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Poly-ADP ribosyl polymerase 1 (PARP1) regulates influenza A virus polymerase

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Abstract: Influenza A viruses (IAV) are evolutionarily successful pathogens, capable of infecting a number of avian and mammalian species, and responsible for pandemic and seasonal epidemic disease in humans. To infect new species, IAV typically must overcome a number of species barriers to entry, replication, and egress, even while virus replication is counter-acted by antiviral host factors and innate immune mechanisms. A number of host factors have been found to regulate the replication of IAV by interacting with the viral RNA-dependent RNA polymerase (RdRP). The host factor PARP1, a poly-ADP ribosyl polymerase, was required for optimal functions of human, swine, and avian influenza RdRP in human 293T cells. In IAV infection, PARP1 was required for syntheses of viral mRNA and vRNA progeny, and for synthesis of viral nucleoprotein (NP) in human lung A549 cells. Intriguingly, pharmacological inhibition of PARP1 enzymatic activity (PARylation) by 4-amino-1,8-naphthalamide led to a 4-fold increase in RdRP activity, and a 2.3-fold increase in virus titer. Exogenous expression of the natural PARylation inhibitor PARG also enhanced RdRP activity. These data suggest a virus-host interaction dynamic where PARP1 itself is required, but cellular PARylation has a distinct suppressive modality on influenza RdRP function.

Keywords: influenza A virus; PARP1; RNA-dependent RNA polymerase; host factors; PARylation; 4-amino-1,8-naphthalamide.

1. Introduction

Influenza viruses are segmented, negative-sense single-stranded RNA viruses in the evolutionarily diverse viral family Orthomyxoviridae. Annual seasonal epidemics of influenza A virus (IAV) infections are a considerable health burden in humans. The natural reservoir of IAV is in wild birds, although AIV can infect poultry, and highly pathogenic avian influenza viruses (HPAIV) of H5N1, H5N6 and H7N9 hemagglutinin subtypes, among others, can spread to humans [1,2]. Epizootic IAV infections occur frequently in seals, horses, and swine [1,3-5]. Reassortment of the 8 viral gene segments in IAV genomes can result in emergence of immunologically distinct strains, capable of rapid, virulent spread in susceptible populations, gravely illustrated by the high burden of influenza and mortality of the 1918 H1N1 pandemic in humans [6]. In 2009, a novel reassortant strain of IAV (pdmH1N1), in part a genetic descendant of the 1918 H1N1 strain [7], emerged from swine to spread globally in humans again, causing considerable respiratory disease, particularly in patients with underlying medical conditions [8]. The pdmH1N1 pandemic virus also spread to other species, including elephant seals, and contributed gene segments to novel strains in swine (H3N2v) that are capable of infecting humans [9-11]. Thus, although restricted by natural or vaccine-generated
subtype-specific immune responses against surface hemagglutinin (HA) and neuraminidase (NA) proteins, as a virus group, IAV has proved capable of overcoming host barriers to achieve replication in multiple species [1,2,12]. This suggest that the fundamental processes of the IAV life cycle, including HA-dependent binding and entry into a susceptible host cell, viral RNA and protein syntheses, virion assembly and NA-dependent maturation, can utilize host cell molecular structures and physiological processes that are broadly conserved in multicellular vertebrates.

The IAV RNA-dependent RNA polymerase (RdRP) is a critical determinant of viral pathogenesis and transmission of IAV from avian to mammalian species [7,13-15]. The IAV RdRP, consisting of heterotrimeric PB1, PB2, and PA proteins, in conjunction with viral nucleoprotein (NP), functions as both a transcriptase that binds viral (-) sense vRNA genomes to synthesize viral mRNA, and as a replicase that synthesizes vRNA progeny via a (+) sense cRNA intermediate [16]. Importantly, these processes are modulated by interacting cellular proteins. Critically, host RNA polymerase II aids in initiation, cap-snatching and elongation of viral mRNA syntheses during transcription [17-19]. A large number of other host cell proteins interact with the IAV RdRP complex in nuclei of infected cells [20-22]. Experiments targeting host genes by RNA interference (RNAi) or CRISP knockout [23] showed that a number of cellular factors are required for IAV infection. Among these are host factors regulating the viral polymerase, such as the RNA binding protein DDX17 [24], DDX19 [25] protein kinase C [26], and ANP32A/pp32 [27]. Alternately, antiviral factors, such as NF90 [28] and MXA [29], antagonize RdRP functions. Influenza RdRP activity is typically examined in cell culture infection by co-transfection of luciferase reporters and plasmids encoding PB1, PB2, PA, and NP, in an optimized viral polymerase minigenome reporter assay, and by primer extension for detection of viral RNA species [24].

The poly-ADP ribosyl polymerase 1 (PARP1) protein was identified as an interacting partner of influenza A virus polymerases [20]. Poly-ADP ribosyl polymerases contain an enzymatic active site domain that adds ADP ribosyl polymeric oligonucleotides to target molecules (PARylation). ADP ribosyl polymers affect the activity of a number of proteins, in turn modulating cellular pathways including cell cycle, DNA replication, apoptosis and metabolic cell viability [30]. PARP1 restricts replication of Kaposi’s sarcoma-associate herpesvirus (KSHV) by PARylation of the KSHV immediate-early transactivator RTA, but is blocked by viral processivity factor PF-8 [31]. Other PARP family members, including PARP7, PARP10, and PARP12L, are interferon (IFN)-induced proteins that have antiviral functions by limiting translation [32]. Interestingly, complete CRISPR/Cas9 knockout of PARP1 leads to induction of type I IFN, possibly due to aberrant cellular RNA species [33], and PARP12 activation [34]. Isoforms of PARP13 are antiviral [35], including ZAPS which activates RIG-I [36], while ZAPL specifically disrupts the PB2 and PA proteins of the influenza RdRP complex, but is counter-acted by the viral PB1 protein [37].

Our previous work has suggested that PARP1 is required for IAV replication [24], however, the mechanism of this regulation is poorly understood. We observed that PARP1 has been found to interact with a network of cellular transcriptional regulatory proteins that also modulated IAV infection, including NCL, NPM1, DDX21 [38], HSP90 [39], RBM14 and the DNA-PK/Ku70/Ku86 (XRCC1/5/6) complex [24]. Experiments in cell culture infections showed a >50% reduction in infectious IAV titer released from cells depleted of PARP1 by RNAi [20],[24]. We also showed that PARP1 is specifically required for the activity of the viral RdRP of human H1N1 and avian-derived H5N1 viruses [24], although the mechanism of this requirement was unknown.

However, the PARP1 homozygous null (+/--) mouse exhibits accelerated aging and spontaneous tumorigenesis [40], and human HCT116 colon carcinoma cells that are deficient in both PARP1 alleles by CRISPR/Cas9 knockout exhibited reduced growth rates and increased cellular senescence [33]. These findings highlight the importance of ablating PARP1 by other methods such as those explored in this study, including partial ablation of PARP1 protein by RNAi knockdown targeting PARP1 mRNA [41], and inhibition of PARylation activity with small molecule inhibitors [42]. To understand
how PARP1 modulates influenza virus life cycle, we studied the relationship between PARP1, cellular PARylation, and activity of the IAV RdRP. We found that the dependence of viral polymerase activity on poly-ADP ribosyl polymerase and its enzymatic activity is complex, reflecting the many roles of PARP1 in cellular physiology.

2. Materials and Methods

2.1. Cell cultures, drug treatments, and viruses. Human embryonic kidney (HEK 293T) fibroblasts, human lung adenocarcinoma (A549) cells, and Madin-Darby Canine Kidney (MDCK) cells were cultured at 37ºC in a 5% CO2 atmosphere in DMEM (Corning Inc., Manassas, VA), supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO) and antibiotics (1X penicillin/streptomycin). Cells were treated with pharmacological agents doxorubicin or 4-amino-1,8-naphthalalamide (4-AN), obtained from Sigma-Aldrich (St. Louis, MO), after optimization by measurement of cell viability using CellTiter-Glo assay (Promega, Madison WI). Influenza A/WSN/1933 (H1N1) and A/PR/8/34 (H1N1) viruses were grown as described previously [24],[43]. Attenuated avian-derived influenza A/Viet Nam/1203/2004 (H5N1) HALo virus was generated by reverse genetics 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. As this virus plaques poorly in absence of exogenous trypsin, titers of attenuated, avian-derived H5N1 HALo influenza viruses were determined by limiting-dilution immunofluorescence assay on A549 cells, with NP-staining cells counted in triplicate for titration, averaged from ten-fold dilutions. All experiments with live H5N1 HALo virus were conducted at the Icahn School of Medicine at Mount Sinai, under biosafety regimen described previously [24], with review of protocols under Dual-Use Research of Concern (DURC) framework.

2.2. RNA interference experiments. Small interfering RNA (siRNA) pools containing 1-4 distinct siRNA (Ambion/Life Technologies, Carlsbad, CA; Dharmacon, Lafayette, CO) were used to target and knockdown host factor transcripts, including PARP1, NPM, DDX17, IMPDH2, and Ku70/Ku86 (simultaneously), and compared to non-target (scrambled) siRNA, as previously described [24],[43]. Briefly, siRNA (10-15nM) were transfected into cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) 24-36 hours prior to transfection of viral polymerase reporter cDNA for minigenome experiments in 293T cells, or infection of A549 cells with influenza viruses. The effect of siRNA transfection on cell viability was measured by CellTiter-Glo assay, and target gene knockdown efficiency was validated by quantitative RT-PCR or Western blotting, as described previously [24].

2.3. Minigenome reporter assays. To assay IAV RdRP activity, an optimized polymerase minigenome luciferase reporter assay was used, as described previously [24]. Briefly, in minigenome reporter assays, viral polymerase and NP plasmids (total 375ng), 100ng of a vRNA-promoter reporter encoding firefly luciferase, and 25ng of a constitutive Renilla luciferase internal control plasmid, were co-transfected into cells in 24-well format using Lipofectamine 2000. Optimized plasmid (ng) ratios of 10:2:1:2 (NP:PB1:PB2:PA), or 5:2:1:2 for experiments adding exogenous cDNA of host factors, were determined experimentally to maximize luciferase RLU/ng plasmid. Dual luciferase assay (DLR; Promega) was used to measure polymerase activity. The vRNA reporter encoding firefly luciferase alone (100ng) was transfected into A549 cells for assessment of authentic, infected cell IAV RdRP activity [44], one day prior to infection, and analyzed for luciferase assay after 20 hours post-infection.

2.4. PARylation Assay. Total cellular poly-ADP ribosyl polymerization (PARylation the enzymatic activity of PARP proteins) was analyzed by HT chemiluminescent PARP/ Apoptosis Assay kit (Trevigen, Gaithersburg, MD), was measured from A549 cell extracts, according to the manufacturer’s instructions. HRP chemiluminescence of PARylation of a histone substrate was measured in a BioTek Synergy HT plate reader set on the luciferase channel, with averaged results of gain 80 and gain 100 for each condition compared to standard curve. An unpaired, 2-tailed t-test was used to estimate a statistical significance (p-value).
2.5. Other analyses of IAV infection in cells. Transfection of cDNA encoding host factors using Lipofectamine 2000 was performed as described previously [24]. Immunofluorescence microscopy, primer extension assay, and Western blotting were performed as described previously [24].

3. Results

3.1. PARP1 is required for influenza A virus RNA-dependent RNA polymerase function. Our earlier studies indicated that PARP1 interacts with, and is required for activity of the influenza RNA-dependent RNA polymerase (RdRP) [20],[24]. These experiments showed a reduction in polymerase activity during infection by H1N1 (57%) and avian H5N1 (83%) viruses [24]. Therefore, to more study IAV polymerase genotypes more comprehensively, we examined the requirement for PARP1 for activity of the polymerase for human, swine, and avian-derived influenza strains using an optimized influenza polymerase minigenome reporter assay. We compared the requirement for PARP1 to knockdown of the DNA damage repair complex proteins Ku70/86 that was also found to be required for the influenza viral polymerase [24], and interacts with PARP1 in DNA-damage repair (DDR) proteome network [45]. Similar to Ku70/86, PARP1 was required for optimal activity of polymerases from five different influenza strains (Fig. 1A): seasonal human H1N1 derived from the 1918 pandemic H1N1 (WSN and PR8), the polymerase from the swine triple reassortant (TRIG) A/swine/Texas/4199-2/98 (H3N2) [46], the human pandemic 2009 H1N1 polymerase A/California/04/2007 (pdmH1N1) [44],[47], and avian-derived A/Viet Nam/1203/2004 (H5N1) polymerase from a fatal human case of highly pathogenic avian influenza [48]. The degree of dependence of the influenza polymerases on PARP1 varied among strains (34-85% of polymerase activity, average 58%) but were significant (p<0.05), with the exception of PR8 that did not indicate as significant dependence on PARP1 in this assay (p=0.07). Knockdown of PARP1 by siRNA in this assay leads to an observable decrease in PARP1 protein in the cells, however, depletion is not complete (Fig. 1B). Thus, although not optimal, it is possible that the residual PARP1 provides functions required for limited influenza polymerase activity.

![Fig. 1. PARP1 is required for optimal activity of the influenza A virus RNA-dependent RNA polymerase. (a) Host factors are required for influenza A virus polymerase function. For minigenome reporter assays, cDNA encoding influenza polymerase proteins (PB1, PB2, PA; and NP), a firefly luciferase reporter driven by a virus RdRP-binding site promoter, and a constitutive Renilla luciferase internal reference, were transfected into human HEK 293T cells targeted with siRNA against human PARP1, Ku70 and Ku80/86 (Ku70-86), or scrambled siRNA control (Nontgt). Polymerases from influenza A virus strains included human A/WSN/33 (H1N1) (WSN), human A/PR/8/34 (H1N1) (PR8), A/swine/Texas/4199-2/98 (H3N2) (swTX98), human A/California/04/09 (pdmH1N1) (CA0409), or avian-derived A/Viet Nam/1203/04 (H5N1) (VN1203). Polymerase activity of the negative control was normalized for each strain to 1.0; significance estimated by 2-tailed t-test, with p-values p<0.05 (**) or as indicated (*). (b) Immunoblot showing PARP1 protein depletion with GAPDH protein as internal reference.](image-url)
3.2. PARP1 is involved in synthesis of viral RNA by the RdRP. To better understand the mechanism by which PARP1 regulates influenza RdRP functions, we examined PARP1’s role in synthesis of viral mRNA and vRNA species during infection. Viral RNA species were analyzed by primer extension assay in human 293T cells targeted by siRNA against PARP1, and infected with attenuated H5N1 HALo virus (Fig. 2A). Although the magnitude of differences were not large ($p<0.09$), a trend where PARP1 was required for normal synthesis of viral mRNA ($p=0.002$, one-way ANOVA) and vRNA ($p=0.008$, one-way ANOVA) encoding NP and HA was observed (Fig. 2B). It should be noted that siRNA-mediated knockdown only partly depleted PARP1 protein in the cells (Fig. 2C), and viral cRNA were not measured in this assay. However, in light of the minigenome reporter assay results showing a similar trend (Fig. 1A), these data further suggest that PARP1 is required, to a moderate degree, for both major functions of the influenza RdRP: transcription of viral (+) sense mRNA, and complete replication of (-) sense vRNA through a (+) sense cRNA intermediate. In sum, these experiments provide additional evidence that PARP1 is required for influenza A virus replication and optimal transcription and replication functions of the influenza virus RdRP complex.

![Fig. 2](https://example.com/fig2.jpg)

**Fig. 2.** Knockdown of PARP1 moderately decreases viral mRNA and vRNA syntheses. (a) Human 293T cells were treated with nontarget siRNA (lanes labelled N) or siRNA pool targeting PARP1 (lanes labelled P). Total RNA was harvested for primer extension analysis with specific primers for HA or NP viral mRNA and vRNA synthesis, as indicated, for mock-infection or 12h.p.i. after infection with attenuated, avian-derived influenza virus strain A/Viet Nam/1203/04 (H5N1) HALo (MOI = 1, ref. mBio2011). 5S, rRNA loading control; nsp, nonspecific background band. (b) Viral RNA quantification by densitometry, normalized to 5S ribosomal RNA and background band. Significance of differences was estimated by unpaired, 2-tailed t-test from density histograms ($p<0.09$ for PARP1 siRNA conditions for all viral RNA species) and one-way ANOVA ($p=0.002$ for NP and HA mRNA species, and $p=0.008$ for NP and HA vRNA species). (c) Immunoblot showing PARP1 protein depletion with actin protein as internal reference.

3.3. PARP1 is required for synthesis of NP during infection. To understand how the influenza virus polymerase’s requirement for PARP1 affected expression of viral nucleoprotein (NP), we ablated expression of PARP1 by transfection of human cells with pooled short interfering RNA (siRNA), for
specific gene silencing. When PARP1 was targeted, expression of viral nucleoprotein (NP) during infection of human lung A549 cells with attenuated, avian-derived influenza virus strain A/Viet Nam/1203/04 (H5N1) HAlO was considerably reduced (Fig. 3), to a degree similar to the RNA binding proteins NPM1 and DDX17, that are also known to be required for influenza virus polymerase activity [24]. The ribonucleotide synthesis enzyme inosine monophosphate dehydrogenase 2 (IMPDH2), a cellular factor that interacts with the PARP1 and PARP2 DDR proteome network [45],[49], was also required for influenza NP synthesis.

3.4 Inhibition of PARP enzyme PARylation enhances activity of the influenza polymerase. As poly-ADP ribosyl polymerases encode an enzymatic activity that adds polymeric ADP ribosyl oligonucleotides to target molecules, affecting numerous cellular transcriptional processes, we investigated the role of this enzymatic activity in influenza infection. To assess the requirement of the enzymatic activity of PARP1 in influenza RdRP function, we directly inhibited PARylation using small molecule inhibitor 4-amino-1,8-naphthalamide (4-AN). The majority of cellular PARylation activity is catalyzed by PARP1 (85%-90%) with the remainder mostly by PARP2 [30]; 4-AN inhibits the enzymatic activity of both PARP1 and PARP2, the most abundant active PARP enzymes. We measured PARylation activity of total A549 cell extracts by in vitro PARylation of a histone substrate, essentially measuring the total activity of PARP1, PARP2, and other PARP enzymes. Treatment 4-AN (20uM) effectively reduced total cell PARylation by approximately 90% within two hours of treatment, with a weak recovery (to <20%) after 1 day of treatment (Fig. 4A). Next, total PARylation activity was assessed in cells over the course of influenza A virus life cycle. In A549 cells infected with influenza A/WSN/33
(H1N1) virus (MOI = 1). PARylation activity was relatively stable over the course of infection (Fig. 4B). A mild, transient loss of PARylation activity at early timepoints was not significant, although interesting, as this was also reflected in non-stochastic variation in cellular PARP1 protein abundance (Fig. S1). However, influenza infection itself did not drastically alter cellular PARylation, suggesting that the activity of PARP1, PARP2, and other PARP enzymes is not significantly targeted by viral proteins. This contrasted with treatment with the specific drug inhibitor 4-AN that drastically reduced PARylation activity in infected cells (Fig. 4B) as it does in uninfected cells (Fig. 4A).

3.5. Inhibiting PARylation enhances polymerase activity. As RNAi knockdown experiments showed that PARP1 is required for influenza RdRP function and virus replication, we next sought to examine the influenza polymerase’s requirement for cellular PARylation, using the optimized viral polymerase minigenome reporter assay. For these experiments, 293T cells were used because they exhibit high transfectability for viral cDNA and minigenome plasmids [24]. Interestingly, although PARP1 itself is required for viral polymerase function, treatment of cells with the PARylation inhibitor 4-AN (2-20uM) resulted in increased viral polymerase activity in a linear (R²=0.99013), dose-dependent manner (Fig. 4C). At 20uM 4-AN treatment, polymerase activity was significantly increased (4.2±0.4 fold over DMSO vehicle, p<0.01, 2-tailed t-test). This data suggests that cellular PARylation is directly refractive to the assembly or enzymatic activity of the influenza RdRP, namely, synthesis of viral mRNA, reducing subsequent translation and expression of viral protein.

In human 293T cells pre-treated with 4-AN (25uM) and infected with attenuated, H5N1 HALo virus (MOI = 1), authentic viral polymerase activity was increased 1.8±0.4 fold over DMSO vehicle (p=0.06, 2-tailed t-test), as measured by a firefly luciferase reporter of influenza RdRP activity [24],[44] that is active in infected cells (Fig. 4D). In addition, a corresponding increase in single-step growth titer of this virus in A549 cells pre-treated with 4-AN (10uM) was moderate but significant (2.3±0.03 fold, p=0.04), analyzed by limiting dilution assay (Fig. 4E). However, treatment with 25uM 4-AN in the single-step growth assay led to a mild but not significant increase in virus titer (1.7±0.2 fold, p=0.3). Taken together, the results from the minigenome assay, infected-cell polymerase reporter, and virus growth measurement indicate that pharmacological inhibition of cellular PARylation with 4-AN licensed a significant increase in influenza virus polymerase activity, leading to enhanced growth of influenza A virus in human cells.

A. Uninfected A549 cells

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20uM 4-AN: hours post-treatment

B. Influenza infected A549 cells

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<th>Normalized PARP Activity</th>
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Hours post-infection

C. Influenza minigenome reporter
Fig. 4. Inhibitor of PARP1/2 proteins PARylation activity increases IAV RDRP activity. (a) Assay of cellular PARylation after treatment by 4-amino-1,8-naphthalimide (4-AN) for 20h in A549 cells. (b) Poly-ADP ribosylation (PARP) enzymatic activity was analyzed in protein lysates from A549 cells treated with DMSO vehicle, 20μM 4-AN, or 1μM doxorubicin (DOXO), and infected 20h with IAV (A/PR/8/34 H1N1, MOI = 1); lysates were analyzed by PARylation assay. (c) HEK 293T cells were transfected with NP and polymerase cDNA plasmids in IAV minigenome reporter assay and untreated, treated with vehicle (DMSO), or increasing doses of PARP inhibitor drug (4-AN). (d) HEK 293T cells were transfected with RdRP firefly luciferase reporter construct one day prior to vehicle (DMSO) or treatment with 25μM 4-AN, and infected with attenuated, H5N1 HALo virus (MOI = 1). Infected cell viral polymerase activity was analyzed after 20h by luciferase assay; two-tailed t-test (*) p=0.06. (e) Single-step growth of H5N1 HALo virus (MOI = 1) in A549 cells pre-treated with 4-AN or vehicle (DMSO) as indicated. Infectious titer (IU) released 1d.p.i. measured by limiting dilution assay for NP-positive cells. Two-tailed t-test for significance in comparison to vehicle: **, p<0.05; ns, not significant.

3.6. Role of PARG. As a mechanism of cellular homeostasis and transcriptional control [30], the endogenous enzyme PARG removes poly-ADP ribosyl moieties from cellular macromolecules. To examine the role of PARylation in influenza virus polymerase function, we transfected 293T cells with cDNA to overexpress host factors, along with the firefly luciferase reporter for authentic influenza RdRP activity, and infected cells with attenuated, H5N1 HALo virus. Overexpression of NPM, a known positive regulator of influenza polymerase led to a mild but significant (1.4-fold, p<0.05) increase in activity of the viral polymerase activity during infection (Fig. 5). A similar increase in polymerase activity for PARG overexpression was observed at a high multiplicity of infection (MOI = 1 and MOI = 5), but this was only marginally significant (p<0.1), likely reflecting the incomplete efficiency of cDNA transfection. However, IMPDH2, that interacts with and supplies PARP1 and PARP2 with NAD+ substrates for enzymatic reactions [49], significantly enhances polymerase activity during infection (Fig. 5). These results illustrate the complexity of the IAV interaction with cellular PARylation pathways on the infected cell.
Fig. 5. Expression of PARG, the endogenous enzyme counteracting PARP1/2 proteins, increases RdRP activity in influenza-infected cells. HEK 293T cells were transfected with an RdRP reporter plasmid, and empty vector or cDNA expressing NPM, PARP1, PARG, or IMPDH2. After 24h, cells were infected with with attenuated, H5N1 HALo virus at MOI indicated, and viral polymerase activity was analyzed 20h.p.i. by luciferase reporter assay.

3.7. Localization of PARP1 and NP in the nucleus. The influenza polymerase complex and NP localize to the cell nucleus where it synthesizes viral RNA species. To better understand the interaction of PARP1 protein with the influenza polymerase and NP, we analyzed the subcellular localization of PARP1 in influenza A virus-infected cells. In resting A549 cells, PARP1 is a nuclear protein as it contains a N-terminal NLS [50]. Nuclear localization of PARP1 is maintained in cells infected with IAV even by 12h.p.i., when viral NP has translocated from nucleus to cytoplasm (Fig. 6), in contrast to other host factors such as DDX17 [24] and NF90 (data not shown).

Fig. 6. PARP1 localizes to the nucleus in cells infected with influenza A virus. Human HeLa cells were infected with attenuated, avian-derived influenza virus strain A/Viet Nam/1203/04 (H5N1) HAlo (MOI = 0.5). Infected cell cultures were fixed for immunofluorescence 3h.p.i. and 12h.p.i. with anti-PARP (red) and anti-NP (green), and DAPI chromatin counterstaining indicating cell nuclei (blue).

However, although PARP1 localized to the nucleus throughout infection, it only co-localized with the viral nucleoprotein early in infection in cells expressing abundant NP distributed throughout the nucleoplasm. This may correspond to an earlier phase of virus life cycle, where NP is involved primarily in viral mRNA synthesis. NP that has bound viral RNA has been reported to localize to the nuclear periphery prior to export to the cytoplasm [51]. The NP accumulated at the nuclear periphery by 3h.pi. did not significantly overlap with PARP1 (Fig. 6), suggesting that the phenotypes observed in knockdown of PARP1, and inhibition of PARylation, may result from transient interactions between PARP1 and viral proteins, and indirect effects on RdRP functions.
4. Discussion.

4.1 PARP1 is an antiviral protein and facilitator of IAV infection. Poly-ADP ribosylation (PARylation) is central to cellular viability, gene expression, and metabolism, and is mediated by the (PARP) proteins. Small-molecule inhibition of PARP leads to disruption of DNA-damage repair (DDR) pathways and has been explored as potential anti-cancer chemotherapy [33],[52]. Because of its role in DDR, and potent ability to target chromatin modifiers, PARP1 was explored in the regulation of viruses [32] with a DNA genome stage in their life cycle. PARP1 functions as an antiviral protein in a pre-integration step of avian retrovirus infection [53], hepatitis B virus transcription [42], and lytic replication of gammaherpesviruses EBV [54] and KSHV [31]. Unlike PARP1’s antiviral activity observed in DNA viruses, we showed that PARP1 is a co-factor in the activity of the influenza A virus RdRP. PARP1 was required for optimal IAV polymerase activity, mRNA and vRNA synthesis, and viral nucleoprotein synthesis. These results are consistent with previous studies showing that PARP1 knockdown limits viral polymerase activity in a minigenome reporter assay [24]. However, we found that PARP1’s role in influenza infection appears to be more complex: inhibition of the PARylation enzymatic activity of PARP1 and PARP2 leads to increased IAV polymerase activity and greater replication of IAV in human cells. We also found that the endogenous protein PARG, which counter-acts cellular PARylation, had a similar effect to enhance IAV RdRP activity. The mechanisms by which PARP1 and PARylation function in an antiviral fashion against retroviruses, HBV, EBV, and KSHV are virus-specific; in general, PARP1 appears to act as a transcriptional repressor of viral gene expression. While IAV has no DNA stage in its replication, its RdRP is highly dependent on cellular RNA polymerase II and co-factors [17-19]. Thus, akin to the transcriptional repression of DNA viruses by PARP1, it is possible that PARP1 is required for function of other host cell factors that in turn facilitate IAV RdRP functions. Meanwhile PARylation of PB2 and PA may inhibit assembly of RdRP [37], and also activate antiviral proteins in a manner similar to other PARP family members [32],[33],[36]. Indeed the IAV PB1 protein antagonizes one of these PARP proteins, PARP13/ZAP [37]. Notably, however, PARP1 may be cleaved and deactivated during apoptosis in IAV-infected cells [55]. Thus, PARP1’s multitude of cellular functions could both facilitate, and inhibit, the IAV polymerase and other steps in the virus life cycle.

4.2. PARP1 is not a strain-specific host factor of IAV. The influenza polymerase minigenome reporter assay suggested that PARP1 is required for optimal function of avian, swine, and human RdRP (Fig. 1A). Notably, the dependence of polymerase activity on PARP1 for attenuated, avian-derived H5N1 HALo virus match the reduced expression of NP (Fig. 3A) and reduction in viral titer (53% reduced) in infected cell cultures [24]. This analysis suggests that polymerase activity is a useful, biosafe proxy assessment for replicative capacity and host factor dependence of highly pathogenic avian influenza virus (HPAIV) strains. Moreover, in previous studies, we found that PARP1 was also required for polymerase function for avianized revertant PB2/K627E, suggesting that the mammalian adaptive mutations of the H5N1 HPAIV polymerase are not PARP1-dependent [24]; rather, RNA binding and signaling factors such as DDX17 [24], DDX19, protein kinase C, and ANP32A/pp32 [24-27] mediate this virus-host relationship. However, the precise mechanisms of PARP1’s interaction with IAV in vivo may vary with the many hosts and virus strains extant in nature.

4.3. Model of PARP1 role in influenza infection. Given the interaction of PARP1 with the viral RNP [20], the requirements PARP1 for RdRP function (Fig. 1) and viral RNA synthesis (Fig. 2), and an inhibitory effect of PARylation itself on viral polymerase function (Fig. 4), a transient intranuclear interaction of PARP1 with viral polymerase and/or NP can be proposed as a mechanism. We observed that PARP1 remains in the nucleus during IAV infection (Fig. 6A). Preliminary experiments suggest that PARP1 was pulled down by NP in the presence of viral RNA, but associated most strongly with RdRP complexes containing NP but treated with RNase to remove viral RNA (Fig. 52). We also observed that NPM, a binding partner of another polymerase interactor NCL [56], could effectively displace PARP1 from interaction with NP (Fig. 52). Consequently, our data suggests a model in which PARP1 transiently interacts with NP to facilitate polymerase function, but akin to the
displacement of antiviral PARP13/ZAP by antagonistic action of PB1 [37], a prolonged interaction of PARP1 through PARylation inhibits RdRP functions. Consistent with this model, overexpression of PARP1 cDNA weakly inhibited influenza polymerase activity (~45%) at a low MOI infection (MOI = 0.2, \( p<0.1 \)) (Fig. 5). PARP1 overexpression also appeared to reduce polymerase activity in the minigenome assay, an effect could be in part titrated away by increased NP (Fig. S3), but results were not statistically significant (\( p<0.1 \)). This data suggests that cellular PARylation is directly refractive to the assembly or enzymatic activity of the influenza RdRP, namely, synthesis of viral mRNA, reducing subsequent translation and expression of viral protein. A study of PARP1 with influenza proteins using bimolecular fluorescent complementation [57] may shed additional light on the molecular mechanisms of this dynamic virus-host interaction.

5. Summary.
Taken together, our results have demonstrated the importance of PARP1 in influenza RdRP function and IAV replication. Thus, PARP1 and other proteins in the virus-host interaction network are attractive targets for deeper study of host factors that regulate influenza virus infection and pathogenesis, and development of new virus-host targeted molecules as antiviral therapy against severe influenza infection.

Supplementary Materials: The following are available online at www.mdpi.com/link
Figure S1: Expression of PARP1 protein in IAV H1N1-infected cells.
Figure S2: PARP1 associates with influenza A virus NP in an RNA-independent manner.
Figure S3: Exogenous PARP1 expression inhibits influenza polymerase activity.

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Appendix A. Supplemental Data

A.

Fig. S1. Expression of PARP1 protein in IAV H1N1-infected cells. (a) Human 293T cells, and cells infected with IAV (WSN, MOI = 2). (b) Human lung A549 cells infected with IAV (PR8, MOI = 0.5). In both, equivalent protein lysates were analyzed by Western blot for PARP1, viral NP, and where shown, GAPDH control, at indicated timepoints.

B.

Fig. S2. PARP1 associates with influenza A virus NP in an RNA-independent manner. (a) and (b) FLAG-tagged NP, vRNA, and polymerase constructs (vPOL: PB1, PB2, PA) were transfected into 293T cells and immunoprecipitated with anti-FLAG monoclonal (M2) antibody agarose beads (Sigma-Aldrich). Lysates were treated with RNase as indicated, and Western blots probed for PARP1 and NP. (c) HA-tagged NP, vRNA, and FLAG-tagged NPM were transfected into 293T cells and lysates harvested for immunoprecipitation with anti-HA antibody agarose beads (Sigma-Aldrich) and probing for PARP1 and NP.
Fig. S3. Exogenous PARP1 expression inhibits influenza polymerase activity. PARP1, PARG, or vector (pC) cDNA were co-transfected with H5N1 influenza polymerase plasmids, NP, and minigenome reporter in 293T cells. IAV RdRP plasmid ratios were NP:PB1:PB2:PA of 2:2:1:2 (low NP) or 5:2:1:2 (high NP), and PARP and PARG were expressed as 100ng or 200ng of plasmid (concentration triangles). Statistical t-test analyses indicated weak (p<0.1) inhibition of polymerase by 100ng or 200ng PARP1 plasmid only in the low NP condition; all other pairwise p>0.1.

References:


