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PQBP1 is a Proximal Sensor of the cGAS-dependent Innate Response to HIV-1

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Summary

Dendritic cells (DCs) play a critical role in the immune response to viral infection through the facilitation of cell intrinsic antiviral activity and the activation of adaptive immunity. HIV-1 infection of DCs triggers an IRF3-dependent innate immune response, which requires the activity of cyclic GAMP synthase (cGAS). We report the results of a targeted RNAi screen utilizing primary human monocyte-derived DCs (MDDCs) to identify immune regulators that directly interface with HIV-1-encoded features to initiate this innate response. Polyglutamine binding

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Author Contributions

S.M.Y., A.G.-S., R.K. and S.K.C. designed experiments. S.M.Y., M.S., J.S., S.S., R.E.A., P.D.D.J., P.A.R. and E.C. performed the experiments. S.M.Y., M.S., and S.K.C. wrote the manuscript. S.M.Y. and K.C.O. generated the viruses. C.R. performed the LC-MS. D.G. and V.d.P. coordinated and collected patient samples.

protein 1 (PQBP1) emerged as a strong candidate through this analysis. We found that PQBP1 directly binds to reverse-transcribed HIV-1 DNA and interacts with cGAS to initiate an IRF3-dependent innate response. MDDCs derived from Renpenning Syndrome patients, who harbor mutations in the PQBP1 locus, possess a severely attenuated innate immune response to HIV-1 challenge, underscoring the role of PQBP1 as a proximal innate sensor of a HIV-1 infection.

Introduction

Innate immune responses that trigger type-I interferon (IFN) secretion have been implicated in HIV-1 transmission and pathogenesis (Gringhuis et al., 2010; Iwasaki, 2012; Meier et al., 2009). HIV-1 has evolved countermeasures to escape the activities of several IFN-stimulated genes (ISGs), and those mechanisms not disabled by the virus define both cell type and species tropisms (Kirchhoff, 2010). Recent data suggest that one or more intrinsic signaling pathways sense invariant features encoded by HIV-1 and initiate innate immune responses, including IFN secretion (Gao et al., 2013; Jakobsen et al., 2013; Manel et al., 2010; Rasaiyaah et al., 2013). This response is proximally mediated by recognition of specific viral components in infected cells by pattern recognition receptors (PRR), resulting in the activation of transcription factors that participate in ISG expression and IFN synthesis, such as IRF3 (Luban, 2012; Paludan and Bowie, 2013). Dendritic cells (DCs) play a critical role in the immune response to viral infection through the facilitation of cell intrinsic antiviral activity and the activation of adaptive immunity. Human DCs are resistant to HIV-1 infection due to expression of SAMHD1, a phosphohydrolase that functions to deplete cellular nucleotide pools (Berger et al., 2011; Goldstone et al., 2011; Lahouassa et al., 2012). SAMHD1 restriction can be overcome in DCs by the introduction of HIV-2- or SIV-encoded Vpx, which targets SAMHD1 for ubiquitin-mediated degradation (Hrecka et al., 2011; Laguette et al., 2011, 2012). Littman and colleagues (Manel et al., 2010) have demonstrated that co-transduction of DCs with HIV-1 and SIV VLP-Vpx not only enables productive infection, but results in the activation of DCs and in IRF3-dependent production of IFN.

Furthermore, studies have shown that this IRF3-dependent innate immune response requires the activity of cyclic GAMP synthase (cGAS) (Gao et al., 2013; Sun et al., 2013; Wu et al., 2013). cGAS has been identified as a critical mediator of the innate response to cytosolic DNA through synthesis of a cyclic guanosine monophosphate–adenosine monophosphate isomer (cGAMP) (Sun et al., 2013). While cGAS was shown to regulate anti-viral responses to HIV-1 and other retroviruses, its direct association with a retroviral-encoded PAMP has not been shown (Gao et al., 2013; Li et al., 2013). Additionally, the apparent low affinity of cGAS for DNA and the promiscuous response to a broad set of DNA ligands suggests that co-receptors may function to enhance affinity for non-self DNA during cGAS dependent innate signaling (Kranzusch and Vance, 2013). Results from this study indicate that the PQBP1 protein functions as a specific co-receptor for reverse-transcribed HIV-1 DNA and complexes with cGAS to initiate an immune response to retroviral infection.

Results

As previously observed, when MDDCs were co-infected with SIV VLP-Vpx and VSV-G-HIV-1 (hereafter HIV/Vpx), we detected robust expression of IRF3-dependent target genes *ISG54* and *IP-10*, peaking at 16 hours post infection (Figure 1A; S1A). Infection with either HIV-1 or Vpx alone did not result in an appreciable increase of these transcripts (Figure 1A). Expression of *ISG54* in response to HIV/Vpx was accompanied by phosphorylation-dependent activation of IRF3 and its upstream regulator, IKKε (Figure 1B). In addition, *ISG54* induction by HIV/Vpx was IRF3-dependent, as demonstrated by IRF3 silencing; depletion of IRF3, but not p65, attenuated early induction of *ISG54* (Figure 1C and S1B). Consistent with a previous report (Gao et al., 2013), we found that this signaling response required reverse transcription (RT) of HIV-1 RNA but not viral DNA integration treatment with Nevirapine (NVP), an RT inhibitor, reduced *ISG54* expression and phosphorylation of IRF3, but treatment with the integrase inhibitor Raltegravir (Ral) did not (Figure 1A, B). Both treatments were effective as they potently inhibited viral replication, but they did not affect cell viability or innate responses to non-HIV-1 stimuli (Figure 1A, right and S1C). These data confirm that HIV-1 elicits an IRF3-dependent innate immune response in MDDCs through a process that requires viral reverse transcription.

To identify potential co-sensors that regulate this response, we performed a targeted RNAi screen in primary MDDCs. Because our data suggest that a viral DNA-containing species is likely responsible for the initiation of signaling, we reasoned that putative receptors would possess annotated nucleic acid binding activity and be preferentially expressed in myeloid cells. Based on these criteria, we assembled a sub-genomic RNAi library of 351 putative sensors (see Experimental Procedures; SI Table 1). Each candidate gene was individually depleted by transfecting a pool of 4 siRNAs per gene into MDDCs. After 48 hr, the cells were challenged with HIV/Vpx, and induction of innate signaling was measured by monitoring *ISG54* expression (Figure 1D; SI Table 1). We identified 26 genes that when depleted inhibited *ISG54* mRNA induction greater than 3 standard deviations below the scrambled controls (Figure 1D; SI Table 1 $p<0.0001$).

To further validate these results, 4 siRNAs for each putative sensor were tested independently. One of the strongest candidates that reproducibly and potently inhibited *ISG54* induction with at least two sequence-independent siRNAs targeted the gene *PQBP1* (Figure 2A; S2A-C; SI Table 1). PQBP1 is a polyglutamine-binding protein and mutations in the PQBP1 locus are associated with neurodegenerative pathologies, including Renpenning and other syndromes associated with X-linked mental disorders (XLMR; Germanaud et al., 2011; Kalscheuer et al., 2003; Lenski et al., 2004; Marubuchi et al., 2005). RNAi depletion of a putative nucleic acid sensor *LRRFIP1*, a gene that did not meet the 3 standard deviation threshold in the original screen, did not reduce *ISG54* induction when compared to the scrambled controls (Figure 2A), and it was thus used as a negative control in subsequent experiments.

The PQBP1-dependent induction of innate immune signaling after HIV-1 infection was not unique to MDDCs. Knockdown of PQBP1 in the monocyte-like cell line THP-1 also resulted in reduced *ISG54* induction after HIV-1 infection (S2D). We investigated the

possibility that PQBP1 was reducing ISG induction through interference with HIV-1 replication. However, HIV-1 infection was unimpeded in the absence of PQBP1, as compared to cells harboring a control shRNA (S2E). Evaluation of IKKε and IRF3 phosphorylation after HIV/Vpx challenge in PMA-differentiated THP-1 cells (hereafter THP-1) depleted of PQBP1 indicated that PQBP1 acts as a positive regulator of *ISG54* and *IP-10* induction upstream of IKKε/IRF3 phosphorylation (Figure 2B; S2C). To exclude potential off-target siRNA effects, THP-1 lines that stably expressed either wild type PQBP1 or siRNA-resistant PQBP1 cDNAs were generated, transfected with a siRNA targeting PQBP1, and challenged with HIV/Vpx. Expression of the siRNA-resistant PQBP1 (siR) restored *ISG54* and *IP10* expression in response to infection (Figure 2C and S2F). However, those cells harboring wild type PQBP1 displayed attenuated induction of *ISG54* (Figure 2C and S2F). Additionally, we employed a CRISPR-mediated genome editing approach to target *PQBP1* (cPQBP1; Supplemental Information). We were unable to generate lines harboring genetically null mutations for PQBP1, but we were successful in recovering several hypomorphic mutations in the PQBP1 locus that resulted in significantly diminished levels of PQBP1 expression. Similar to transient silencing of *PQBP1* in DCs or THP-1s, we found genome editing of *PQBP1* in THP-1 cells abrogated *ISG54* induction after HIV/Vpx infection, but not after HT-DNA stimulation (Figure 2D). ISG54 induction was rescued after adding a stable, ectopically expressed PQBP1 (Figure 2E).

We subsequently sought to determine if PQBP1 directly associated with HIV-1-encoded PAMPs. To this end, MDDCs were infected with HIV/Vpx for 3 or 16 hr, formaldehyde cross-linked, lysed and subjected to immunoprecipitations (IP) of endogenous PQBP1 or ZC3H3, another nucleic acid binding protein that was identified in our screen (SI Table 1). Specific pulldown of PQBP1 or ZC3H3 proteins was confirmed by western blot (Figure 3A, right), and HIV-1-derived nucleic acids associating with the normal serum, ZC3H3 and PQBP1 IPs were quantitated by qRT-PCR. We found that a significant and specific enrichment of HIV-1-derived nucleic acids was associated with the PQBP1 IP, but not with the ZC3H3 or control IP (normal IgG) (Figure 3A, S3A, B). To further confirm the selectivity of binding, a recombinant GST-tagged PQBP1 protein was added during the IP to displace endogenous PQBP1. Addition of exogenous PQBP1 reduced the level of HIV-1 nucleic acids in the immunoprecipitants (S3C). To understand whether PQBP1 association with HIV-1 DNA required the innate regulator cGAS, cells that stably expressed an shRNA vector targeting either luciferase or cGAS were infected for 6 hr. They were then cross-linked, and PQBP1 was immunoprecipitated from the lysate, as described above. Despite the absence of cGAS, PQBP1 was still able to associate with HIV-1 nucleic acids (Figure 3B). Next, to identify the species of HIV-1-encoded nucleic acid that was involved in this activity, the IPs were treated with either DNase or RNase A/H prior to qRT-PCR or qPCR analysis, respectively (Figure 3C). HIV-1 nucleic acid products were detected only in the samples treated with RNase A/H (Figure 3C). Additionally, PQBP1 binding to the HIV-1 nucleic acids was significantly reduced in the presence of the RT inhibitor NVP but was not affected by the integrase inhibitor Ral (Figure 3D). These data suggest that an HIV-1 DNA intermediate product associates with PQBP1.

To characterize the activity of HIV-1 DNA in the cytosol of infected cells, MDDCs were infected with HIV/Vpx for 6 hr and then fractionated into soluble non-chromatin (SNC) and chromatin (C) fractions (S3F). At this time point after infection, we found that HIV-1-encoded nucleic acids exist primarily in the SNC (S3F). Consistently, electroporation of RNAse A/H resistant nucleic acids isolated from either the SNC or C fractions of HIV-1 infected MDDCs into naïve THP-1 cells harboring a stable shRNA towards PQBP1 (Figure 3E) revealed that DNA, or possibly an RNAseH-resistant RNA-DNA hybrid species, present in the soluble fraction of infected cells is immunogenic, and the immune response to this ligand is dependent upon PQBP1 expression in uninfected target cells (Figure 3F).

We then tested whether the PQBP1-associated HIV-1 DNA product was itself immunogenic. DNA immunoprecipitated from the infected MDDC lysate by the PQBP1 antibody or the control normal IgG was electroporated into THP-1 in the presence or absence of PQBP1. We found that only PQBP1-associated nucleic acids led to the enhancement of *ISG54* expression and that this innate response was sharply attenuated in the absence of PQBP1 in the target cells (Figure 3G). This innate induction was specific to a reverse transcribed DNA product, since the PQBP1-IP product from cells treated with NVP during infection failed to induce *ISG54* expression in target cells (Figure 3H). Taken together, these results suggest that PQBP1 directly associates with immunogenic HIV-1 DNA.

We next evaluated the breadth of nucleic acid stimuli that are governed by a PQBP1-dependent innate response. In contrast to HIV-1, the innate immune response to an RNA virus, Sendai virus, was not dependent upon PQBP1 in THP-1 (S4A). Further, the dependence on PQBP1 for HIV-1 DNA recognition did not extend to all cytoplasmic DNA, as response to B-DNA (poly(dA:dT)) and Herring testis (HT)-DNA was not reliant on PQBP1 in MDDCs or THP-1 cells (Figure 4A, 4B; S4B, S4C). Consistent with these results, PQBP1 depletion did not dampen the immune response to the double-stranded DNA virus MHV-68 (Figure 4C), and MHV-68 DNA did not associate with PQBP1 after infection, despite a greater abundance of MHV-68 in the lysate (Figure 4D, S4D). Feline Immunodeficiency Virus (FIV) and Equine Infectious Anemia Virus (EIAV) are both retroviruses that are divergent from HIV-1 but maintain the requirement of a reverse transcription step for viral propagation. Both VSV-G pseudotyped retroviruses induced an innate response in THP-1. However, in the absence of the PQBP1 protein, the response was sharply reduced (Figure 4E-F, S4E). Taken together, this data indicates that PQBP1 specifically recognizes a retroviral DNA product.

Similar to previous reports (Gao et al., 2013), we found that depletion of cGAS in MDDCs resulted in decreased *ISG54* induction by both HT-DNA and HIV-1 (Figure 5A; S5A). Since PQBP1 is dispensable for the innate response to HT-DNA and MHV-68 (Figure 4A-C), both of which have been reported to be sensed by cGAS (Schoggins et al., 2014; Sun et al., 2013), our data suggest that PQBP1 regulates the response to a more restricted set of cGAS-dependent DNA ligands. However, because both PQBP1 and cGAS were required for response to a RT DNA PAMP produced by HIV-1 infection (Gao et al., 2013), we sought to determine whether PQBP1 and cGAS modulate the response to HIV-1 through coincident or parallel pathways. We evaluated whether PQBP1-depleted cells could respond to

exogenously added synthetic cGAMP. When cGAMP molecules were incubated with perfringolysin O (PFO)-treated THP-1 cells harboring PQBP1 shRNA, as previously described (Wu et al., 2013), we did not observe a reduction of cGAMP-induced p-IRF3 in comparison to control cells (Figure 5B), but there was a reduction of cGAMP-mediated activation in the absence of STING (Figure 5C). This result suggested that PQBP1 activity is not required for cGAMP-mediated activation of IRF3. We next evaluated the impact of PQBP1 depletion on the synthesis of cGAMP after HIV-1 challenge. Both qRT-PCR analysis and western blot analysis confirmed that depletion of PQBP1 does not reduce cGAS expression (S5B). MDDCs or THP-1 were transfected with siPQBP1 and infected with HIV/Vpx. cGAMP levels in the infected cell lysates were indirectly measured through incubation of PFO-treated THP-1 cells with those lysates, and phosphorylation of IRF3 was assessed (Figure 5D). cGAMP levels were also directly quantified by LC-MS (Figure 5E, F; S5C-E). In both MDDCs and THP-1, the loss of PQBP1, even in the presence of functional cGAS, resulted in a significant reduction of cGAMP production upon HIV/Vpx infection (Figure 5D, E). As cGAS is the only enzyme known to generate cGAMP in mammalian cells, we conclude that PQBP1 is required for activation of cGAS during HIV-1 infection of myeloid cells.

Consistent with other studies (Waragai et al., 1999; Kunde et al., 2011), immunofluorescent staining of endogenous PQBP1 revealed that it was localized to both the cytosol and nucleus of MDDCs (S3E). To determine if PQBP1, cGAS and STING are in similar cellular compartments during HIV-1 infection, we performed a subcellular fractionation and found that PQBP1 co-fractionates with cGAS and STING (Figure 6A). To determine if PQBP1 physically interacts with cGAS, we performed an IP of THP-1 cells stably expressing V5-PQBP1, immunoblotted for endogenous cGAS, and found that PQBP1 physically associated with cGAS in the absence of immune stimulation (Figure 6B). Next, we expressed eYFP-tagged PQBP1, V5-tagged cGAS and FLAG-tagged STING in HEK293T, which lack both endogenous cGAS and STING, and immunoprecipitated PQBP1. As expected, cGAS co-immunoprecipitated with PQBP1; in contrast, when PQBP1 and STING were expressed together in the absence of cGAS, we observed no interaction between the two proteins (Figure 6C). However, when PQBP1, cGAS and STING were expressed simultaneously, STING was able to associate with PQBP1, indicating that PQBP1 is able to form a complex with STING bridged by cGAS (Figure 6C). To determine the domain necessary for PQBP1-cGAS interaction, two disease-associated mutations were generated: a truncation mutation that shortens the protein at the C-terminus without disrupting the WW domain (delC), and an amino acid substitution that disrupts the WW domain (WW) (Lenski et al., 2004) (Figure 6D). Immunoprecipitation experiments revealed that PQBP1 required an intact WW domain (WW) but not a complete C-terminus (delC), for binding cGAS (Figure 6D). The PQBP1 WW domain has previously been shown to be required for interactions with other proteins (Ikeuchi et al., 2013; Salah et al., 2012).

Next, we sought to determine which domain(s) of PQBP1 were necessary for DNA binding. To this end, we determined that recombinant PQBP1 (rPQBP1) specifically associated with HIV-1 DNA, as there was an enrichment of reverse transcribed HIV-1 DNA but not a HIV-1-encoding plasmid associated with rPQBP1 (Figure 7A). Then, GST-tagged recombinant wild type, C-terminal truncation (GST1-176) or WW domain mutant (GST-

WW) proteins were generated, and each was incubated with non-chromatin DNA from cells infected with HIV/Vpx. A PQBP1 antibody was used to immunoprecipitate the recombinant proteins, and the amount of bound HIV-1 DNA was assessed using qPCR. Whereas wild type and GST-WW bound HIV-1 DNA, GST1-176 associated with significantly less DNA (Figure 7B). Through fractionation, we determined that the WW domain and C-terminal truncation mutations did not affect the localization of PQBP1 (Figure 7C). We then confirmed these *in vitro* binding results using an *in vivo* nucleic acid binding assay. PQBP1-depleted cPQBP1 cells were stably reconstituted with wild type, delC-PQBP1 or WW-PQBP1, and then the cells were infected with HIV/Vpx for 6 hr, formaldehyde cross-linked and immunoprecipitated for PQBP1. Consistent with the *in vitro* studies, wild type and WW-PQBP1 associated with HIV-1 DNA, but delC-PQBP1 did not (Figure 7D). Evaluation of ISG induction in these reconstituted cells following HIV/Vpx infection indicated that both cGAS and DNA binding domains were necessary for an immune response to viral challenge (Figure 7E).

Finally, we evaluated the innate response to HIV-1 infection in MDDCs derived from two Renpenning syndrome patients. The two male patients harbor a genetic mutation in the X-linked PQBP1 locus that results in a C-terminal truncation of amino acids 153-265 in the protein. MDDCs derived from the patients and healthy donors were challenged with HIV/Vpx for 8 hr after which *ISG54* expression was measured (Figure 7F). Although paired healthy and Renpenning patient cells were infected to similar levels (S6A), MDDCs from both Renpenning patients displayed severely impaired induction of *ISG54* in comparison to infected healthy donors (Figure 7F).

Discussion

In this study, we find that the host protein PQBP1 directly binds to an HIV-1 DNA PAMP generated after early infection and initiates innate signaling through cGAS. Our results also indicate that the HIV-1-encoded PAMP is likely based on a unique and discernable secondary structure, which is only generated during reverse transcription in the infected cell. Specifically, while PQBP1 binds reverse transcribed HIV-1 DNA from infected cells, it is unable to associate with a HIV-1 encoding plasmid DNA, *in vitro* reverse transcribed DNA (data not shown), or other DNA viruses (Figure 7A).

Interestingly, we observed that PQBP1 is not required for cGAS-dependent responses to all cytosolic DNAs but only those derived from retroviral infection. These results suggest that PQBP1 and cGAS form a pattern recognition receptor complex that specifically recognizes and responds to retroviral PAMP(s). While cGAS may serve as a common catalytic module for a variety of cytosolic DNA ligands, its specific association with PQBP1 enables cGAS to sense HIV-1 encoded DNA ligands. Further studies are required to understand how PQBP1 modulates the activation of cGAS in response to HIV DNA.

The reported affinity of cGAS for DNA is poor (k_D~20 μM) and recognition is promiscuous (Civril et al., 2013; Kranzusch et al., 2013; Zhang et al., 2014), inconsistent with its ability to specifically respond to small quantities of microbial PAMPs and/or self DAMPs. It is currently unclear if cGAS requires co-sensors analogous to PQBP1 to detect non-retroviral

cytoplasmic DNA, including HT-DNA. However, based on our results, we propose a model in which cGAS, by virtue of its association with specific co-receptors such as PQBP1, increases its ability to recognize distinct molecular patterns encoded by pathogen-associated DNA. Similarly, LGP2 has been proposed to enhance the sensing ability of the cytoplasmic RNA sensor MDA5 (Bruns et al., 2014; Satoh et al., 2010). Therefore this model may apply to multiple innate immune sensors.

The experimental models in this report have relied on the introduction of Vpx, which alleviates the block of early reverse transcription at the minus strand strong stop in highly restrictive cells such as DCs (Hrecka et al., 2011; Laguette et al., 2011). Previous work has indicated that abortive HIV-1 infection results in the exposure of reverse transcribed HIV-1 DNA intermediates to trigger cGAS-dependent IRF3 activation (Lahaye et al., 2013; Rasaiyaah et al., 2013). We hypothesize that circumventing SAMHD1 restriction by Vpx increases the concentration of both productive and abortive (late) RT intermediate products during infection of myeloid cells, with the latter propagating a potent and detectable innate response in these cells. Although HIV-1 infection in the absence of Vpx does not induce a significant innate response in myeloid cells *in vitro*, immune induction during mucosal and acute infection has been observed in humans and non-human primate models (Abel et al., 2005; Katsikis et al., 2011). Based on these observations, we propose that rare abortive infection events are sufficient to trigger an innate immune response *in vivo*, which is likely amplified during the course of natural infection. However, in the absence of Vpx, these occurrences cannot be robustly detected on a cell population level using current *ex vivo* cell culture model systems.

Finally, we find that PQBP1 mutations associated with Renpenning syndrome severely attenuate the innate immune response to HIV-1 infection in patient-derived myeloid cells. Remarkably, each disease-associated PQBP1 mutation resulted in a corresponding defect in the immune response to retroviruses. Although a potential link between the observed innate immune defect and the neurological impairments in Renpenning patients remains uncertain, these data suggest that there may be common features in the signaling architecture of the innate immune response and neural development.

In summary, our results present a molecular understanding of how HIV-1 and other retroviruses are uniquely and specifically recognized to trigger cell-autonomous antiviral responses. Further studies are warranted to better understand the specific molecular characteristics of the HIV-1 DNA intermediates that enable recognition by the PQBP1/cGAS axis and to determine how this recognition event contributes to an antiviral and adaptive immune response to HIV-1. The identification of PQBP1 as an immune regulator required for sensing of retroviral DNA provides an opportunity for the therapeutic development of pharmacological agonists that may improve the efficacies of HIV vaccines and prophylactics.

Experimental Procedures

Cell culture and viral infection

The study was approved by the National Institutes of Health (NIH) through our Institute Biosafety Committee (IBC). Primary monocyte-derived dendritic cells (MDDCs) were subjected to siRNA transfection and two days later they were infected with VSV-G pseudotyped HIV-1 virus harboring a firefly luciferase reporter and VLP-Vpx, as previously described (Manel et al., 2010). Six to 16 hours post infection, RNA was isolated from the cells and subjected to qRT-PCR analysis. For the viral replication assay, the infected MDDCs were harvested 48 to 72 hr post-infection, and assayed for luciferase activity from the integrated provirus. For mutation analysis, PBMCs from both healthy donors and two male Renpenning patients (mutation c.459_462delAGAG resulting in type p.R153fs193X) were analyzed similar to above at the Paul-Ehrlich-Institut. Biological replicates within each experiment represent independent infection and/or treatments. Technical replicates represent qPCR replicates on each biological sample. Replicate experiments were performed with MDDCs derived from different donors. The human monocyte-like THP-1 cell line was differentiated by treating with 5 ng/ml PMA (Phorbol myristate acetate) for 2 days, followed by siRNA transfection and viral infection as described for MDDCs.

Virus production and infection

Preparation of VSV-G-pseudotyped HIV-1 firefly luciferase (luc), FIV and EIAV is described in the Supplemental Experimental Procedures. In general, 2.5 to 5 ng of p24 of HIV-1 virus or 10^4 - 10^5 IU (infectious units) of FIV or EIAV were used per 25,000 PMA-THP. MHV-68 was a gift from Ren Sun at UCLA and used at MOI of 5. VLP-Vpx was generated using the SIV3+ plasmid as previously described (Manel et al., 2010). The Mount Sinai Department of Microbiology Virus Collection provided the Cantell strain of Sendai virus.

siRNAs and qRT-PCR

siRNAs were introduced into either MDDCs or PMA-THP using Stemfect RNA transfection kits. Typically, 2.5-5 pmol of siRNAs and 0.14 – 0.18 μl of Stemfect were used for 2.5×10^4 cells. Forty eight hr after siRNA transfection, cells were infected with indicated virus for 5 to 8 hr, followed by RNA isolation and qRT-PCR analysis. The siRNA that knocked down PQBP1 most consistently was used for all subsequent experiments. A list of the siRNAs and the qPCR primers that were used in these experiments is shown in Supplementary Table 2.

In vivo interaction assay

Lysates prepared from MDDCs infected with HIV/Vpx for 6 hr (unless otherwise indicated) followed by formaldehyde cross-linking (see Supplemental Experimental Procedure for detail) were subjected to immunoprecipitation with 3-5 μg of antibody. The co-precipitated nucleic acids were analyzed by either qRT-PCR or qPCR analysis. Unless indicated otherwise, the samples that were analyzed by qPCR only were treated with RNaseA/H digestion prior to proteinase K treatment.

For the fractionation experiments, the cross-linked MDDCs were lysed in a Triton cell lysis buffer and the cleared supernatants (SNC) were separated from the pellets, which were resuspended in equal volumes of Triton cell lysis buffer supplemented with 0.2% SDS to generate a chromatin fraction (C). The primers used in this assay are shown in SI Table 2.

PMA-differentiated THP-1s or MDDCs, uninfected or infected with HIV/Vpx for 6-8 hr, were fractionated into cytosolic (S1), membrane and organelle (S2), and chromatin (P2) fractions (see Supplemental Experimental Procedures for detail).

In vitro interaction assay

Recombinant GST-tagged wildtype and mutant PQBP1 proteins were incubated with extra-chromosomal DNA isolated from MDDCs or PMA-THP infected with HIV/Vpx using the Hirt extraction protocol (Arad, 1998). Three hundred ng of GST or GST-PQBP1 protein was incubated with 100 ng of either extra-chromosomal DNA or a mixture of pNL43 HIV-1 provirus plasmid and pUC19 in a binding buffer, followed by immunoprecipitation with an antibody against either GST or PQBP1 (as indicated in each experiment). DNA that associated with the recombinant proteins was analyzed by qPCR using the HIV-1 3' LTR primer (SI Table 2).

Co-immunoprecipitation

HEK293T lysates expressing GFP or GFP fused either to wild type, insertion C (delC), or A194G substitution (WW) PQBP1 and with V5-tagged cGAS were subjected to immunoprecipitation analysis.

Immunostimulatory assay

DNA that was isolated from the *in vivo* cross-linking interaction assay, either from the cellular fractions or from the immunoprecipitants were treated as in ***In vivo* interaction** assay, above. DNA was electroporated into THP-1 using the Neon transfection system (Life Technologies). Six to 8 hr post-electroporation, RNA was isolated from the cells and subjected to qRT-PCR analysis.

In vitro assay for cGAMP activity

The cGAMP activity assay was performed as described previously, with some modifications (Wu et al., 2013; see Supplimental Experimental Procedures).

Ethics Statement

The collection of Renpenning patient blood was approved by the Comité de Protection des Personnes (French Research Ministry Approval # DC-2011-1437) and its use for research was approved by the Biobank NeuroBioTec (Centre de Ressources Biologiques des Hospices Civils de Lyon, France, IRB # AC-2013-1867). The collection and research including whole blood obtained from healthy blood donors has been approved by the Ethics Committee of the Medical Faculty of the Goethe-University of Frankfurt (Reference No 385/14) and performed according to the principles expressed in the Declaration of Helsinki.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- PQBP1 is an essential component of cGAS/IRF3-dependent innate response to HIV.
- PQBP1 directly binds to immunogenic reverse transcribed HIV-1 DNA.
- PQBP1 physically associates with and regulates cGAS activity.
- Renpenning patients' cells have an impaired innate immune response to HIV-1.

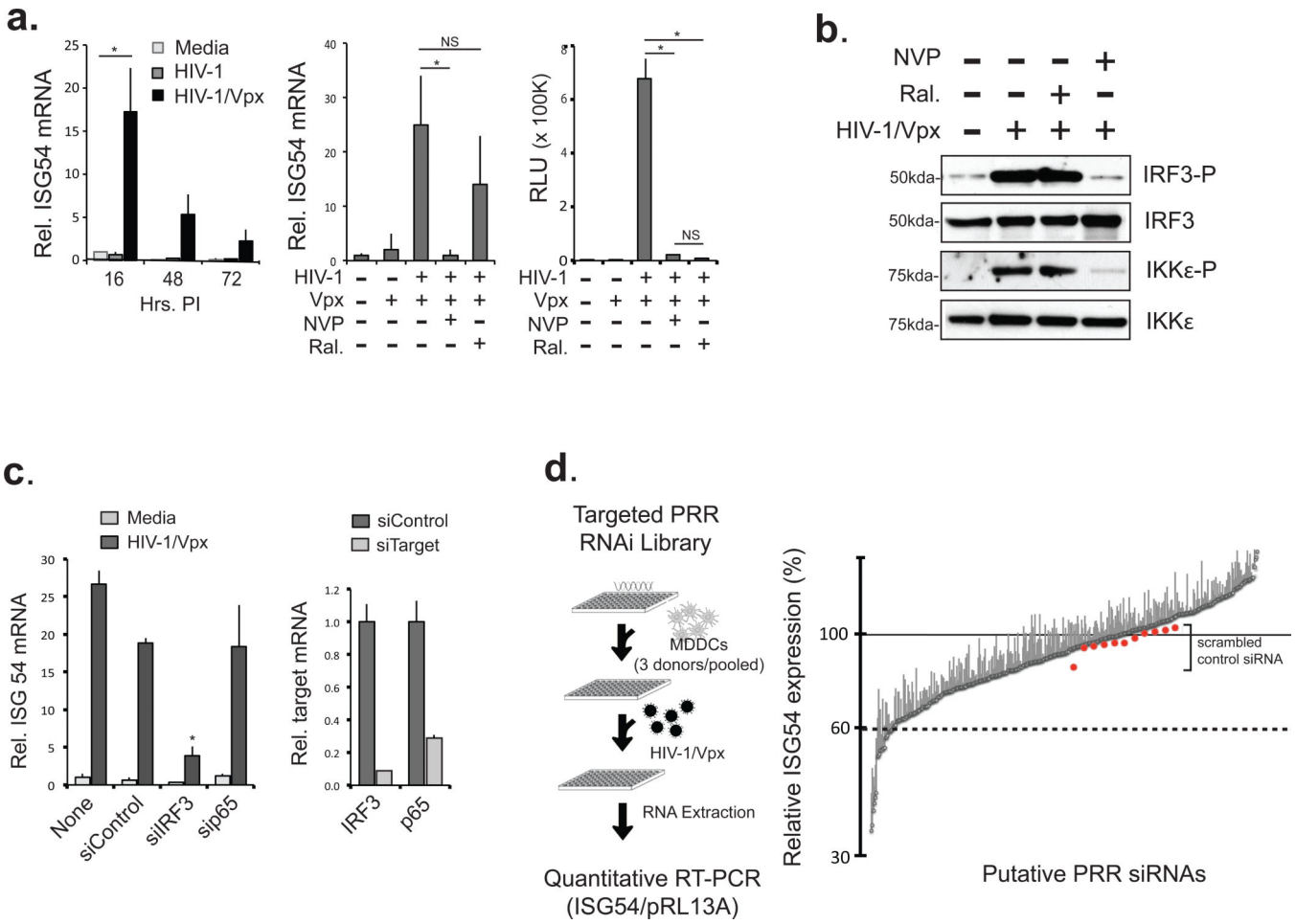

Figure 1. A targeted RNAi screen to identify innate immune sensors of HIV-1 infection

(A) MDDCs were infected with HIV/Vpx encoding luciferase for the indicated duration (left). *ISG54* mRNA levels were measured after infection in the absence or presence of antiviral drugs, NVP (5 μM) or Ral (5μM) (middle). Viral infectivity was evaluated by assessing viral luciferase activity (right). (B) MDDCs treated as in (*A*) were assayed for p-IRF3 and p-IKKε levels by western blot. (C) MDDCs were transfected with siRNAs targeting IRF3 and NF-κB (p65), and relative *ISG54* induction was measured 12 hr post infection. 'None' indicates that the cells were not transfected. (D) A schematic depicting the screening process (left). The average and standard deviation of *ISG54* mRNA levels in MDDCs depleted of each putative co-sensor is shown (right). The scramble control activities are depicted in the large, red circles. The horizontal dotted line reflects the 3 standard deviation cut-off from the controls. The p value was determined by one-tailed, unpaired t-test ($p \leq 0.0001$). This screen was run in biological duplicates. Except in (*D*), all data are shown as the average ± SD of biological triplicates. *$p < 0.05$ as determined by 1 way ANOVA with Tukey's post-test. Panels (*A*) - (*C*) represent data from at least three independent experiments. See also Figure S1 and SI Table 1 for additional validation studies of the screening platform.

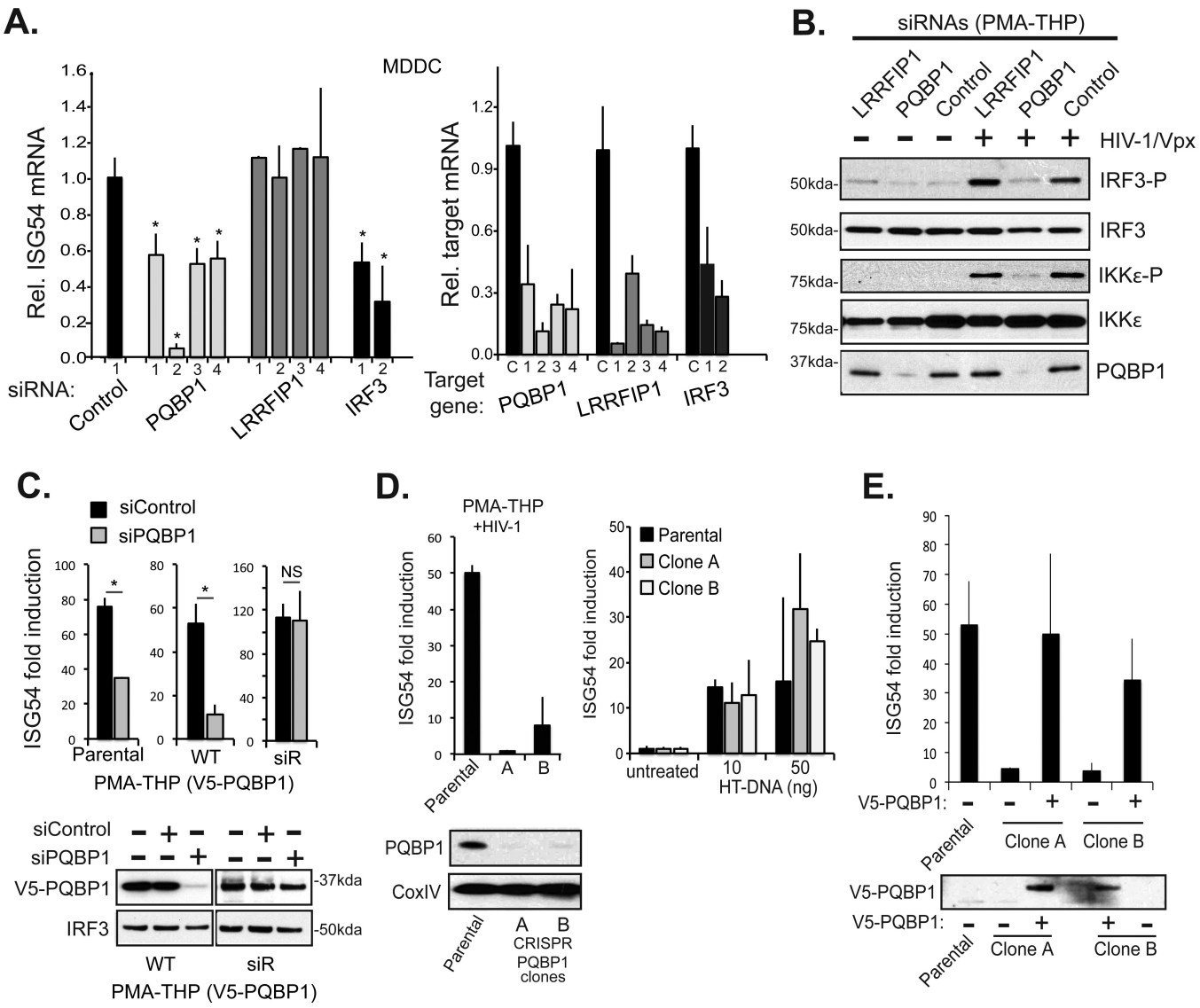

Figure 2. PQBP1 regulates the innate immune response to HIV-1 in primary human DCs and human monocytic cell lines

(A) *ISG54* expression was measured in MDDCs transfected with four independent siRNAs targeting PQBP1 and challenged with HIV/Vpx for 8 hr (left). A scrambled siRNA and a siRNA targeting an unrelated innate regulator (LRRFIP1) were utilized as negative controls, and a siRNA targeting IRF3 was employed as a positive control. Expression of each gene was calculated relative to mRNA levels after transfection with the scramble siRNA (right) (* represents a significant [$p < 0.05$] difference from the scramble). (B) THP-1 were transfected with siRNAs targeting the indicated genes and a scramble control for 48 hr, followed by HIV/Vpx infection; the indicated protein levels were then analyzed. (C) V5-tagged WT-PQBP1-THP-1 (expressing wild type PQBP1) or siR-PQBP1-THP-1 (expressing siRNA-resistant PQBP1) cells were transfected with either a control or PQBP1-targeting siRNA and infected with HIV/Vpx for 8 hr, then the fold induction of *ISG54* in comparison to uninfected sample was measured (top panel). V5-tagged PQBP1 protein

expression is shown (bottom panel). (D) CRISPR-generated PQBP1 THP-1 cells were PMA-differentiated and infected with HIV/Vpx or stimulated with HT-DNA for 8 hr, then *ISG54* induction was measured (top). Loss of PQBP1 protein in cPQBP1 cells was confirmed by western blot (bottom). (E) cPQBP1 clones were stably reconstituted with V5-tagged wild type PQBP1 and infected with HIV/Vpx, and the *ISG54* fold induction was compared in all lines (top). Expression of V5-PQBP1 protein in the rescue lines is shown (bottom). Panels from (*A*) and (*B*) represent data from at least three independent experiments. Panel (*C*) – *(E)* is a representative of two independent experiments, and the data are shown as the mean ± SD of biological triplicates. See also Figure S2.

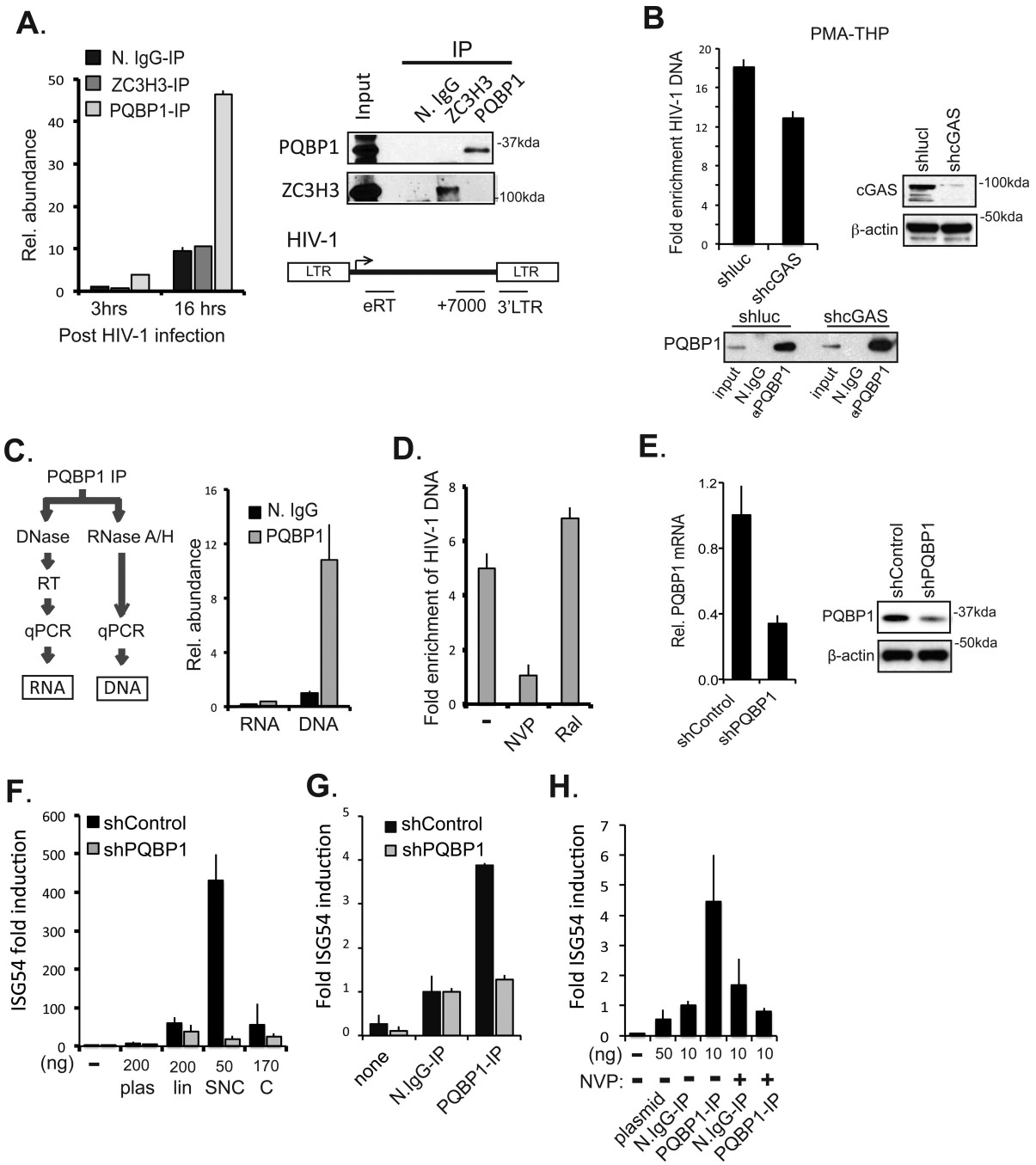

Figure 3. PQBP1 associates with immunogenic HIV-1 DNA

(A) MDDCs were challenged with HIV/Vpx for 3 or 16 hr, followed by formaldehyde cross-linking. The abundance of HIV-1-encoded nucleic acids associating with the PQBP1 IP or the ZC3H3 IP was evaluated using qRT-PCR using primers for the HIV-1 strong stop sequence (left) (eRT primers, Supplementary Data Table 2). The values are relative to the level of nucleic acids that precipitated by normal IgG from the 3 hr infected lysate. Binding specificity of the antibodies was confirmed by western blot of the immunoprecipitants (right). The schematic depicts the HIV-1 genome with the three sets of primers used to

measure the amount of HIV-1 nucleic acid. See also Figure S3C. (B) THP-1 cells that stably expressed shRNA targeting luciferase or cGAS were infected with HIV/Vpx for 6 hr, formaldehyde cross-linked, lysed and immunoprecipitated with a PQBP1 antibody. The fold enrichment of HIV-1 DNA (3'LTR) pulled down by the PQBP1-IP over normal IgG-IP is indicated (top). A western blot of cGAS protein in shcGAS cells compared to shLuc control (right). A western blot shows levels of PQBP1 protein immunoprecipitated from both samples (bottom). (C) Immunoprecipitants described in (*A*) were treated with either DNase or RNaseA/H, as shown in schematic, and HIV-1 nucleic acid levels (3'LTR) were measured as described. (D) MDDCs were challenged with HIV/Vpx for 6 hr in the absence or presence of NVP (5μM) or Ral (5μM). Fold enhancement of HIV-1 sequences (3'LTR) from the PQBP1 IP over the control normal IgG IP measured by qPCR. (E) Knock down efficiency of PQBP1 in THP-1 cells expressing shRNAs against a scramble control or the PQBP1 protein was measured by qRT-PCR (left) and western blot (right). (F) Infected MDDC lysates were separated into soluble non-chromatin (SNC) and chromatin (C) fractions. DNA isolated from each fraction, a plasmid encoding HIV-1 proviral DNA (circular or linearized) was electroporated into THP-1 cells harboring a shRNA control or shRNA targeting PQBP1 and *ISG54* expression was measured. (G) The co-immunoprecipitated DNA as in (*A*) was eluted and electroporated into PMA-differentiated shControl or shPQBP1 THP-1 cells and *ISG54* expression was assessed. (H) MDDCs in the absence or presence of NVP were infected with HIV/Vpx for 6 hr and treated as in (*A*), and the immunoprecipitated DNA was electroporated into PMA-THP and *ISG54* expression was assessed. Data in panels (*A*) - (*D*) are shown as the average ± SD of technical quadruplicates. Panels (*A*) – (*D*) are representative of at least three independent experiments and (*F*) – (*H*) are representative of two independent experiments. $*p < 0.05$ as determined by 1 way ANOVA with Tukey's post-test. See also Figure S3.

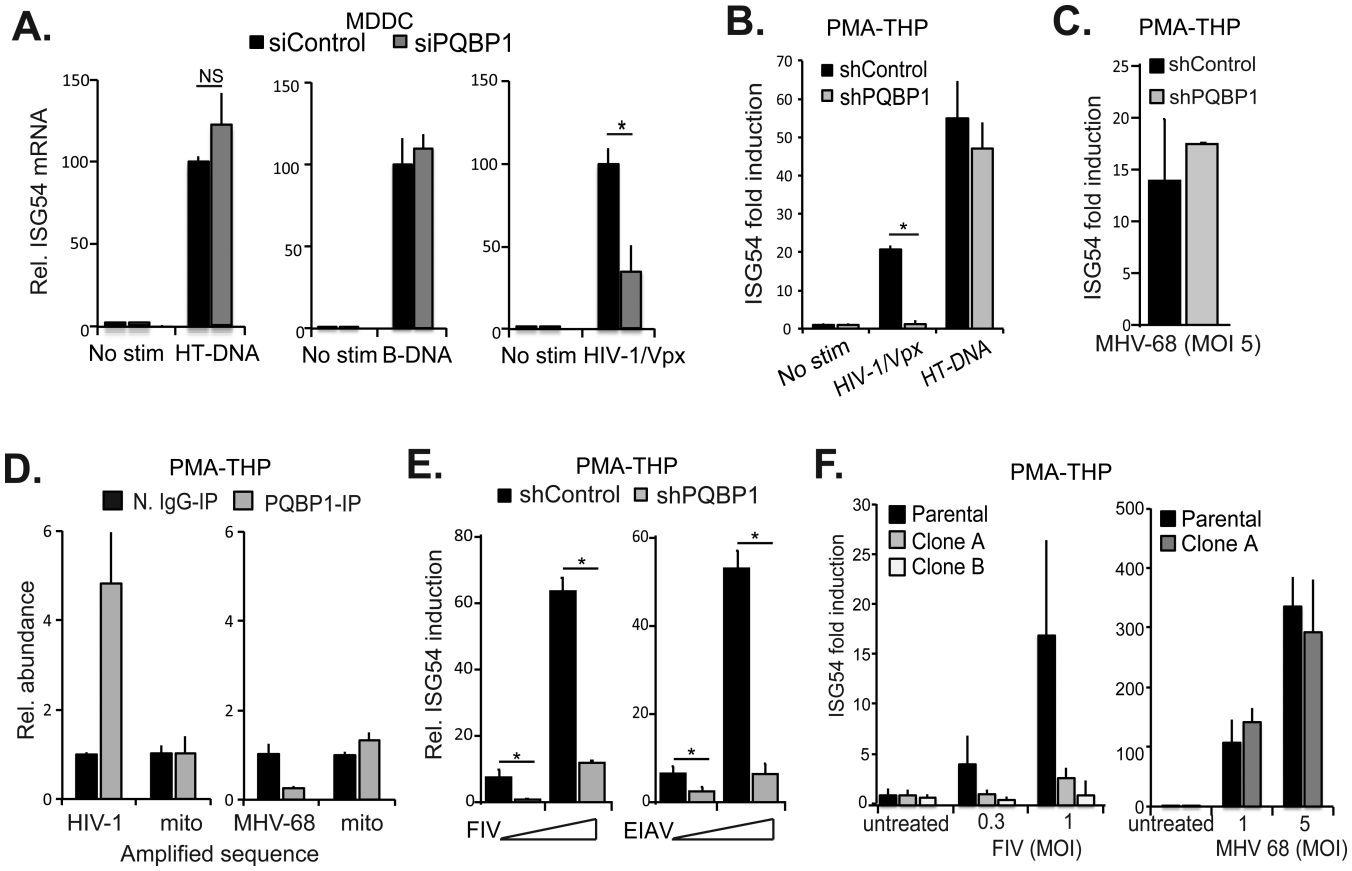

Figure 4. PQBP1 is a specific sensor of retroviral DNA

(A) MDDCs were transfected with a control siRNA or siPQBP1 and challenged with HIV/Vpx, B-DNA, or HT-DNA, then *ISG54* expression was measured. (B) PMA-differentiated shControl or shPQBP1 THP-1 cells were infected with HIV/Vpx or transfected with HT-DNA for 8 hr, followed by measuring *ISG54* induction. Both infection and transfection were performed in parallel. (C) PMA-differentiated shControl or shPQBP1 THP-1 cells were infected with MHV-68 virus at an MOI 5, and *ISG54* induction was measured. (D) THP-1 were challenged with HIV/Vpx or MHV-68 for 6 hr, followed by formaldehyde cross-linking. The abundance of HIV-1- or MHV-68-encoded nucleic acids or mitochondrial DNAs associated with the PQBP1 IP was evaluated (SI Table 2). (E) PMA-differentiated shControl or shPQBP1 THP-1 cells were infected with VSV-G pseudotyped FIV or EIAV, followed by measuring *ISG54* induction. (F) CRISPR PQBP1 clones were infected with increasing MOIs of FIV (left) or MHV-68 (right) and *ISG54* induction was measured. All data except (*D*) are shown as the average ± SD of biological triplicates and are representative of at least three independent experiments. For the THP-1 infected with MHV-68 in (*D*), the lysates were pooled from 6 biological replicates. *$p < 0.05$ as determined by 1 way ANOVA with Tukey's post-test (*A*, *B*), or by an unpaired Student's t-test (*E*). See also Figure S4.

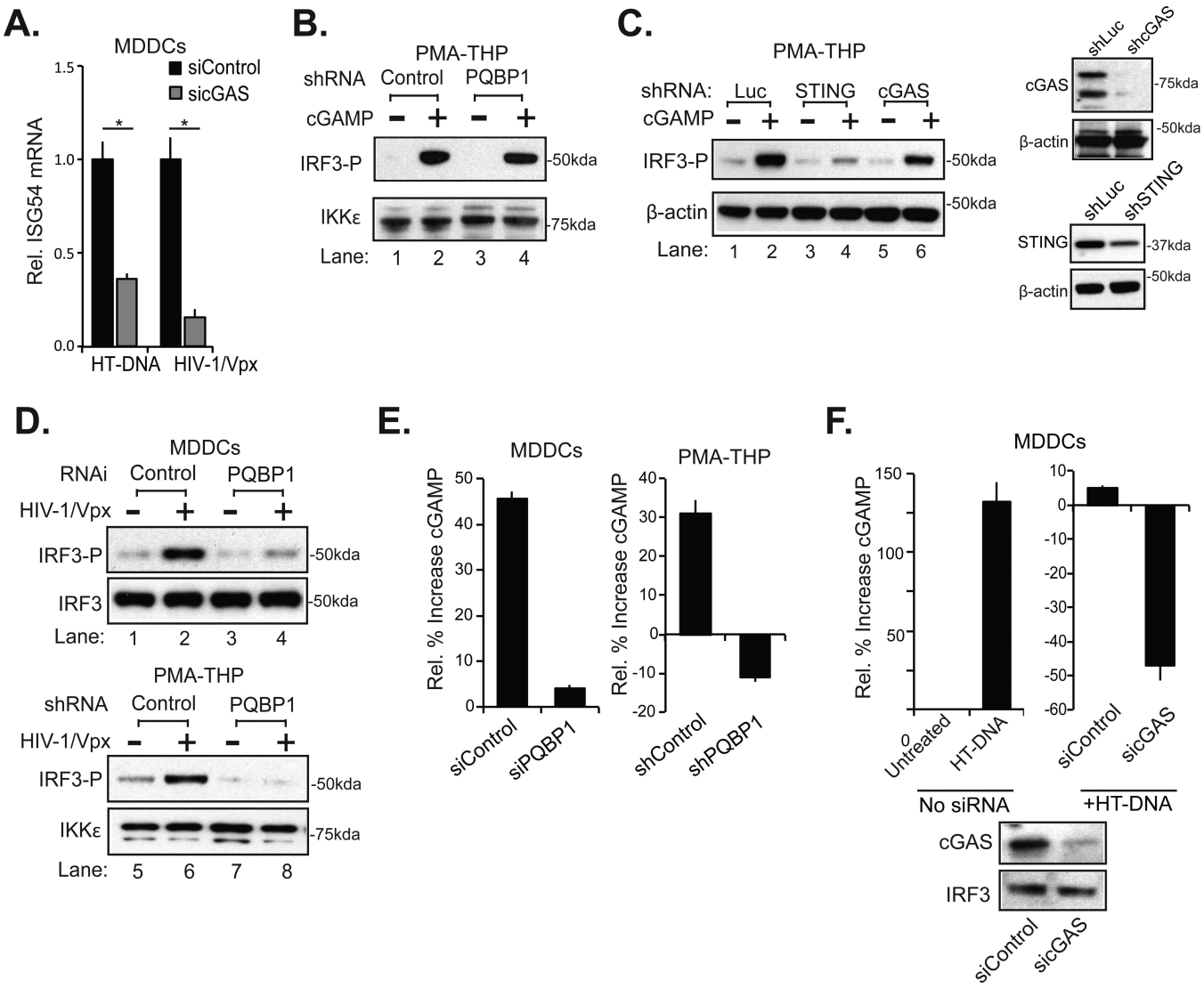

Figure 5. PQBP1 functions upstream of cGAS and regulates cGAS-STING-dependent IRF3 activation upon retroviral infection

(A) MDDCs were transfected with siRNAs targeting cGAS or a scramble control, challenged with either HIV/Vpx or HT-DNA for 8 hr, and assayed for relative *ISG54* mRNA levels. (B) Synthetic cGAMP was incubated with PFO-treated shControl or shPQBP1 THP-1 cells and induction of p-IRF3 was analyzed. (C) Synthetic cGAMP was incubated with PFO-treated shcGAS, shSTING and shLuciferase THP-1 cells and induction of p-IRF3 was analyzed. Knock down efficiency of the target proteins in shcGAS and shSTING lines compared to the shLuc line is shown (right). (D-E) cGAMP levels in the lysates of MDDCs transfected with indicated siRNAs (top) or PMA-differentiated shRNA THP-1 cells (bottom) were assayed prior to and after HIV/Vpx infection. Infected cellular lysates were incubated with PFO-treated THP-1 cells, followed by western blot analysis to detect p-IRF3 (D) or directly quantified using LC-MS (E). The level of cGAMP was graphed as a % increase in HIV-1 infected cells over the untreated samples either in the presence (si/shControl) or absence of PQBP1 protein (si/shPQBP1). (F) cGAMP levels in

the lysates of MDDCs were assayed prior to and after HT-DNA stimulation. cGAMP was directly quantified using LC-MS. The level of cGAMP was graphed as a % increase in HT-DNA stimulated cells over the untreated samples either in the presence (siControl) or absence of cGAS protein (sicGAS). Knock down efficiency of cGAS protein in the lysate is shown at the bottom. Panel (*A*) is shown as the average ± SD of biological triplicates. The data in (*A*)– ((*E*)) are representative of at least two independent experiments. In panel (*D*) - (*F*) the data were from 6 biological replicates pooled to generate the lysate. For the MDDCs, a single donor was used for the biological replicates in a single experiment. The data are representative of four independent experiments. For the experiments using THP-1, two independent experiments were performed. *$p < 0.05$ as determined by an unpaired Student's t-test. See also Figure S5.

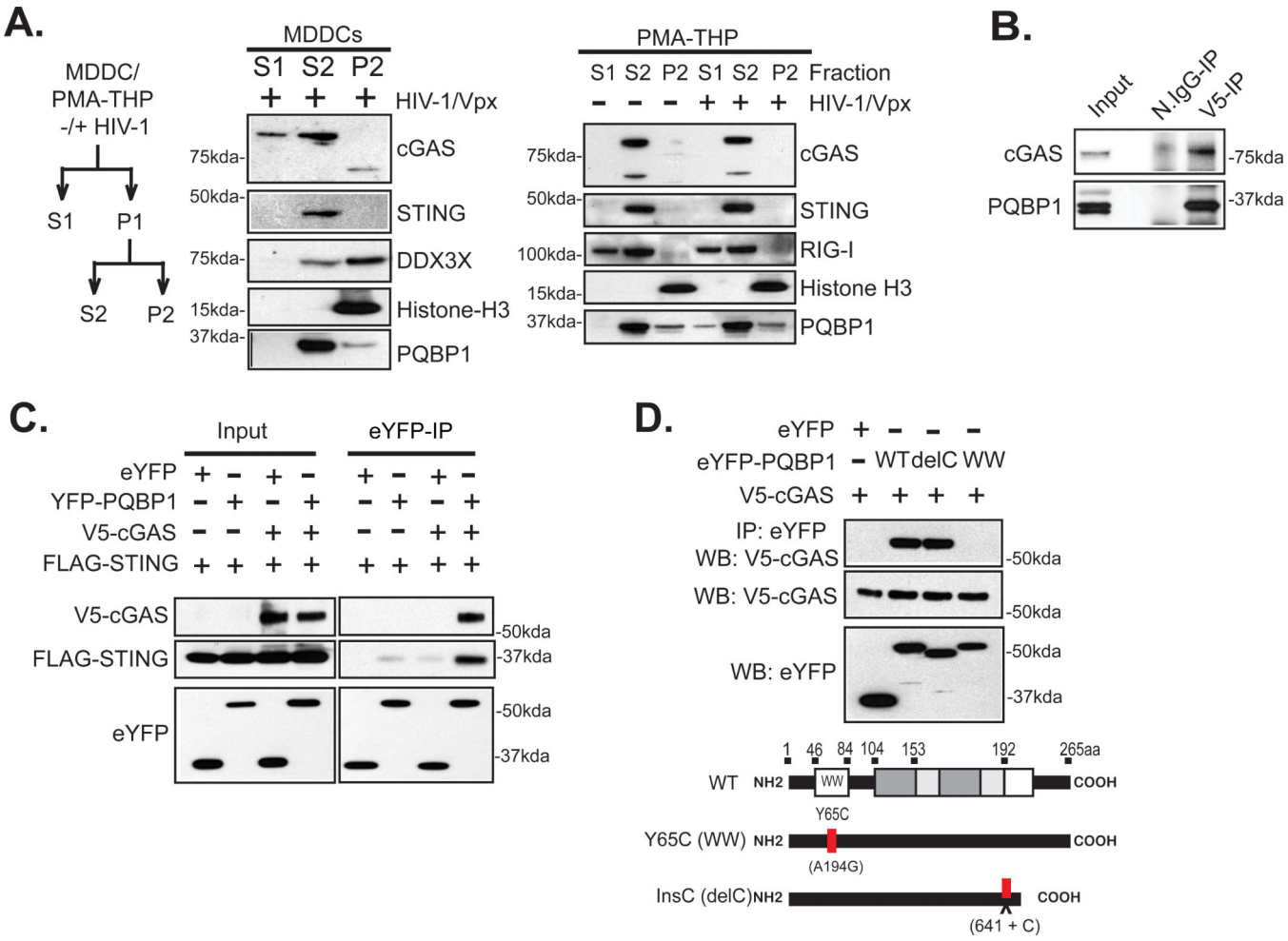

Figure 6. The WW domain of PQBP1 directs interaction with cGAS

(A) MDDCs (left) and THP-1 (right) were fractionated, as shown in schematic (left), yielding a soluble cytosolic component (S1), a membrane- and organelle-associated component (S2) and a chromatin component (P2). Presence of cGAS, STING, DDX3X, Histone-H3 and PQBP1 in specific fractions were measured by western blot. (B) THP-1 cells stably expressing V5-tagged WT-PQBP1 were lysed and immunoprecipitated for V5 or a normal serum IgG, then probed for endogenous cGAS. (C) HEK293T cells were transfected with eYFP-tagged PQBP1 or eYFP, V5-tagged cGAS and/or FLAG-tagged STING. The cells were lysed and eYFP was immunoprecipitated, followed by a western blot for V5 or FLAG. (D) HEK293T cell lysates expressing V5-tagged cGAS and eYFP-tagged wild type (WT), C-terminal truncation mutant (delC) or WW domain mutant (WW) PQBP1 were immunoprecipitated with an antibody against eYFP and probed for V5. (*A*) – (*D*) are representative of at least two independent experiments.

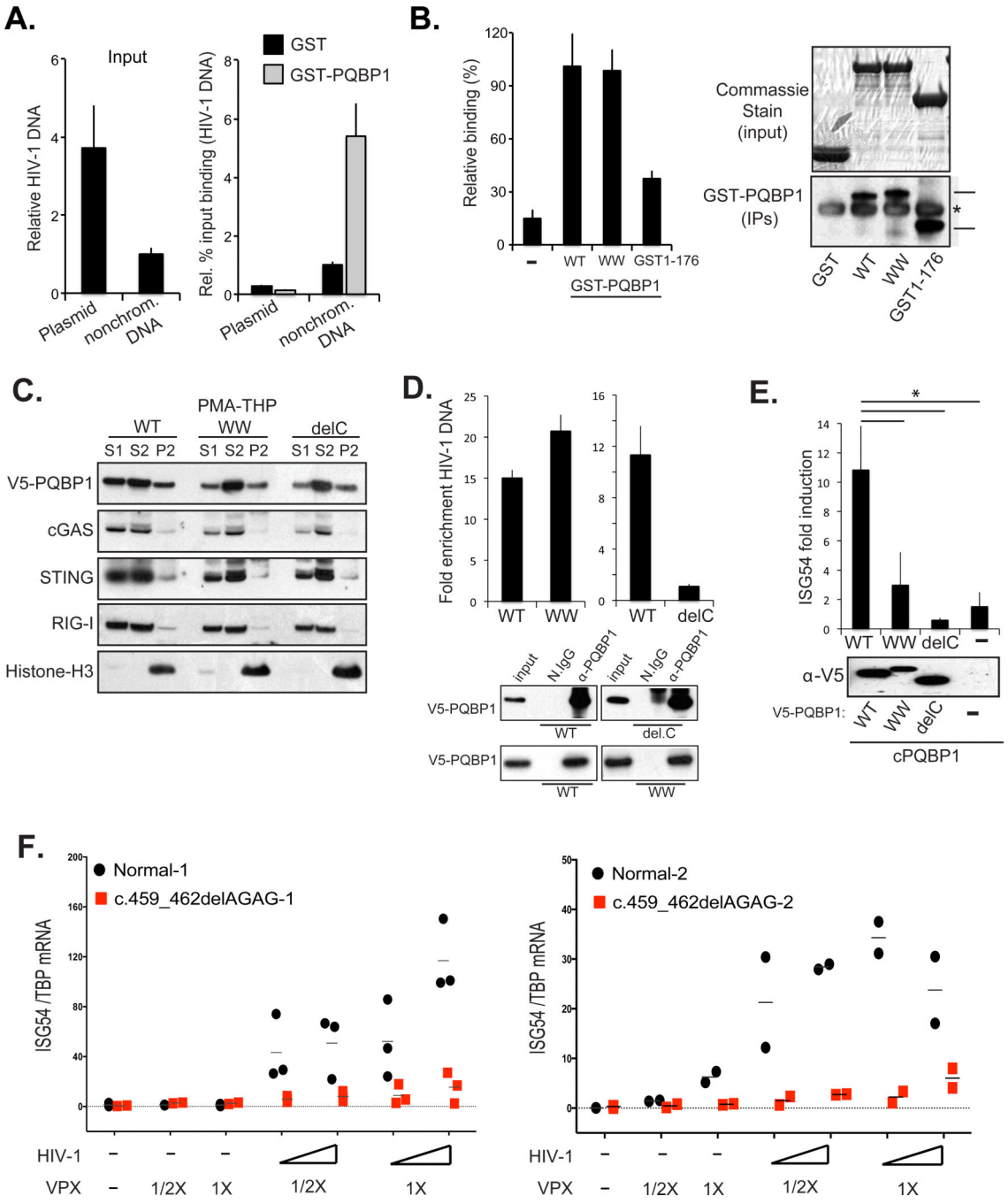

Figure 7. The C-terminal domain of PQBP1 binds HIV-1 DNA and is required for the innate immune response to HIV-1 infection

(A) Recombinant GST or GST-tagged PQBP1 proteins were incubated with either an HIV-1 proviral plasmid or extra-chromosomal nucleic acids (non-chromatin DNA) derived from HIV/Vpx infected MDDCs, and were then immunoprecipitated by GST antibody. The relative abundance of HIV-1 DNA in the input (left) and the IPs (right) is indicated, as measured by qPCR against HIV-1 3' LTR sequences. (B) Recombinant GST or GST-PQBP1 proteins (WT, WW mutant or C-terminal truncation (1-176) mutant) were incubated with non-chromatin DNA, as in (*A*), followed by immunoprecipitation with a PQBP1

antibody. The graph at left depicts the relative binding of HIV-1 DNA to the mutant proteins compared to the wild type PQBP1 protein. The blots at right show input and IP'ed GST and/or GST-PQBP1 proteins used in the assay. – indicates GST-PQBP1 proteins, both wild type and mutants. "*" indicates a non-specific band. (C) cPQBP1 THP-1 cells reconstituted with V5-tagged wild type (WT), WW or delC PQBP1 were fractionated as in Figure 6A, and the subcellular fractions were analyzed for the indicated proteins. (D) cPQBP1 THP-1 cells, stably reconstituted with wild type (WT), delC or WW mutant PQBP1, were infected with HIV/Vpx for 6 hr and were subjected to formaldehyde cross-linking followed by assaying for fold enrichment of HIV-1 DNA immunoprecipitated with the reconstituted PQBP1 protein over normal IgG control. Levels of PQBP1 protein immunoprecipitated are shown by western blot (bottom). (E) cPQBP1 THP-1 cells, stably reconstituted with wild type (WT), delC or WW mutant PQBP1, were infected with HIV/Vpx and fold induction of *ISG54* over an uninfected sample was measured by qRT-PCR. All panels are representative of at least two independent experiments. (F) MDDCs from two healthy donors and two male Renpenning syndrome patients were challenged with HIV/Vpx. *ISG54* induction after 8 hr of infection was evaluated by qRT-PCR and normalized to TBP mRNA levels. *ISG54* values for each independent biological replicate are shown. $p < 0.0001$ (two way ANOVA) comparing HIV-1 challenged samples of healthy and Renpenning cells for each matched set. See also Figure S6.