideration both the total number of genes from the analyzed dataset and the total number of genes that is linked to the same ontology term, as well as multiple testing corrected p values, calculated using the Benjamini & Hochberg False Discovery Rate (FDR) correction, for the enrichment of each GO term (in our case, Biological Process). Golorize is a Cytoscape plugin that highlights the class-members of the enriched-categories identified by BiNGO using a color-code within the cytoscape-built network (16, 50). The cross-validation of our Y2H interaction dataset revealed a false-positive rate of 15%. To take into account of this rate in the functional
enrichment analysis, we generated 50 different clusters of 93 randomly chosen Y2H interactors (representing 85% of the 109 Y2H interactors) + all the literature cellular partners. For each cluster, we performed a GO analysis using BiNGO. We considered as positive the over-represented classes that were scored positive for all the 50 clusters. 9 functional categories were selected and the class-members were colorized within the FluPol network using GOlorize. Note that we did not change the network layout after the GOlorize analysis.

FluPol network visualization

We used Cytoscape Web API for visualizing and manipulating the FluPol network graph, allowing a dynamic network display (http://flupol.lyon.inserm.fr, 31). All the interaction data can be exported through this web page.

Bi-functional Fluorescence Complementation assay

A/WSN/33 PA, PB1 and PB2 were cloned into Polyc vector, where they are fused upstream of the C-term moiety of YFP. Cellular partners genes were amplified from the human cDNA Y2H libraries then *in vitro* cloned into pDONR207 as viral ORFs (see above), or were retrieved from hORFeome collection (48). They were then cloned by recombination in pGWEN, in fusion downstream of the N-term moiety of Venus (modified YFP, 26). HEK-293T cells were cotransfected using JetPei (Polyplus) with each combination of plasmid pair in a 96-well plate. 48h after transfection, cells were resuspended in 100 µl PBS and YFP signal was measured using a FACSArray (BD). The percentage of fluorescent cells was measured (mean of three independent experiments) and interaction was considered as positive when more than 5% of cells were fluorescent, according to results obtained with negative controls.
Flow-cytometry protein interaction assay (FCPI assay)

The FCPI assay was set up using two couples of interacting proteins (see Supplementary Figure 1). Viral ORFs (A/WSN/33 strain) were cloned by in vitro recombination into pCMV-BioEase-Cherry-gw, where they are fused downstream of the BioEase tag (Invitrogen) and the fluorescent protein mCherry. Cellular genes were transferred to pCMV-eGFP-gw (kindly provided by Y. Jacob), to be fused downstream to eGFP. Human HEK-293T cells were cotransfected using JetPei (Polyplus) with each combination of plasmid pair (1 µg of each vector) in a 12-well plate and lysed 48h after transfection. 60 µg of protein extracts were suspended in 200µl lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8, 1 mM EDTA, 0.5 % NP40 + complete mini protease inhibitors cocktail, Roche) containing 1 µl of streptavidin conjugated microspheres (Polysciences) on a multiwell filter plate (Pall) and incubated for 2h at 4°C under gentle agitation. Each affinity purification was performed in triplicate. The beads were washed twice with 200 µl of Wash Buffer (same as lysis buffer, without the protease inhibitors cocktail) using a vacuum manifold, then were resuspended in 100 µl of Wash Buffer and beads-associated GFP signal was analysed using a FACSArray (BD). The analysis of results is based on the relative mean fluorescence intensity (MFI) associated to the beads compared to MFI of an empty bait vector. An interaction is considered as positive when the difference is higher than 5%.

Cells and viruses

A549 and HEK-293T cells were maintained in DMEM containing 1% Penicillin/Streptomycin and 10% fetal bovine serum at 37°C and 5% CO2. Influenza A virus A/New Caledonia/2006 (H1N1) (clinical isolate) was propagated in MDCK cells. Viral stocks were titrated by PFU assay on MDCK cells.
Co-immunoprecipitation and protein detection

Cellular genes were transferred to pCIneo-3Flag-gw (kindly provided by Y. Jacob), to be fused downstream to 3xFlag epitope (Sigma). A549 cells were transfected in 6-well plates using Turbofect (Fermentas). 24h after transfection, cells were infected by H1N1 A/New Caledonia/2006 virus at MOI of 1 and lysed 24h after infection. 300 µg of protein extracts were suspended in 1 ml lysis buffer containing 20 µl of anti-Flag M2 magnetic beads (Sigma) and incubated for 2h at 4°C under gentle agitation. The beads were washed three times with 1 ml of Wash Buffer on a magnetic rack, then precipitates were eluted in 20 µl SDS loading buffer without reducing agents and boiled 5 minutes at 95°C. Total cell extracts (20 µg) and immunoprecipitates were separated by SDS-PAGE and analysed by western-blotting using antibodies against PB1 (rabbit polyclonal antibody kindly provided by J. Ortin) and Flag (HRP-conjugated mouse monoclonal antibody, Sigma). The secondary anti-rabbit antibody was also HRP-conjugated (Santa Cruz). eGFP-PRKRA was detected with mouse monoclonal anti-GFP antibody (Sigma).

Influenza replication assay and virus titer determination using MUNANA

Human A549 cells were transfected with siRNA targeting each gene (a pool of 2 siRNA at a concentration of 40 nM was used, Invitrogen) in a reverse transfection procedure using Lipofectamine RNAiMAX® (Invitrogen) in a 96-well plate. 48h after siRNA transfection, cells were infected by H1N1 A/New Caledonia/2006 virus at a MOI of 0.5 in DMEM containing 1% penicillin/streptomycin and 0.25 µg/ml TPCK-treated trypsin (Sigma). Cell culture supernatants (25 µl) were collected at 24h and 48h post infection and virus titers were determined by quantifying the neuraminidase activity using a MUNANA assay, as described in (59), with minor modifications. Briefly, cells supernatants were transferred into a 96-well black flat-bottom plate, mixed with 25 µl of PBS with Ca²⁺/Mg²⁺ (Invitrogen) and 50 µl of
MUNANA stock-solution (20 µM, Sigma). Plates were incubated 1h at 37°C and reaction stopped by adding 100 µl of Stop Solution (glycine 0.1 M pH10.7, 25 % ethanol). The amount of fluorescent product released by MUNANA hydrolysis (4-MU) was measured in a Tecan spectrophotometer with excitation and emission wavelengths of 365 and 450 nm, respectively. A reference curve was established in parallel with serial dilutions of a titrated stock of influenza virus.

To assess potential cellular toxicity induced by siRNAs, a cell viability assay was performed using the resazurin-based fluorometric assay at 48h post-transfection (Figure 2C). Resazurin (1 mg/ml of medium, Sigma) detects cell viability by converting to a red fluorescent dye in response to reduction of growth medium resulting from cell growth. The fluorescent signal generated is proportional to the number of living cells. After an incubation of 2h at 37°C, the fluorescence was measured with a Tecan spectrophotometer with excitation and emission wavelengths of 550 and 590 nm, respectively. A reference curve was established with known serial dilutions of growing cells.

**Influenza minigenome replicon assay**

- Overexpression of human partner proteins. Human HEK-293T cells (plated in a 96-well plate) were cotransfected using JetPei (Polyplus) with a combination of plasmids expressing a cellular partner (100 ng), vectors encoding the A/WSN/33 polymerase subunits (PA, PB1 and PB2) and NP (50 ng each plasmid), a reporter plasmid (pPOLI-Luc-RT, encoding the firefly luciferase in the negative-sense orientation flanked by the non-coding regions of the segment 8 of A/WSN/33, driven by a PolI promoter, kindly provided by M. Shaw (19), 50 ng) and a vector constitutively expressing the *renilla* luciferase (pRL-SV40-Rluc, Promega, 5 ng). 24h and 48h after transfection, luciferase activities were determined using the Dual-Glo® luciferase assays system (Promega). As positive control, cells were transfected by the
complete set of vRNP and an empty cellular protein expression vector. As negative control, cells were transfected with the same reaction mix but without the plasmid expressing PB1.

- Depletion of human partner genes. Human HEK-293T cells were transfected with siRNA targeting each gene as described above. Knockdown was allowed to proceed for 48h, and then cells were transfected with the complete set of vRNP components (plasmids expressing NP, PA, PB1 and PB2 and the reporter vector encoding firefly luciferase) as described above. As positive control, cells were transfected by a scrambled control siRNA, and then with the complete set of the vRNP. As negative control, cells were treated with the same mix but without PB1 expressing plasmid.
Results and discussion

1. Building of an influenza virus polymerase cellular interactome network

We generated a comprehensive protein-protein interaction network between the viral influenza A virus polymerase (FluPol) and its host-cell partners using the yeast two-hybrid system (Y2H). For this purpose, human cDNA libraries were screened with the RdRP subunits and NP from different virus strains (H1N1, H3N2 and H5N1). PB2 fragments that were isolated by the ESPRIT technology and whom structures were defined were also screened individually (17, 54, 55, 60). Human cDNA libraries from three different tissues (spleen, fetal brain and respiratory epithelium) were used, covering a wide range of the human proteome as well as a tissue that is specifically targeted by the influenza A virus. Altogether, we discovered 112 viral-human protein interactions involving 109 human proteins, including 5 interactions with NP, 30 with PA, 18 with PB1 and 59 with PB2. Among them, 3 human proteins interact both with PA and PB2 (Supplementary Table 1, Figure 1A and 1B).

To assess the confidence of the yeast two-hybrid results, two validation methods in human cells were used. 34 cellular partners (31 % of the Y2H interactors) were randomly retested for their interaction with the viral protein bait that trapped them in Y2H either by BiFC (Bi-functional Fluorescence Complementation, 26), or a flow cytometric method developed in our lab to measure protein-protein interactions (FCPIA, similar to the assay developed by the Neubig lab 4). While the bait (in our case, the viral protein) is fused both to the BioEase tag, an in vivo biotinylated sequence that can be trapped using streptavidin beads, and to the fluorescent protein mCherry (allowing its detection on beads), the prey is fused to eGFP. This assay allows detection of co-purified preys on streptavidin beads using a flow cytometer (material and methods and Supplementary Figure 1). KPNA1 was used as positive control as it interacts with PB2 and NP (39, 54). Among the 34 host-cell partners tested, 29 were validated using either BiFC, FCPIA or both assays (in green in Table 1 and supplementary...
Table 5). We identified eighteen new connections through these validation assays, by performing an array where the cellular proteins were *a priori* tested against different viral baits (in blue in Table 1). It is of note that we did not validate the interactions identified in Y2H for three cell partners but discovered interactions with different viral proteins (EIF3S6IP, FANCG and ZCCHC17). 29 of the 34 Y2H interactors subset tested were validated, giving a false-positive rate of 15%, which is in accordance to previous studies (9).

Most of the cellular proteins interact with one FluPol subunit (17 of the 29 validated), like C14orf166 that only interacts with PA, or EEF1A1 with PB1. We also observed that a few host cell partners interact both with PB1 and PB2 (5 cellular proteins: CES1, CHAF1A, DDX54, MNAT1, NMI). PCNA and PSMA7 interact with the FluPol subunits (PA, PB1, PB2), whereas EEF1D interacts with all the vRNP protein constituents.

We also retrieved virus-host protein interactions from literature, and added them to the VirHostNet knowledge base (37). In total, 26 interactions were identified involving 23 human proteins (all the papers describing individual interactions can be retrieved using supplementary Table 1, where we indicated the PubMed ID). In addition, Shapira *et al.* identified recently 90 interactions involving 69 cellular proteins in their Y2H screens with the viral polymerase subunits (51). Moreover, we added 49 cellular proteins that were identified as vRNP or polymerase complex host-cell partners by proteomic approaches, even though we don’t know precisely with which viral protein they actually interact (23, 33). The overlap between these four datasets (Y2H from this report and further referred as I-MAP dataset for Infection Mapping Project, Y2H from Shapira *et al.*, literature curated (LC) and proteomics (AP)) is low, but not surprising (Table 2). In a previous work where we performed a comprehensive analysis of the Hepatitis C Virus interaction network, we obtained such results (among 278 human proteins interacting with HCV proteins identified in Y2H, only 10 were already described in literature, representing 3.6 % overlap, 9). This is due to the different
methods that were used (e.g. Y2H for I-MAP vs affinity purification-MS for AP, representing 1.8 % overlap). The low overlap of our results and those from Shapira et al. (0.9 %), although both were conducted using Y2H, could be explained by different screening procedures that were used, including different libraries (a normalized library of 12,000 full-length ORFs for Shapira et al. vs 3 human cDNA libraries encompassing a large fraction of the human proteome, but including many truncated coding sequences for I-MAP), screening techniques (mating of mini-pool of prey strains for Shapira et al. vs mating of one bait strain against a whole library of prey strains for I-MAP), Y2H strains (Y8800 and Y8930 for Shapira et al. vs AH109 and Y187 for I-MAP), and reporter genes (ADE2 and HIS3 for Shapira et al. vs HIS3 for I-MAP, 51). This is supported by a comprehensive interactome analysis conducted by the Vidal lab (29), where Y2H screens were conducted in parallel against both cDNA and ORFeome libraries, and then compared for protein interactions identified. In total, 1,517 interactions were identified with the cDNA library, and 3,263 with the ORFeome library. Only 250 interactions were discovered using both libraries, thus representing 5.5 % overlap.

Furthermore, the viral polymerase genes used as baits came from different influenza strains when comparing the results from Shapira et al. and our I-MAP dataset.

The FluPol interactome we reconstructed is thus composed of 4 viral proteins and 234 human partners (H_{Flu}) forming 279 viral-human protein interactions (Figure 1, for a dynamic version of the network, see http://flupol.lyon.inserm.fr, Supplementary Table 1). Using VirHostNet (37), we found that among the 234 H_{Flu}, 111 are connected with each other through 204 interactions within the human interactome (H_{Flu}-H_{Flu}, Figure 1 and Supplementary Table 2). We also found through VirHostNet that 69 H_{Flu} are targeted by 47 other viruses (Supplementary Table 3). This suggests that cellular pathways that are connected to the influenza polymerase (see below) represent common virus targets. Furthermore, 24 influenza polymerase interactors are also connected to other influenza A virus proteins (mainly NS1,
Supplementary Table 4), centering the polymerase partners in a more comprehensive influenza network.

2. **Functional enrichment of the influenza polymerase interactome.**

To visualize which cellular functions are targeted by the viral polymerase, the enrichment of H_{Flu} for Gene Ontology (GO) terms corresponding to “biological processes” was determined by using the BiNGO plugin in Cytoscape (1, 16, 32, 50). It is biased, since not all human proteins have yet been annotated (in our data set, 208 of the 234 H_{Flu} possess a GO “biological process” annotation), but it remains a powerful way to incorporate conventional biology to systems-level datasets. We took into account the false-positive rate obtained during the validation of the Y2H interactors and performed 50 tests by removing randomly 15% of the Y2H cellular partners. We retained only GO terms that were significantly enriched in all these 50 tests. Functions related to DNA synthesis (DNA replication and DNA repair), transcription, RNA processing and translation were over-represented. An enrichment for cell cycle, nucleocytoplasmic transport, response to unfolded proteins and viral infectious cycle was also found (Figure 1C). Among these over-represented processes, several were mainly retrieved from literature, e.g. nucleocytoplasmic transport (10/14 cellular interactors were mined from literature) or DNA replication (6/10 interactors from literature), denoting functions already known to be associated with the influenza polymerase (11, 13, 25, 34, 35, 39, 46, 57). Others processes contain several interactors discovered in our Y2H screens, like RNA processing (11/27 proteins identified from Y2H), DNA repair (8/18 proteins identified from Y2H), unraveling physical links between the influenza polymerase and these cellular functions. They may be mandatory for the function of influenza polymerase within the host cell.
3. Functional implication of the FluPol partners

On the basis of the enriched cellular functions that are targeted by the influenza polymerase, we selected eight validated cellular interactors to test their ability to affect the viral replication: C14orf166 (not annotated in GO but known as a transcription elongation factor, 44), COPS5 (implicated in transcription and in translation), EIF3S6IP (translation), FANCG (DNA repair), MNAT1 (member of 4 overrepresented classes : cell cycle, RNA processing, transcription and DNA repair), NMI (transcription), NUP54 (nucleocytoplasmic transport), and PRKRA (RNA processing and also implicated in innate anti-viral response and known to interact with NS1, our unpublished data and 39) (Figure 1C).

To further confirm observed interactions, we tested whether these cellular partners interact with the viral polymerase in influenza virus-infected cells. For this purpose, we transfected cells with each protein fused to the 3xFlag epitope, then infected them with H1N1 A/New Caledonia/2006 virus (clinical isolate). 24h after infection, Flag-tagged cellular proteins were immunoprecipitated, and western-bLOTS were performed with an antibody raised against PB1 to detect the presence of the polymerase in the immunoprecipitates. As shown in Figure 2, COPS5, EIF3S6IP, FANCG, NMI, NUP54 and PRKRA immunoprecipitated the viral polymerase (detection of PB1 in the immunoprecipitates). We did not detect PB1 in the C14orf166 and MNAT1 immunoprecipitates. Nevertheless, we noticed that C14orf166 interacts with PA but not with the other polymerase subunits (Table 1). It might reflect that C14orf166 only interacts with free PA protein. In summary, these results indicate that 6 of the 8 host-cell factors tested specifically associate with the RdRP in an infected cell context.

We then monitored the virus replication in human cells where these 8 influenza polymerase interactors were depleted by specific siRNA. We added to this set POLR2A that we identified by Y2H and that was previously described in literature as a RdRP interactor (14). For this
purpose, siRNA-depleted cells were infected by H1N1 virus and 48h after infection, virus titers were determined by quantifying the neuraminidase activity using a MUNANA assay (59, see material and methods). The individual depletion of 8 genes decreased the virus replication to less than 50% as compared to a siRNA scrambled control: C14orf166, COPS5, EIF3S6IP, FANCG, MNAT1, NMI, NUP54 and POLR2A (Figure 3B). Conversely, PRKRA appeared to have potent antiviral activity, as its inhibition by siRNA increased the virus replication 3.5 times as compared to the control scrambled siRNA. As control, we showed that tested siRNA had no side effects on cell survival as assessed by a resazurin assay (Figure 3C).

We speculated that these cellular proteins specifically regulate the viral polymerase activity. This was tested in a minigenome replicon assay either by overexpressing (by transient transfection of a plasmid) or by depleting (using directed siRNA) each cellular partner gene. HEK-293T cells were cotransfected with vectors encoding the A/WSN/33 polymerase subunits and NP, and a reporter plasmid encoding an RNA template for the viral polymerase that expresses the firefly luciferase, together with a plasmid expressing each cellular partner. Luciferase activity was determined 48h after transfection. For the depletion assay, human cells were first treated by siRNA targeting each gene for 2 days, and then were transfected with the complete set of the vRNP (plasmids expressing NP, PA, PB1 and PB2 and the reporter plasmid). Results are represented in Figure 4. The eight genes required for virus replication reveal an increased polymerase activity when overexpressed and/or a decreased activity when depleted. COPS5, EIF3S6IP, FANCG and MNAT1 overexpression enhanced the RdRP transcriptional activity from 1.25 to 3 times as compared to control. The depletion of C14orf166, COPS5, EIF3S6IP, NUP54 and POLR2A reduced more than 2 times the viral polymerase transcription. We did not observed a drastic change in the RdRP transcription when overexpressing or depleting NMI. This cellular protein could contribute to viral
replication at specific steps that cannot be detected with the minigenome system like virus assembly or trafficking (2, 62). These human proteins are thus host cell factors required for influenza polymerase activity, a mandatory step for influenza replication. On the contrary PRKRA acts as an antiviral cellular factor, since its overexpression inhibits the viral polymerase activity and its knock-down favors the influenza polymerase transcriptional activity as well as the virus replication.

Among the eight pro-viral cellular proteins, 5 are implicated in mRNA transcription: C14orf166, COPS5, MNAT1, NMI and POLR2A. This latter, the largest PolII subunit, interacts with the viral polymerase through its CTD (Carboxy-Terminal Domain) phosphorylated at the serine 5, i.e. when the transcription initiates and where the capping enzymes are recruited and activated (14). This interaction inhibits the transcription elongation and leads to the degradation of POLR2A (6, 47). We noticed in a very recent paper that influenza polymerase interacts with the P-TEFb complex (CDK9/Cyclin T1), which phosphorylates the PolII CTD at the onset of transcription elongation (61). This further connects the influenza polymerase complex to the RNA PolII at the beginning of the transcription elongation, i.e. when cellular pre-mRNA are capped (reviewed in 8). We have shown that the POLR2A CTD specifically and directly interacts with PA: the clones isolated in Y2H all correspond to the POLR2A CTD and in the FCPI assay, it interacted only with PA (and not with NP, PB1 or PB2, Table 1). In biochemical fractionation experiments, the actively transcribing PolII form, as well as many proteins involved in cellular transcription and RNA processing, are associated to an insoluble nuclear fraction, the nuclear matrix (36). Interestingly, newly synthesized viral RNA and components of the vRNP complex have been found associated with this nuclear matrix in influenza virus infected cells, and protein interactions identified in this study probably participate in this process (14, 21).
PRKRA, that we identified as a protein required for the host anti-viral response, is a dsRNA (double-strand RNA) binding protein that activates PKR by interacting through their dsRNA binding domains (41). PKR, a key mediator of the interferon antiviral pathway, is a kinase that phosphorylates the translation initiation factor EIF2α, leading to protein synthesis shut-off (7). The overexpression of PRKRA in mammalian cells leads to PKR activation and inhibition of translation and, under cellular stress, to apoptosis (40, 41). It has already been shown that PRKRA interacts with viral proteins, as the dsRNA binding protein Us11 of HSV1. This interaction inhibits PKR activation and the PRKRA-induced apoptosis (45).

Recently, VSV replication was monitored in a PRKRA-dependent context, showing that the virus production is enhanced in PRKRA-depleted cells and that PRKRA overexpression protects cells against VSV infection (3), like we observed in influenza virus replication assay. Furthermore, it has also been shown that the influenza virus NS1 protein interacts with both PRKRA and PKR, leading to the inhibition of PKR and stress-induced cell death mediated by PRKRA (30). It appears thus that the PRKRA-PKR pathway is very specifically targeted by the influenza virus and it might be an important way for the virus to escape the innate immune response in infected cells.

In their comprehensive influenza infection regulatory study, Shapira and colleagues identified the viral polymerase subunits as modulating factors of the host antiviral response, like the interferon production pathway (51). Furthermore, influenza RdRP was recently shown to interact with Stau1, a dsRNA-binding protein and well-characterized target of NS1 (10).

This global approach paves the way to a new landscape of the influenza polymerase cellular neighborhood. The influenza polymerase interaction network will help us to better decipher the virus biology and molecular mechanisms of viral replication. By adding functional correlation between the influenza polymerase and cellular co-factors, it will facilitate the
development of new antiviral drugs that target the host cell factors instead of viral proteins, the latter being more prone to mutate and become drug resistant.
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References


30. **Li, S., J. Y. Min, R. M. Krug, and G. C. Sen.** 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology **349:**13-21.


of RNA polymerase II is associated with splicing complexes and the nuclear matrix.


ViralORFeome: an integrated database to generate a versatile collection of viral ORFs.


Figures Legends

Figure 1: The influenza polymerase (FluPol) interaction network.

A. Graphical representation of the FluPol interactome map. Black nodes: viral proteins (V: NP, PA, PB1 and PB2. Pol corresponds to the polymerase complex and RNP to the ribonucleoparticle, composed of all 4 proteins and vRNA), grey nodes: human proteins (H_{flu}), red, orange and green edges: interactions between viral and human proteins (V-H_{flu}, identified by Y2H (I-MAP dataset) in red, literature mining in orange and affinity purification in green), black edges: interactions between viral proteins (V-V), blue edges: interactions between human proteins (H_{flu}-H_{flu}). The FluPol interaction network is available online, for a better visualization and dynamic display: [http://flupol.lyon.inserm.fr](http://flupol.lyon.inserm.fr). Human proteins of enriched classes are colored according to table C.

B. Number of FluPol interactors. Data are given for Y2H, literature curated interactions (LC) and by affinity purification approach (AP) by viral protein, the polymerase and the vRNP (for the affinity purification approaches).

C. Table of the over-represented classes (GO biological process) in the FluPol interactome. The cooected p-value column corresponds to a Benjamini-Hochberg FDR correction. Also indicated are the number of targeted (that are detailed in the H_{flu} column) and the total proteins within each class. A colour was assigned to each over-represented class, allowing their positioning on the interactme map.

Figure 2: interaction of the influenza polymerase and host cell partners in infected cells.
A549 cells were transfected with indicated proteins fused to 3-Flag. 24h after transfection, cells were infected with H1N1 A/New Caledonia/2006 at a MOI of 1. Cells were lysed 24h after infection and protein lysates immunoprecipitated with anti-Flag magnetic beads. Total
cell extracts and immunoprecipitates were separated by SDS-PAGE and analysed by western-blotting using antibodies against PB1 and Flag.

**Figure 3: FluPol-host cell interactors regulate virus replication.**

A. Schematic representation of the replication assay. Human A549 cells treated with targeted siRNA were infected 48h after depletion by H1N1 A/New Caledonia/2006 at a MOI of 0.5. Cell culture supernatants were harvested 48h after infection and viral titers were determined by a MUNANA assay (measuring the neuraminidase activity).

B. Results (mean of 2 independent experiments, each performed in triplicate) are expressed as relative replication efficiency as compared to cells treated by a scrambled siRNA control.

C. To assess potential cytotoxicity induced by the siRNAs, a cell viability assay was performed using the resazurin-based fluorometric assay 48h after siRNA transfection. Cells were counted and expressed as relative to control cells (treated by a scrambled siRNA control).

**Figure 4: FluPol-host cell interactors regulate the viral polymerase activity.**

A. Schematic representation of the viral polymerase transcriptional activity using a minigenome replicon assay. Human 293T cells were eitehr transfected by a vector encoding the cellular interactor (1, overexpression test) or treated with a siRNA targeting the cellular gene (2, depletion test). For the overexpression assay (in blue), 293T cells were transfected with a plasmid encoding each cellular protein together with NP, PA, PB1, PB2, a minigenome replicon reporter plasmid coding for firefly luciferase and a renilla control vector. 48h post-transfection, the luciferase activities were determined. For the depletion assay (in red), 293T cells were firstly treated with specific siRNA targeting cellular partners genes. 48h after depletion, cells were transfected with the complete set of vRNP (NP, PA, PB1 and PB2 and
the luciferase reporter plasmid) and the *renilla* control vector, and proceeded as for the overexpression test.

B. Results (mean of 2 independent experiments, each performed in triplicate) are expressed as relative effect of overexpression (in blue) or knock-down of FluPol interactors (in red) compared to control (empty vector for overexpression or scrambled siRNA for depletion). As negative control, cells were transfected with the same reaction mix without PB1. * and **: $P < 0.05$ and $P < 0.01$, respectively, as compared to the positive control, based on a Student’s $t$ test.
### Table 1: List of consolidated FluPol interactors

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<td>Gwen/(^b)</td>
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Green: interaction found in Y2H and validated in either BiFC or FCPIA

Yellow: interaction that was tested either in BiFC or FCPIA but was negative.

Blue: novel interaction detected in either BiFC or FCPIA.

\(^a\): the peGFP-empty vector was used as negative control in FCPIA.
b: the pGwen-empty vector was used as negative control in BiFC.

Raw figures are given in Supplementary Table 5.

Table 2: Overlap in FluPol interactors between different datasets

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<td></td>
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<td>Y2H I-MAP</td>
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<td>/</td>
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<tr>
<td>LC</td>
<td>23</td>
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<tr>
<td>AP</td>
<td>49</td>
<td>2 (4.1%)</td>
</tr>
</tbody>
</table>

a: the overlap is represented as percentage to total \( H_{Flu} \) dataset analyzed (horizontally); e.g., the overlap of 1 interactor between Y2H I-MAP and Y2H Shapira \( et al. \) corresponds to 0.9% of the 109 \( H_{Flu} \) identified in Y2H I-MAP.
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