RESEARCH ARTICLE

Host- and Strain-Specific Regulation of Influenza Virus Polymerase Activity by Interacting Cellular Proteins

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ABSTRACT **Highly pathogenic avian influenza A (HPAI) viruses of the H5N1 subtype have recently emerged from avian zoonotic reservoirs to cause fatal human disease. Adaptation of HPAI virus RNA-dependent RNA polymerase (PB1, PB2, and PA proteins) and nucleoprotein (NP) to interactions with mammalian host proteins is thought to contribute to the efficiency of viral RNA synthesis and to disease severity. While proteomics experiments have identified a number of human proteins that associate with H1N1 polymerases and/or viral ribonucleoprotein (vRNP), how these host interactions might regulate influenza virus polymerase functions and host adaptation has been largely unexplored. We took a functional genomics (RNA interference [RNAi]) approach to assess the roles of a network of human proteins interacting with influenza virus polymerase proteins in viral polymerase activity from prototype H1N1 and H5N1 viruses. A majority (18 of 31) of the cellular proteins tested, including RNA-binding (DDX17, DDX5, NPM1, and hnRNPM), stress (PARP1, DDB1, and Ku70/86), and intracellular transport proteins, were required for efficient activity of both H1N1 and H5N1 polymerases. NXP2 and NF90 antagonized both polymerases, and six more RNA-associated proteins exhibited strain-specific phenotypes. Remarkably, 12 proteins differentially regulated H5N1 polymerase according to PB2 genotype at mammalian-adaptive residue 627. Among these, DEAD box RNA helicase DDX17/p72 facilitated efficient human-adapted (627K) H5N1 virus mRNA and viral RNA (vRNA) synthesis in human cells. Likewise, the chicken DDX17 homologue was required for efficient avian (627E) H5N1 infection in chicken DF-1 fibroblasts, suggesting that this conserved virus-host interaction contributes to PB2-dependent host species specificity of influenza virus and ultimately to the outcome of human HPAI infections.**

IMPORTANCE **Highly pathogenic avian influenza A (HPAI) viruses have recently emerged from wild and domestic birds to cause fatal human disease. In human patients, it is thought that adaptation of the viral polymerase, a complex of viral proteins responsible for viral gene expression and RNA genome replication, to interactions with mammalian rather than avian host proteins contributes to disease severity. In this study, we used computational analysis and RNA interference (RNAi) experiments to identify a biological network of human proteins that regulates an H5N1 HPAI virus polymerase, in comparison to a mammalian H1N1 virus. Of 31 proteins tested, 18 (58%) were required for polymerase function in both HPAI and H1N1 viruses. Remarkably, we also found proteins such as DDX17 that governed the HPAI virus polymerase's adaptation to human cells. These virus-host interactions may thus control pathogenicity of HPAI virus in humans and are promising therapeutic targets for antiviral drugs in severe influenza infections.**

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Influenza A viruses (*Orthomyxoviridae*) cause seasonal epidemics in humans and occasionally lethal global pandemics, such as those of 1918-1919 (H1N1), 1957 (H2N2), 1968 (H3N2), and 2009 (swine-origin H1N1). Currently, highly pathogenic avian influenza A (HPAI) virus strains of the H5N1 subtype circulating in wild and domestic birds are capable of crossing the species barrier into humans and causing significant pulmonary (1–3) and systemic (4) disease. Reverse-genetics studies have demonstrated that the viral polymerase (a heterotrimeric complex consisting of the PB1, PB2, and PA proteins) and nucleoprotein (NP) contribute to the pathogenicity of H5N1 HPAI viruses in humans and other mammals (5–7). Experimental evidence also suggests that adaptation of polymerase proteins and/or NP to interact with mammalian rather than avian host proteins contributes to efficient viral mRNA, cRNA, and/or viral RNA (vRNA) genome synthesis (8, 9). For avian viruses, adaptations in PB2 (e.g., E627K, D701N, and Q591K) allow efficient replication in mammalian systems (10, 11) and are correlated with disease severity in mam-

mals (5–7, 12, 13). These mutations appear to be selected to facilitate H5N1 HPAI virus replication in humans (14).

In humans, the influenza A virus polymerase-host interactome consists of more than 50 host cell proteins identified primarily by mass spectrometry in protein complexes associated with H1N1 influenza A virus heterotrimeric polymerase, NP, or a viral ribonucleoprotein (vRNP) comprised of these proteins and a vRNA template (8, 15–21). Although the direct or indirect nature of many of these host protein interactions with viral polymerase subunits, NP, or viral RNAs requires experimental validation, the discovery of such a large number of interacting proteins suggests that the processes of influenza A virus transcription, replication, and vRNP import/export are potentially dependent on a wide spectrum of host factors over the course of the virus life cycle. In a few cases, notably those of RNA-binding proteins (16, 19, 21, 22) and molecular transporters (8, 15, 20, 23), the importance of these virus-host interactions to the activity of H1N1 polymerases has been characterized. However, the importance of the majority of newly identified factors for polymerase function is unknown, particularly with respect to H5N1 HPAI. Recent RNA interference (RNAi) analyses of a large number of human genes regulating the influenza A H1N1 virus life cycle considerably expanded the list of host factors that regulate virus replication (24–27). However, these high-throughput studies did not specifically examine host factors with respect to H1N1 or H5N1 viral polymerase RNA synthesis (viral gene expression and genome replication) functions.

The present study sought a deeper understanding of influenza virus polymerase-host interactions that regulate polymerase activity in human cells. We hypothesized that host factors interacting with H1N1 polymerase might regulate H1N1 and potentially H5N1 polymerase functions. Using a functional genomics approach, we describe a network of human proteins that modulate the polymerases of influenza A/WSN/1933 virus, a mammalian H1N1 strain, and A/Viet Nam/1203/2004 (A/VN/1203/04), an H5N1 HPAI virus isolated from a fatal human case (6). Given the importance of the polymerase genotype for HPAI infection in mammals, we also searched for a polymerase-interacting host factor(s) that governs adaptation of H5N1 polymerase to human cells. We identified host factors within the network that differentially regulated H5N1 HPAI viral polymerase in human cells depending on the PB2 amino acid 627 genotype, such as DEAD box RNA helicase 17 (DDX17).

RESULTS

H1N1 polymerase-interacting human proteins modulate H1N1 and H5N1 polymerase activity. To discover how interacting host proteins regulate influenza virus polymerases, we first analyzed the H1N1 influenza A virus polymerase-host interactome (see Fig. S1 in the supplemental material). Using biological network analyses (27), we selected 31 of 54 human factors for RNAi phenotype studies (Fig. 1A). These 31 factors formed an interconnected network of proteins (Benjamini-Hochberg estimate of false discovery probability [P_{BH}] < 0.011) encoding functions known to regulate influenza virus replication, including RNA processing, transcription and translation, molecular transport, cell cycle, and cell death (17, 26–28).

We then measured the effects of short interfering RNA (siRNA) knockdown of each of the 31 host factors on A/WSN/33 (H1N1) and A/VN/1203/04 (H5N1) viral polymerase activity in human cells using minigenome reporter assays (Fig. 1A). We optimized minigenome assays to study host factor requirements for the polymerase by itself (VPOL-mg assay, including NP; see Fig. S2 in the supplemental material) and during influenza virus infection (infection-mg assay) (29). For each host factor targeted by siRNA (see Table S1), polymerase activity was expressed as a ratio to the nontarget (control) siRNA value (Fig. 1B), and phenotypic differences were tested for significance (see Table S2). Cell viability assays excluded only KPNA4 and IDH2 from subsequent analyses (see Fig. S3).

Our data rejected the null hypothesis that most host proteins identified in the interconnected network were bystanders, perhaps interacting but not affecting viral polymerase function. Instead, both A/WSN/33 (H1N1) and A/VN/1203/04 (H5N1) viral polymerases depended on a common set (18/31, or 58%) of human host factors (enhancers) for optimal function in both assays (Fig. 1C). Many enhancer factors were RNA binding or processing proteins (e.g., DDX17, NPM1, and heterogeneous nuclear ribonucleoprotein M [hnRNPM]), molecular transport or chaperone proteins (KPNA1, KPNA3, HSP90, HSP70, and RanBP5), or proteins responding to DNA damage or cellular stress (PARP1, the Ku70/86 complex, and DDB1). Only simultaneous knockdown of both the Ku70 and Ku86 subunits showed a significant phenotype, suggesting redundant functions for Ku proteins vis-à-vis the influenza virus polymerase (Fig. 1D). Viral polymerase activity significantly increased when NXP2 or NF90 was targeted by siRNA, suggesting these host proteins are antagonists, or suppressors, of both H1N1 and H5N1 polymerases. Eleven more factors displayed variability depending on the assay and/or strain (see Discussion). Hsc71/HSPA8 was required only for optimal polymerase function in infected cells, maybe due to this protein's function in cellular stress (see Fig. S2 in the supplemental material).

A protein network that regulates H1N1 and H5N1 polymerases. To provide a more coordinated picture of how H1N1 and H5N1 polymerases are regulated by host factors in human cells, we mapped RNAi viral polymerase phenotypes onto the interconnected network of host proteins extracted from the H1N1 influenza A virus polymerase-host interactome (see Fig. S1 in the supplemental material). This functionally enriched regulatory network was expanded to encompass additional proteins reported to affect influenza virus replication (Fig. 2A): DBT and RPS5 (25), hnRNPU (24), hnRNPF (30), KPNB1 (26), CRM1/XPO1 (31), the EIF4A complex (24, 32), host RNA polymerase II (28), and PABPC1 (32). Moreover, three proteins in the regulatory network (ARRB1, hnRNPK, and RBM14) that shared a high degree of connection ($k > 3$ edges) with H1N1 polymerase interactors also displayed significant polymerase phenotypes (Fig. 2A). This suggests that proximal or "hub-like" interactors may contribute to polymerase functions and in part could explain the relatively low overlap in gene hits among global influenza virus RNAi studies (33).

Host factors that differentially regulate H5N1 polymerase according to PB2 genotype at mammalian-adaptive residue 627. We then explored the hypothesis that host factors in the regulatory network could modulate not only functions of H1N1 and H5N1 influenza virus polymerases but also adaptation of HPAI viruses to human cells. We examined host factor RNAi phenotypes for two A/VN/1203/04 (H5N1) polymerases side-by-side: that of the wild-type (wt), human isolate, with a lysine (K) at position 627 in PB2, and a mutant with a change to glutamic acid (E), the consensus residue found in most avian H5N1 isolates (11, 34). Avianized

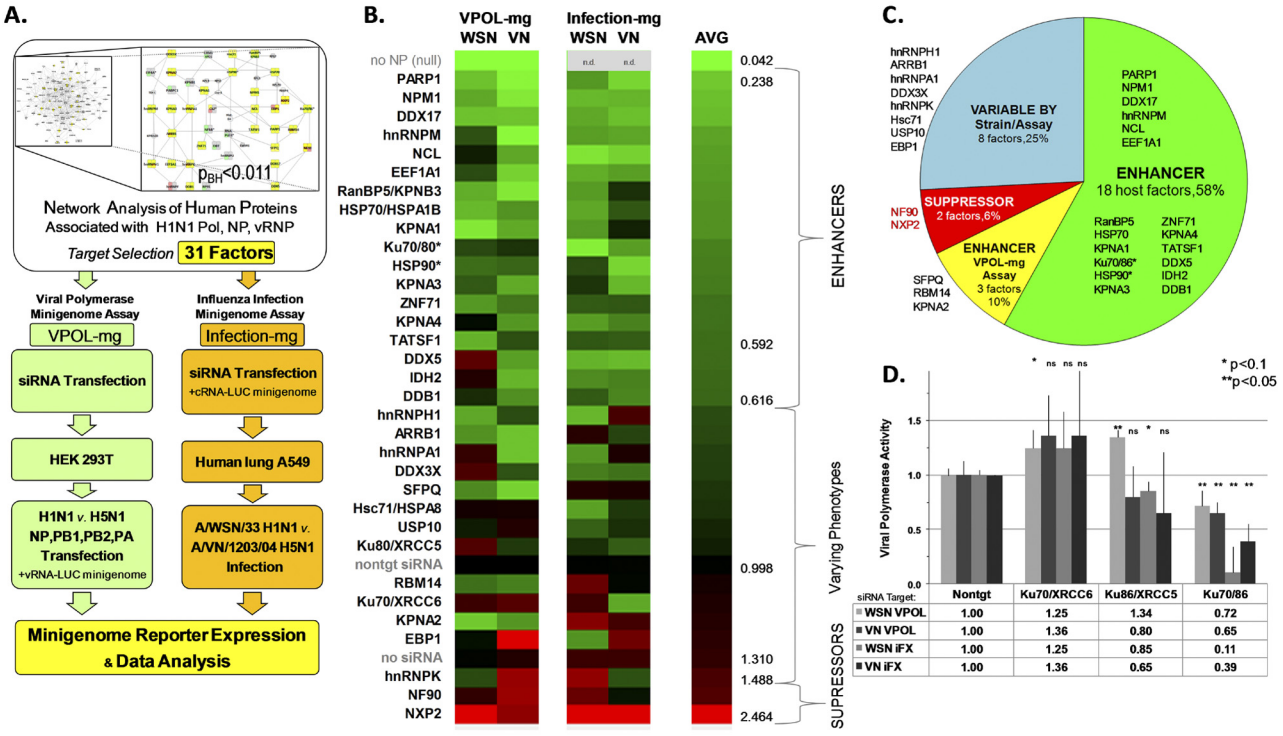


FIG 1 Host factors regulate H1N1 and H5N1 influenza virus polymerase activity in human cells. (A) Host proteins interacting with influenza A virus polymerase, NP, and/or vRNP were analyzed by network bioinformatics. A subset of 31 factors was selected to analyze their roles in H1N1 and H5N1 influenza virus polymerase activity. For assays for viral polymerase phenotypes, siRNAs targeting specific host factor transcripts were transfected into HEK 293T cells or human lung A549 cells. For the viral polymerase minigenome assay (VPOL-mg), after knockdown, HEK 293T cells were transfected with A/WSN/33 (H1N1) or A/VN/1203/04 (H5N1) nucleoprotein and polymerase expression plasmids in optimized ratios, a vRNA promoter minigenome luciferase reporter, and a constitutive *Renilla* internal control. For the influenza virus infection assay (infection-mg), A549 cells were cotransfected with siRNA, a cRNA promoter minigenome luciferase reporter, and a *Renilla* internal control and, after knockdown, infected with influenza A/WSN/33 (H1N1) or A/VN/1203/04 (H5N1) HALo virus (MOI = 0.5). Viral polymerase activity was assessed and normalized by dual-luciferase assay. (B) Heat map of average A/WSN/33 (H1N1) (WSN) or A/VN/1203/04 (H5N1) (VN) viral polymerase activity in a ratio to the nontarget siRNA value in VPOL-mg and infection-mg assays for the 31 RNAi targets, nontarget (nontgt) siRNA, and "no siRNA" controls. Targets were ordered and classified by averaging data from all four conditions (AVG). *, simultaneous targeting of both subunits of HSP90 or Ku70/86; n.d., not done. (C) Functional classification of phenotypes for the 31 host RNAi targets, reflecting composite data from all four experimental conditions. (D) Ku70/XRCC6 and Ku86/XRCC5 targeted individually or simultaneously (Ku70/86) by siRNA in VPOL-mg (VPOL) and infection-mg (IFX) assays for the A/WSN/33 (H1N1) (WSN) and A/VN/1203/04 (H5N1) (VN) strains, with significance indicated by an unpaired, 2-tailed *t* test.

(PB2|627E) polymerase displayed approximately 75-fold-lower activity than that of the wt/human isolate (PB2|627K) in human 293T cells but still provided sufficient dynamic range for VPOL-mg assays (Fig. 2B). In the regulatory network, 13/25 (52%) host factors conserved enhancer phenotypes for both wt/human isolate and avianized H5N1 polymerases (Fig. 2C). Surprisingly, 12/25 (48%) of factors that had been enhancers for the wt/human isolate showed no significant phenotype, or even antagonist phenotypes, for the avianized H5N1 polymerase. RNA-binding/processing proteins ($P < 0.004$, group enrichment score) and molecular transport/chaperone proteins had differential phenotypes.

Exogenous cDNA expression of four RNA-binding host proteins. To complement siRNA experiments on RNA-binding proteins in the regulatory network, we overexpressed cDNA plasmids encoding human NPM1, DDX17, NF90, and hnRNPA1. As expected from VPOL-mg optimization experiments (see Fig. S2 in the supplemental material), increasing NP increased viral polymerase activity (Fig. 3). NF90 suppressed, and both DDX17 and NPM1 enhanced, H1N1 and wt/human isolate H5N1 polymerase activity in comparison to results with an empty DNA vector. Thus, for mammalian-adapted H1N1 and H5N1 polymerases, overexpression phenotypes for DDX17, NPM1, and NF90 were generally consistent with RNAi phenotypes (i.e., opposite to VPOL-mg phenotypes in Fig. 1B). The exception was hnRNPA1, which showed disparate phenotypes in cDNA assays. Along with results of siRNA experiments (Fig. 1B), these data suggest that hnRNPA1 may have pleiotropic functional interactions with influenza virus polymerase in human cells.

Impact of host factors in H5N1 virus replication. We validated the phenotypes of eight representative RNA-binding proteins from the regulatory network and two stress-related proteins, PARP1 and USP10, in H5N1 infection of human lung A549 cells. As with PARP1, knockdown of hnRNPM, DDX17, DDX5, DDX3X, and NPM1 each led to a significant reduction in the titer of A/VN/1203/04 (H5N1) HALo virus (i.e., virus modified by removal of the hemagglutinin [HA0] protein's polybasic cleavage site) released from A549 cells (Fig. 4A), in concordance with phenotypes in both the VPOL-mg and infection-mg assays (Fig. 1B). Knockdown of NXP2 or hnRNPH1 allowed a mild increase in the

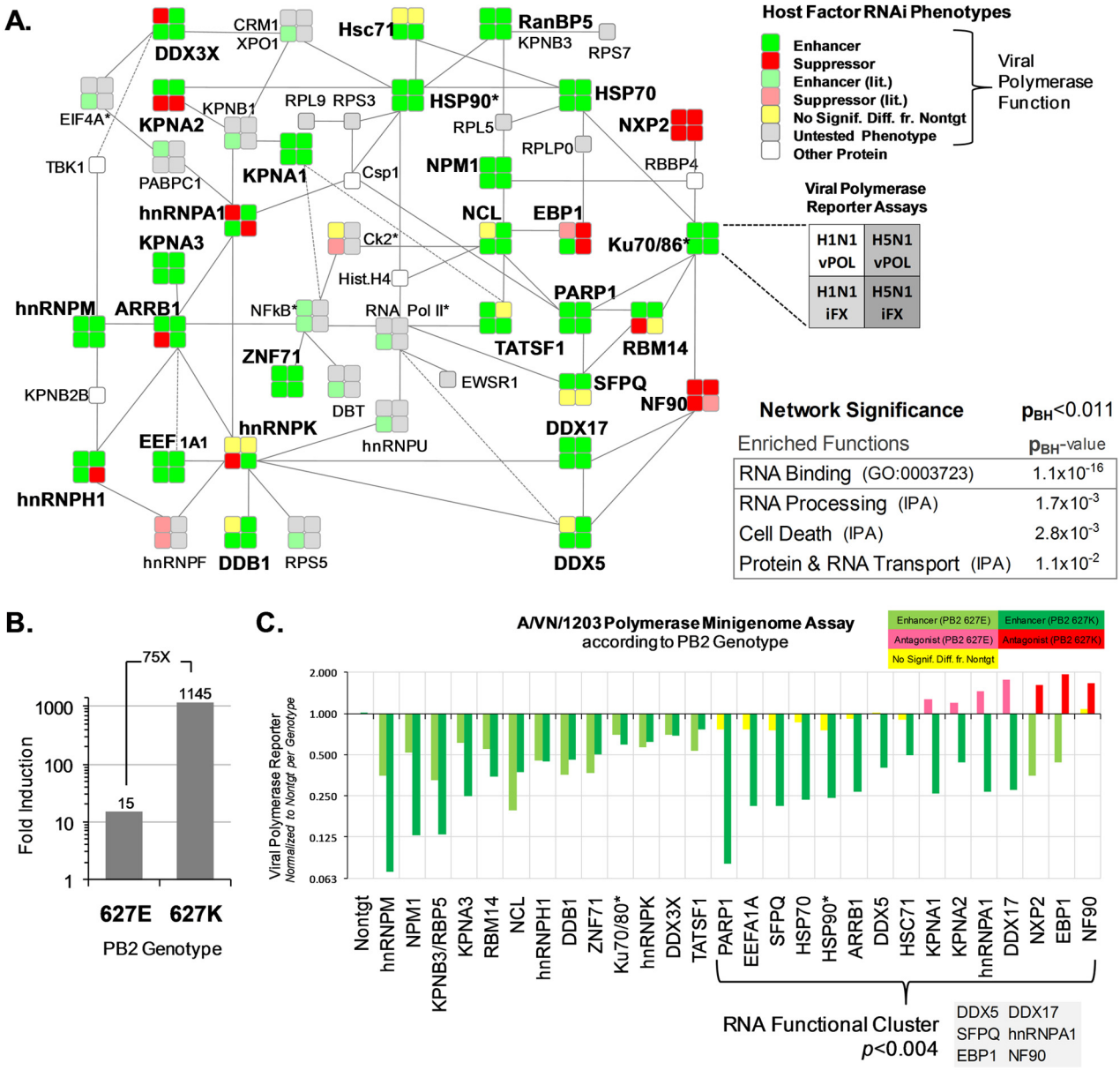
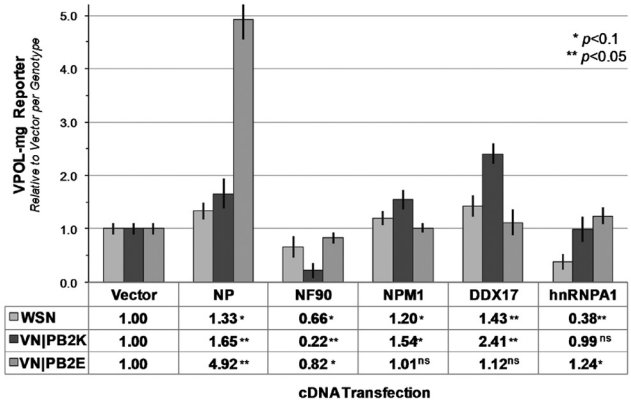


FIG 2 Regulatory network modulating H1N1 polymerase and H5N1 polymerase according to PB2 genotype at amino acid position 627. (A) Protein interaction network comprising influenza virus polymerase-associated cellular proteins and proximal nodes extracted from the human protein interactome by Ingenuity pathways analysis (IPA), with significance estimated by Benjamini-Hochberg test (P_{BH}). H1N1 and H5N1 polymerase-host RNAi phenotype data from VPOL-mg (vPOL) and infection-mg (iFX) assays are indicated by color according to this study and published references (lit.; Refs. 16, 22, 24–28, 30–32). Edges between nodes are reported protein-protein interactions; Untested, phenotypic assay(s) not performed for interactor; Other, connecting protein; dashed links, indirect association reliant on proteins not shown. Inset table: enriched functions found by IPA and Gene Ontology (GO) analyses. (B) Viral polymerase minigenome reporter assays with 293T cells for two A/VN/1203/04 (H5N1) viral polymerase PB2 genotypes (627K, wild type/human isolate; 627E, avianized mutant). Fold induction, ratio to results of mock VPOL-mg assay. (C) Average VPOL-mg activity for PB2 627K and 627E genotypes, expressed as a ratio to the corresponding nontarget siRNA (Nontgt) value for 28 principal network host factors. Phenotypes are classified by significance estimated by an unpaired, 2-tailed *t* test (enhancer or suppressor, $P_{max} < 0.15$; no significant difference from nontarget control, $P_{max} \geq 0.15$). Targets are ordered by magnitude/phenotype; *y* axis, $\log_2$ scale. The Gene Ontology functional cluster of RNA-binding/processing proteins (*P* value, group enrichment score) among indicated targets exhibiting differential phenotypes is shown.

virus titer, in agreement with the siRNA experiments, while targeting SFPQ, a protein involved in mRNA processing, resulted in a more significant increase in titers. For PARP1 and four RNA-binding proteins tested, reduced accumulation of NP in H5N1-infected A549 cells was observed, consistent with the hypothesis that these factors are required for optimal polymerase function (Fig. 4B). In A549 cells with substantial knockdown of PARP1, NPM1, or DDX17 at the protein level, viral nucleoprotein expression was still reduced 20 h after H5N1 infection (Fig. 4C). Since A/VN/1203/04 (H5N1) HALo virus undergoes only one life cycle



	Vector	NP	NF90	NPM1	DDX17	hnRNPA1
WSN	1.00	1.33*	0.66*	1.20*	1.43**	0.38**
VN|PB2K	1.00	1.65**	0.22**	1.54*	2.41**	0.99 ns
VN|PB2E	1.00	4.92**	0.82*	1.01 ns	1.12 ns	1.24*

FIG 3 Exogenous expression of host factor cDNA modulates influenza virus polymerase activity. Subconfluent 293T cells were transfected with a vRNA promoter luciferase minigenome, a constitutively active *Renilla* luciferase, cDNA expression plasmids encoding indicated host factors, or empty vector, and A/WSN/33 (WSN) or A/VN/1203/04 (H5N1) (VN) with either PB2 627K (PB2K, wt/human isolate) or 627E (PB2E, avianized mutant) polymerase plasmid. Viral polymerase activity was assessed as a ratio of normalized polymerase activities to the empty vector value, with significance estimated by an unpaired, 2-tailed *t* test. ns, not significant.

in this system, these results suggest that the reduction in NP protein results from reduced viral gene expression and/or amplification of viral genomes.

DDX17 is required for efficient H5N1 mRNA and vRNA synthesis during infection of human cells. For DDX17 knockdown in the VPOL-mg assay, the divergence between H5N1 PB2|672E antagonist and PB2|627K enhancer phenotypes was approximately 6.4-fold (Fig. 2B). DDX17 thus exhibited characteristics of a species specificity factor for H5N1 HPAI polymerase, so we investigated mechanisms by which DDX17 allows optimal viral gene expression in human cells. Surprisingly, DDX17 interacted with avianized H5N1 polymerase complexes at least as well as, if not better than, the wt/human isolate in immunoprecipitation experiments (see Fig. S4 in the supplemental material). When DDX17 was targeted by siRNA in human 293T cells, expression of the NP and M1 proteins was reduced during A/VN/1203/04 (H5N1) HALo infection (Fig. 5A). Primer extension analysis showed an approximately 25% loss in synthesis of both hemagglutinin (HA) and NP vRNA segments and a 30% loss in HA mRNA synthesis, when DDX17 was targeted by siRNA (Fig. 5B). The decrease in NP mRNA was not significant at this time point (12 h p.i.). These data indicate that DDX17 is required for maximal viral mRNA and vRNA synthesis in human cells infected by H5N1 virus. However, it is also possible that DDX17 has an additional posttranscriptional role(s) facilitating viral gene expression.

Avian DDX17 is required for optimal H5N1 replication in avian cells. While human DDX17 was required for optimal replication of the wt/human isolate H5N1 virus in human cells, we asked whether this host factor is required for optimal replication of avianized H5N1 virus in avian cells. The domestic chicken (*Gallus gallus*) has emerged as a host for H5N1 HPAI viruses (1). After identifying a DDX17 ortholog in the chicken genome by sequence comparison, we synthesized siRNAs specifically targeting the *chkDDX17* transcript (see Table S1 in the supplemental material). Knockdown of the chkDDX17 protein in chicken DF-1 fibroblasts (Fig. 6A) resulted in a 3-fold-decreased viral titer (Fig. 6B) and significantly less NP expression (Fig. 6C) during A/Viet Nam/1203/04 (H5N1) HALo (PB2|627E) infection. The wild-type/human isolate (PB2|627K) virus showed a 2-fold decrease in viral titer (data not shown). Thus, a DDX17-like protein in chicken cells is required for optimal avian (PB2|627E) H5N1 virus replication.

DDX17 and NPM1 colocalize with H5N1 NP in the cytoplasm late in infection. Finally, we studied the subcellular interaction of DDX17 and NPM1 with viral NP during the H5N1 virus life cycle in human cells. According to the current understanding of viral ribonucleoprotein (vRNP) export and virion assembly (31, 35, 36), NP first accumulates in the nucleoplasm as a viral protein, and RNA syntheses progress. An unidentified signal induces NP to relocalize inside the nuclear periphery for subsequent export with viral RNA, mediated by NS2/NEP, to the cytoplasm for microtubule-mediated transport to the plasma membrane, where new virions bud. In uninfected, interphase HeLa cells, endogenous DDX17 exhibited a punctate, predominantly intranuclear distribution that was altered by mitosis; NPM1, an abundant RNA chaperone, was mostly cytoplasmic but also was present in the nucleoli and nucleoplasm (see Fig. S5 in the supplemental material). Early in the spatial context of H5N1 infection, NPM1 did not overlap with nuclear NP (Fig. 7A). However, by 12 h p.i., NPM1 closely associated with NP in the nuclear periphery and perinuclear region and in discrete punctae in the cytoplasm (Fig. 7B). DDX17 initially associated with NP in the nucleus and at micropunctae in the cytoplasm (Fig. 7A) and showed extensive colocalization with NP in the cytoplasm by 12 h p.i. (Fig. 7C). The relocalization of NPM1 and DDX17 in infected cells contrasted with the nuclear localization of another enhancer, PARP1 (Fig. 7A). These results suggested that both DDX17 and NPM1 associate with vRNP that have been exported to the cytoplasm late in the viral life cycle.

DISCUSSION

Host protein regulation of influenza virus polymerases. Virus-host interactions modulating the influenza A virus polymerase have the potential to govern the replication and species specificity of emerging HPAI virus strains. We found that a majority (58%) of the virus-host interactions explored herein positively regulated (enhanced) activities of both A/WSN/33 (H1N1) and A/VN/1203/04 (H5N1) polymerases (Fig. 1C). Significant phenotypes in the VPOL-mg assay (Fig. 1B; see also Table S2 in the supplemental material) suggested that many human host factors can directly regulate viral polymerase activity in the absence of other viral proteins or cellular pathways induced by infection. Though precise mechanisms remain to be resolved, one can hypothesize that by interacting with the enhancer proteins in the regulatory network (Fig. 2A), influenza virus polymerases interact with enriched cellular processes (e.g., RNA processing; see Table S3) to achieve a high level of intranuclear RNA synthesis. Conversely, antagonist proteins could activate antiviral or RNA stress pathways that inhibit polymerase functions, viral protein expression, or RNA traffic. Molecular transport and RNA-related cellular functions were also identified by functional genomics (RNAi) surveys (24–27, 33), suggesting the critical nature of these processes, at least for H1N1 virus infection.

RNA-associated cellular proteins have been found to positively or negatively affect viral RNA syntheses (16, 19, 21, 22). Indeed,

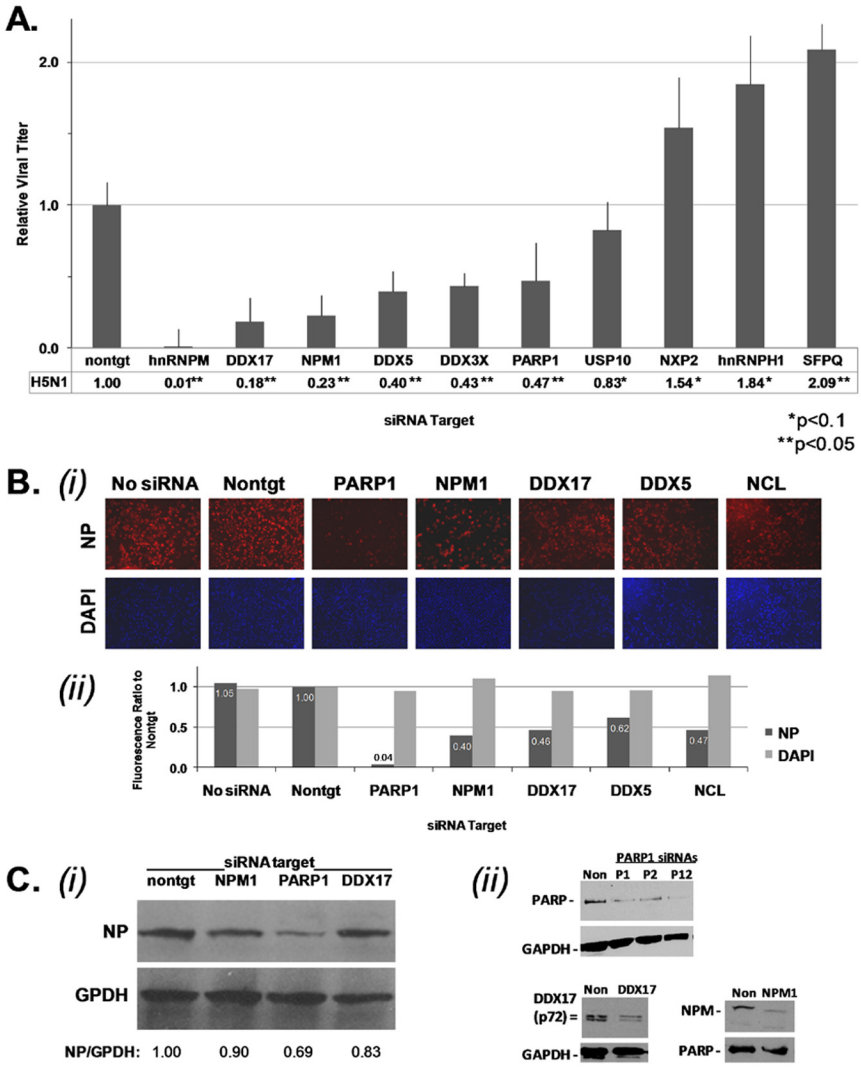


FIG 4 Host factor RNAi silencing alters progression of the H5N1 virus life cycle. (A) Average relative titers of virus released 20 h p.i. from A549 cells targeted 36 h prior by siRNA transfection and infected by influenza A/VN/1203/04 (H5N1) HALo virus (MOI = 0.1). Values shown are averages of plaque and limiting dilution assay ratios for each host target siRNA relative to nontarget (nontgt) siRNA values, with significance estimated by an unpaired, 2-tailed *t* test. (B) Viral nucleoprotein (NP) expression in A549 cells mock-treated or transfected with nontarget (Nontgt) or siRNA-targeting selected host factors 24 h prior to infection with A/VN/1203/04 (H5N1) HALo virus (MOI = 2). Cultures were fixed 6 h p.i. for IFA with monoclonal anti-NP and Alexa-555-labeled secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining (*i*). Fluorescence intensity quantification of Alexa-555 in representative images as a ratio to the nontarget siRNA value (*ii*). (C) NP expression (20 h p.i.) in A549 cells infected with A/VN/1203/04 (H5N1) HALo (MOI = 0.5). Infected cell lysates were separated by SDS-PAGE and immunoblotted, and separated strips were probed with anti-NP or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. Densitometry depicting the ratio of NP to GAPDH for equal protein samples is indicated (*i*). A549 cells transfected with siRNA (Non, nontarget; P1 and P2 indicate two different siRNAs, while P12 indicates both pooled, targeting PARP1; DDX17, siRNA pool targeting DDX17; NPM1, siRNA pool targeting nucleophosmin) were lysed and run on an SDS-PAGE gel, and Western blots were probed with antibodies as indicated (*ii*).

we found that RNA-binding proteins, particularly heterogeneous nuclear ribonucleoproteins (hnRNPs) and DEAD box (DDX) RNA helicases, regulated both H1N1 and H5N1 polymerases (Fig. 2A and 3) and H5N1 infection in a human lung cell line (Fig. 4). NF90 (DRBP76/ILF3), an antiviral RNA-binding protein that restricts H1N1 and H5N1 NP from interacting with vRNA (22), antagonized polymerase (Fig. 1B and 3). Six RNA-associated proteins (EBP1 [16], hnRNPK [25], hnRNPH1, hnRNPA1, RBM14, and DDX3X) and ARRB1 (25), a signaling modulator, displayed significantly differential phenotypes between H1N1 and H5N1 viruses (Fig. 2A). The possibility that H1N1 and H5N1 viruses differ in functional adaptation to an RNA-related process (see Table S3 in the supplemental material) highlights the importance of exploring strain differences in antiviral drug development.

Host factors regulate adaptation of an HPAI virus polymerase to human cells. For an H5N1 HPAI virus, wild-type/human isolate (PB2|627K) and avianized (PB2|627E) polymerases differed considerably in their reliance on host factors in the regulatory network (Fig. 2B). Evidence has suggested that HPAI polymerases containing a mammal-adapted genotype at adaptive residues (e.g., PB2 591, 627, and 701) are able to interact with a host factor(s) to facilitate efficient RNA synthesis activity (11, 37). Host factors that exhibited a differential phenotype for avianized polymerase (Fig. 2B) included chaperone/transport proteins, consistent with models suggested in the literature (8, 38), and RNA-binding proteins, such as DDX17. This suggests that an RNA-related process may also contribute to the mechanistic basis of phenotypes related to the PB2 residue 627 genotype.

DDX17, a protein critical for H5N1 polymerase adaptation to human cells. DDX17 (p72) and a related protein, DDX5 (p68), are abundant nuclear ATP-dependent DEAD-box RNA helicases that can heterodimerize (39) and catalyze RNA unwinding and rearrangement (40). DDX17 and DDX5 have been implicated in ribosomal biogenesis (41). Human DDX17 and DDX5 exhibited consistent, strong enhancer phenotypes in polymerase assays (Fig. 1B) that were confirmed in H5N1 infection, independently of mini-genome reporters. DDX17 and DDX5 were required for A/VN/1203/04 (H5N1) wt/human isolate (PB2|627K) but not avianized mutant (PB2|627E) polymerase activity (Fig. 2B). When overexpressed in human cells, DDX17 also enhanced activity of wt/human isolate but not avianized H5N1 polymerase (Fig. 3). Mechanistically, human DDX17 facilitated both mRNA and vRNA synthesis during wt/human isolate H5N1 infection (Fig. 5B). DDX17 closely colocalized with NP in the cell nucleus early (3 h p.i.) in H5N1 infection (Fig. 7A), consistent with a role in viral RNA synthesis.

Proteomics and immunoprecipitation experiments have

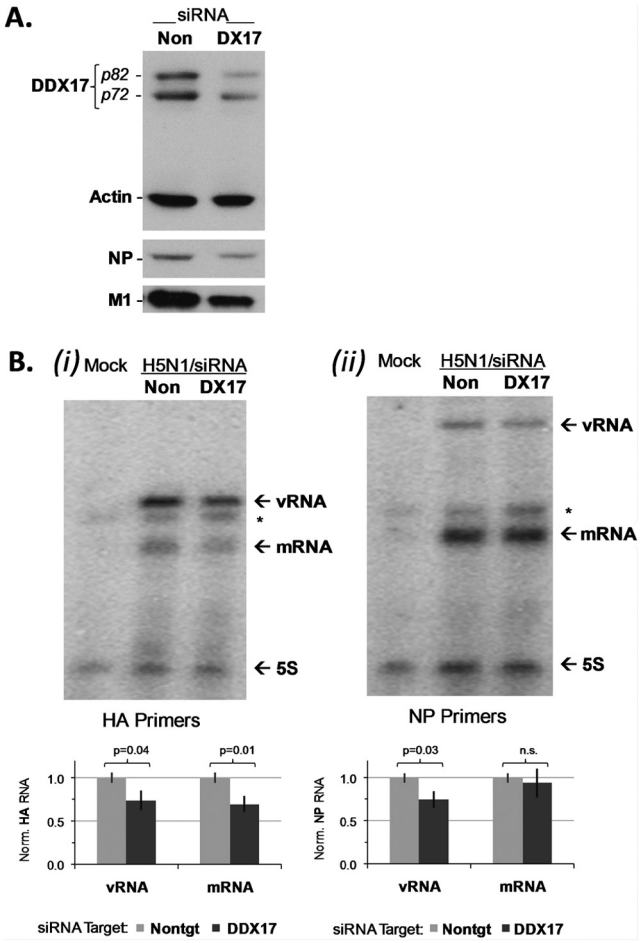


FIG 5 DDX17 facilitates viral RNA synthesis in H5N1 infection of human cells. (A) 293T cells were targeted by Nontarget (Non) or DDX17 (DX17) siRNA. After 24 h of knockdown, cells were infected by A/VN/1203/04 (H5N1) HALo virus (MOI = 1). Cell protein lysates were analyzed 12 h p.i. for the DDX17, NP, M1, and actin proteins by Western blotting. (B) Primer extension analysis with specific primers for HA (*i*) or NP (*ii*) mRNA and vRNA synthesis, as indicated, for mock-infected control or 293T cells 12 h p.i. with A/VN/1203/04 (H5N1) HALo virus (MOI = 1). 5S, rRNA loading control; *, nonspecific background band. Graphs show RNA quantification by densitometry normalized to 5S rRNA and background; significance was estimated by an unpaired, 2-tailed *t* test from density histograms and standard deviations.

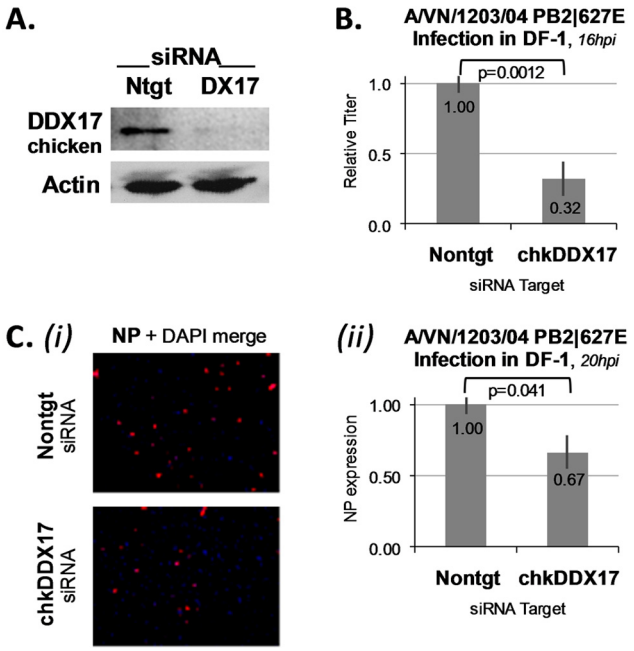


FIG 6 DDX17 is required for optimal H5N1 infection in chicken fibroblasts. (A) Targeted knockdown of chicken DDX17. Nontarget siRNA (Ntgt) or specific siRNA targeting a *Gallus gallus* DDX17 ortholog (*DX17*) was synthesized, purified, pooled, and transfected into DF-1 cells. After 48 h of knockdown, cells were lysed and analyzed by Western blotting for chicken DDX17 and actin with cross-reactive, polyclonal antisera. (B) Decreased H5N1 virus replication with knockdown of chicken DDX17. Chicken DF-1 fibroblasts transfected by nontarget (Nontgt) or chicken DDX17 (*chkDDX17*) siRNA were infected after 48 h with A/VN/1203/04 (H5N1) HALo (PB2|627E) virus (MOI = 0.5). Viral titers (16 h p.i.) were estimated by limiting dilution relative to the nontarget siRNA control value, and significance was tested by an unpaired, 2-tailed *t* test. (C) DF-1 cells were infected with avianized A/VN/1203/04 (H5N1) HALo (PB2|627E) virus (MOI = 0.5) and fixed 20 h p.i. NP expression in infected DF-1 cells was analyzed by IFA with monoclonal anti-NP-labeled and Alexa-555-labeled anti-mouse secondary antibody and DAPI nuclear staining (*i*), and fluorescence intensity was quantified relative to the nontarget siRNA value, with significance tested by an unpaired, 2-tailed *t* test (*ii*).

found that human DDX5 (17), DDX17, and NPM1 (18; data not shown) associate with WSN (H1N1) polymerase. Surprisingly, we found that DDX17 also associated with either wt/human isolate (PB2|627K) or avianized (PB2|627E) HPAI (H5N1) polymerase complexes and with either PB2 alone (see Fig. S5 in the supplemental material). However, the presence of another enhancer, NPM1, was greater in the wt/human isolate PB2 complexes. Thus, the failure of avian H5N1 polymerase to take advantage of DDX17's ability to facilitate viral RNA synthesis is apparently not due simply to an inability of human DDX17 to interact with a heterotrimeric polymerase containing PB2|627E but perhaps involves association with another protein(s), such as NPM1 or DDX5. We also found that while the chicken DDX17 homologue was required for H5N1 protein expression (Fig. 6C) and viral replication (Fig. 6B), human DDX17 antagonized avianized polymerase in human cells (Fig. 2B). Thus, physical association of PB2|627E with avian DDX17 in avian cells might mechanistically enhance replication, while in contrast, interaction with human DDX17 could inhibit unadapted HPAI virus polymerase in human cells. This hypothesis might explain why avian influenza virus polymerases are blocked from efficient RNA synthesis in human cells by an antagonistic host factor(s) (42). Taken together, H5N1 virus requires a DDX17-like protein in both avian and human cells, suggesting conservation of a polymerase-host interaction critical for H5N1 survival in different host species.

Possible roles for RNA chaperones in vRNP export from the nucleus. We found that two RNA-associated proteins (DDX17 and NPM1) may have additional functions during influenza virus vRNP export from the nucleus. Nuclear export of vRNP occurs in a CRM1-dependent manner (31). DDX17 and DDX5 function together in ribosomal pre-rRNA processing, and rRNA is also exported from the nucleus in a CRM1-dependent manner (41, 43). Meanwhile, the ribosomal protein RPL5, which associates with influenza virus vRNP (18), requires NPM1 for nuclear export (44). Late in H5N1 virus infection, DDX17 and NPM1 had redistributed in the cytoplasm in conjunction with NP (Fig. 7), likely signifying export of nascent vRNP from the nucleus (35). Thus, we

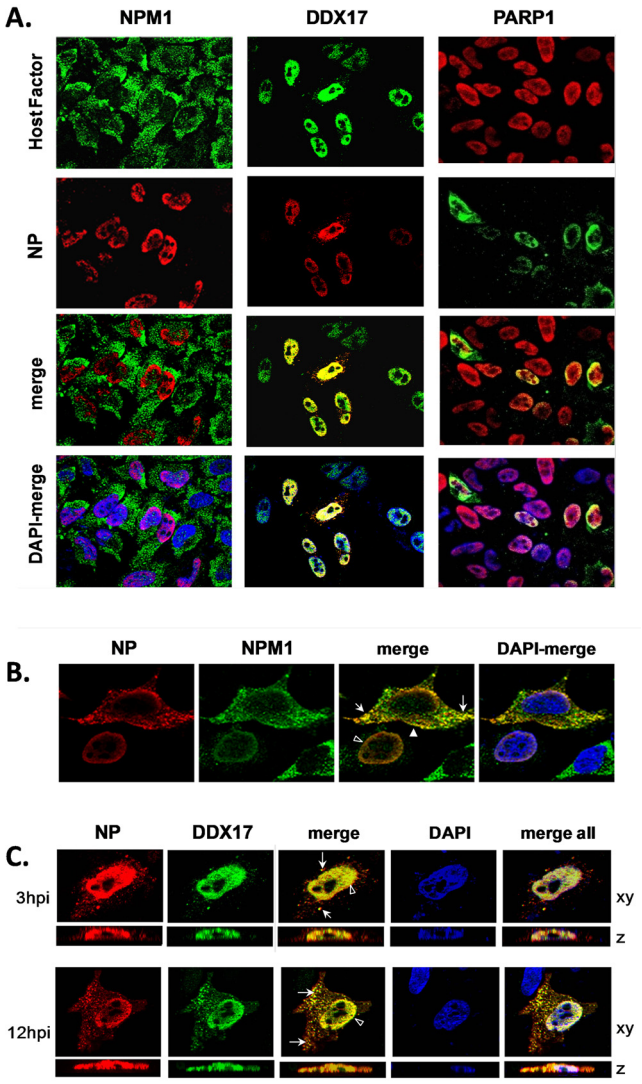


FIG 7 Colocalization of H5N1 NP with DDX17 and NPM1 during infection. (A) IFA of HeLa cells infected with influenza A/VN/1203/04 (H5N1) HALo virus (MOI = 0.5) were fixed 3 h p.i. and incubated with anti-NP monoclonal and anti-NPM1 or -DDX17 polyclonal antibodies or anti-PARP1 monoclonal and anti-NP polyclonal antibodies, Alexa-555-labeled (red) or Alexa-488-labeled (green) secondary antibodies, and DAPI nuclear stain (blue). Images were captured by structured illumination, providing defined submicron vertical (*z*-plane) optical sections imaged through the cell, and merged as indicated. (B) IFA of HeLa cells infected with A/VN/1203/04 (H5N1) HALo virus (MOI = 0.2) fixed 12 h p.i. Colocalization (yellow) of NP and NPM1 in the nuclear periphery and perinuclear region (open arrowhead), in discrete punctae in the cytoplasm (arrows), or in proximity to the plasma membrane (filled arrowhead) is indicated. (C) IFA of HeLa cells infected with A/VN/1203/04 (H5N1) HALo virus (MOI = 0.2) fixed at 3 h p.i. or 12 h p.i. Colocalization (yellow) of NP and DDX17 in the nucleus (open arrowhead) or in discrete punctae in the cytoplasm (arrows) is indicated. Structured illuminations of horizontal optical sections (*xy* plane) were stacked to reconstruct lateral (*z*-plane) images of infected cells.

are investigating the hypothesis that influenza viruses have evolved to hijack aspects of the CRM1-dependent export mechanism of another large ribonucleoprotein complex, the ribosome, in order to facilitate their own vRNP nuclear export.

Mapping functions in the influenza virus polymerase-host interactome. Genomics experiments, from proteomics and genome-wide RNAi surveys to more targeted studies examining particular aspects of virus life cycles (e.g., influenza virus polymerase regulation), allow for the first time systematic analysis of the complexity of host-pathogen regulatory networks. While the present study identifies host factors important for influenza virus polymerase function and host species tropism, a comprehensive, dynamic model of how host interactions regulate influenza virus RNA synthesis, gene expression, and genome replication is far from complete. Adoption of common standards for RNAi data submission (45) would allow for more accurate meta-analysis and integration of multiple genomics data types to model complex virus-host interactions (27). We have demonstrated that functional genomics analysis of host proteins provides a novel means to study the phenotypes of influenza virus polymerase mutants and to map adaptation of emerging H5N1 HPAI polymerases to human cells. This approach has the potential to identify novel targets for therapeutic intervention in severe influenza virus infections.

MATERIALS AND METHODS

Viruses. Attenuated influenza A/Viet Nam/1203/2004 (H5N1) HALo viruses (PB2|627K and PB2|627E) were generated by reverse genetics as described previously (13, 46), with removal of the hemagglutinin (HA0) protein's polybasic cleavage site (GenBank accession no. CY077101); the virus undergoes only one round of replication in the absence of exogenous trypsin. All other wild-type viral gene segments were unmodified. Titers of H5N1 influenza viruses were determined by plaque assay on MDCK cells (46) or by limiting-dilution assays on A549 or chicken DF-1 cells. Titers of A/WSN/33 (H1N1) virus were determined as described previously (18, 26).

Bioinformatics analyses. The H1N1 polymerase-host interactome was initially analyzed using Ingenuity pathways analysis (IPA7.6; Ingenuity Systems, Redwood, CA). The top-scoring, interconnected subnetwork was extracted, expanded by literature curation, and tested for significance by a Benjamini-Hochberg (P_{BH}) estimate for false discovery probability, i.e., the probability that the linkages and functions assigned to the derived node-edge sets are due to chance alone. The DAVID software program (47) analyzed Gene Ontology (GO) term enrichment and functional group clustering.

Targeting host genes with siRNA. Nontarget (scrambled) siRNA or siRNA targeting specific human or chicken transcripts (see Table S1 in the supplemental material) was transfected into 293T, A549, or DF-1 cells at 10 to 15 nM. For a subset of siRNA, efficacy of RNAi knockdown was evaluated by Western blotting, reverse transcription-PCR (RT-PCR), or siRNA targeting green fluorescent protein (GFP) (see Fig. S2). Cell viability (see Fig. S3) was measured by a *Renilla* reporter, CellTiter-Glo, and caspase-Glo 3/7 assays (Promega Corp., Madison, WI).

Minigenome reporter assays. The VPOL-mg (vRNA-luciferase reporter) assay with HEK 293T cells (18) was optimized with plasmid ratios of 10:2:1:2 (NP/PB1/PB2/PA) to assay autonomous polymerase function (see Fig. S2 in the supplemental material). For cDNA experiments, ratios were 5:2:1:2. VPOL-mg and siRNA conditions were tested for two known polymerase interactors, PARP1 (18) and RanBP5 (15) (see Fig. S2). The infection-mg (cRNA-luciferase reporter) assay (29) was adopted to measure polymerase function in infected A549 cells. In minigenome reporter assays, reporter gene expression ("viral polymerase activity") is dependent on viral polymerase-mediated transcription and replication of a model influenza virus RNA, measured by a Dual-Luciferase reporter assay (Promega Corporation, Madison, WI). Significance of differences relative to nontarget siRNA were estimated by an unpaired, 2-tailed *t* test to calculate *P* values, with a P_{max} value of ≥0.15 regarded as no significant difference (see Table S2). Data for each RNAi target will be available in PubChem under *M*inimum *I*nformation *A*bout an RNAi *E*xperiment (MIARE) data standards (45).

IFA and microscopy. Infected A549 or DF-1 cells were fixed for immunofluorescence assay (IFA) and probed with specific primary antibodies (described in Text S1 [Materials and Methods] in the supplemental material) and Alexa Fluor-linked (488-nm anti-rabbit and 555-nm anti-mouse) IgG secondary antibodies (Molecular Probes, Eugene, OR). For subcellular localization studies, HeLa cells were infected with A/VN/1203/04 (H5N1) HALo virus (multiplicity of infection [MOI] of 0.5), and imaged by structured illumination on an Axioplan 2 microscope (Zeiss, Göttingen, Germany) providing optical, submicron vertical (*z*-plane) sections through the cell.

Molecular biology assays. Primer extension assays were performed as described (23), using ^{32}P-labeled DNA oligonucleotides to A/VN/1203/04 HA and NP, detecting vRNA segments and mRNA, and a 5S rRNA loading control. Immunoblot and immunoprecipitation assays were performed as described previously (18), with specific protocols described in Text S1 in the supplemental material (supplemental Materials and Methods).

Refer to Text S1 (supplemental Materials and Methods) for detailed descriptions of all methods and reagents.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00151-11/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.6 MB.
Figure S2, PDF file, 0.4 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 0.2 MB.
Figure S5, PDF file, 1.2 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.04 MB.

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