HIV-1 Vpr antagonizes innate immune activation by targeting karyopherin-mediated NF-κB/IRF3 nuclear transport

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Abstract

HIV-1 must replicate in cells that are equipped to defend themselves from infection through intracellular innate immune systems. HIV-1 evades innate immune sensing through encapsidated DNA synthesis and encodes accessory genes that antagonize specific antiviral effectors. Here we show that both particle associated, and expressed HIV-1 Vpr, antagonize the stimulatory effect of a variety of pathogen associated molecular patterns by inhibiting IRF3 and NF-κB nuclear transport. Phosphorylation of IRF3 at S396, but not S386, was also inhibited. We propose that, rather than promoting HIV-1 nuclear import, Vpr interacts with karyopherins to disturb their import of IRF3 and NF-κB to promote replication in macrophages. Concordantly, we demonstrate Vpr dependent rescue of HIV-1 replication in human macrophages from inhibition by cGAMP, the product of activated cGAS. We propose a model that unifies Vpr manipulation of nuclear import and inhibition of innate immune activation to promote HIV-1 replication and transmission.

Key words: HIV-1, Vpr, DNA sensing, cGAS, Karyopherin, IRF3, NF- κB, nuclear transport

Introduction

Like all viruses, lentiviruses must navigate the hostile environment of the host cell in order to infect, produce new viral particles, and transmit to new cells. A principal feature of cellular defences is detection or sensing of incoming viruses and subsequent production of inflammatory cytokines, particularly type 1 interferons (IFNs). All viral infections likely trigger IFN *in vivo* and the degree to which they do this, and their capacity to antagonize IFN activity and its complex effects, are key in determining transmission mechanism, host range and disease pathogenesis. Like other viruses, lentiviruses antagonize specific host proteins or pathways that would otherwise suppress infection. Lentiviruses typically do this through accessory gene function. For example, HIV-1 antagonizes IFN induced restriction factors through accessory genes encoding Vif (APOBEC3G/H), Vpu (tetherin) and Nef (tetherin/SERINC3/5) reviewed in (Foster et al., 2017; Sumner et al., 2017).

The role of the HIV-1 accessory protein Vpr has been less clearly defined. Manipulation of host innate immune mechanisms by Vpr to facilitate replication in macrophages has been suggested by several studies although there is still no clear model of mechanism and understanding of the target proteins that link Vpr to innate immune manipulation is limited (Harman et al., 2015; Liu et al., 2014; Okumura et al., 2008; Trotard et al., 2016; Vermeire et al., 2016). Vpr clearly changes infected cell protein profiles affecting the level of hundreds of proteins in proteomic studies, likely indirectly in most cases, suggesting manipulation of central mechanisms in cell biology (Greenwood et al., 2019). There is also evidence for Vpr interacting with and manipulating many specific proteins including its cofactor DCAF1 (Zhang et al., 2001), karyopherin alpha 1 (KPNA1, importin α) (Miyatake et al., 2016) the host enzyme UNG2 (Wu et al., 2016) as well as HTLF (Lahouassa et al., 2016; Yan et al., 2019), SLX4 (Laguette et al., 2014) and CCDC137 (Zhang & Bieniasz, 2019). Vpr has also been shown to both enhance (Liu et al., 2014; Liu et al., 2013; Vermeire et al., 2016) decrease, NF-κB activation (Harman et al., 2015; Trotard et al., 2016) in different contexts and act as a cofactor for HIV-1 nuclear entry, particularly in macrophages (Vodicka et al., 1998). However, despite this work the mechanistic details of Vpr promotion of HIV replication are poorly understood and many studies seem contradictory. This is partly because the mechanisms of Vpr-dependent enhancement of HIV-1 replication are context dependent, and cell type specific although most studies agree that Vpr is more important for replication in macrophages than in T cells or PBMC (Connor et al., 1995; Dedera et al., 1989; Fouchier et al., 1998; Hattori et al., 1990; Mashiba et al., 2014)

In this paper, we demonstrate that Vpr mutants, unable to recruit to the nuclear envelope, fail to antagonize innate sensing, but retain induction of cell cycle arrest, genetically separating key Vpr functions. We provide evidence that Vpr prevents IRF3 and NF-κB from interacting with karyopherin alpha 1 (KPNA1/importin α), thus inhibiting innate immune activation by viral and non-viral agonists. Our new findings support a unifying model of Vpr function, consistent with much of

the Vpr literature, in which Vpr associated with incoming viral particles suppresses nuclear entry of activated inflammatory transcription factors to facilitate HIV-1 replication in innate immune activated macrophages.

Results

HIV-1 replication in cGAMP-stimulated MDMs requires Vpr

A considerable body of evidence suggests an important role for Vpr in supporting HIV-1 replication in macrophages but the relevant Vpr mechanisms for this function have been enigmatic. We set out to investigate the role of Vpr in manipulating host innate immune mechanisms during HIV-1 infection of primary human cells. We prepared human monocyte-derived macrophages (MDM) by purifying monocytes from peripheral blood by adherence and treating with M-CSF (Rasaiyaah et al., 2013). Macrophages prepared in this way are particularly permissive to HIV-1 replication facilitating study of HIV-1 biology in a primary myeloid cell type. We found that wild type HIV-1 and HIV-1ΔVpr replicated equally well in (MDM)(Figure 1A) (Rasaiyaah et al., 2013) Consistent with previous studies, Wild type HIV-1, and HIV-1 deleted for Vpr replicated equally well in activated primary human CD4+ T cells (Figure S1A) (Dedera et al., 1989; Fouchier et al., 1998).

Vpr has been shown to antagonize innate immune signaling in HeLa cells reconstituted for DNA sensing by STING expression (Trotard et al., 2016), so we hypothesized that Vpr might be particularly important when DNA sensing is activated. To test this, we mimicked activation of the DNA sensor cGAS by treating MDM with cGAMP, the product of activated cGAS. In the presence of cGAMP, HIV-1 replication in MDM was, indeed, Vpr-dependent. 1µg/ml cGAMP specifically suppressed HIV-1ΔVpr more potently than wild type virus and 4µg/ml cGAMP overcame Vpr activity and suppressed replication of both wild type and mutant viruses (Figure 1A). Intriguingly, Vpr did not rescue HIV-1 replication from cGAMP-mediated inhibition in primary human CD4+ T cells, and cGAMP had only minimal effect on HIV-1 replication in Jurkat T cells (Figure S1A). These data demonstrate that HIV-1 replication in cGAMP-stimulated MDM is Vpr dependent. They are consistent with previous observations suggesting Vpr is more important in macrophages than T cells and that the consequences of cGAMP treatment differ between these cell types (Gulen et al., 2017; Xu et al., 2016).

HIV-1 particle delivered Vpr inhibits gene expression stimulated by DNA sensing

We next investigated the effect of particle-associated Vpr on innate immune activation. The myeloid cell line THP-1 expresses cGAS and STING and has a functional DNA sensing pathway (Mankan et al., 2014). We used THP-1 cells expressing the Gaussia luciferase gene under the control of the endogenous *IFIT1* promoter (herein referred to as THP-1 IFIT1-luc) (Mankan et al., 2014) to measure the effect of Vpr on cGAMP-induced IFIT1-luc expression. IFIT1 (ISG56) is a

well-characterized ISG that is highly sensitive to cGAMP and type 1 IFN. Treatment of THP-1 IFIT-luc cells with cGAMP induced IFIT1-luc expression by two orders of magnitude. This activation was significantly suppressed if cells were infected with VSV-G pseudotyped, genome-free, HIV-particles bearing Vpr, (referred to here as virus-like particles or VLP), but not by VLP lacking Vpr, immediately prior to cGAMP addition (Figure 1B). IFIT1-Luc was measured 6, 8 and 24 hours after cGAMP addition/infection.

In this experiment, doses of VLP required to suppress IFIT1-luc expression were high, equivalent to a multiplicity of infection of 20 as measured by correlating VLP reverse transcriptase levels (SG-PERT) (Jolien Vermeire et al., 2012), with HIV-1 GFP titers on THP-1. We assume that such a high dose of Vpr-bearing VLP is required because cGAMP treatment activates numerous STING complexes in most of the cGAMP-treated cells. If this effect of Vpr is relevant to infection, we expect that cGAS/STING activated by the incoming HIV genome should be sensitive to the amount of Vpr contained in an individual particle. To test this, we activated DNA sensing using high dose infection by VSV-G pseudotyped HIV-1 vectors bearing GFP-encoding genome. We used an HIV-1 packaging plasmid, derived from HIV-1 clone R9, encoding Gag-Pol, Tat and Rev (p8.91) or Gag-Pol, Tat and Rev and Vpr, Vpu, Vif and Nef (p8.2) (Zufferey et al., 1997). Strikingly, although Vpr positive and negative HIV-1 GFP stocks infected THP-1 cells to similar levels (Figure 1D), induction of inflammatory cytokine, and ISG, CXCL10 was reduced if the HIV-1 GFP carried Vpr (Figure 1C). This indicates that Vpr can inhibit the consequences of sensing driven by the Vpr bearing virus particles themselves.

Genome-free, non-infectious, HIV-1 particles did not induce CXCL10 expression (Figure 1E, F), evidencing the importance of viral DNA in this response. Furthermore, CXCL10 expression was not induced after infection of THP-1 cGAS knock out cells, consistent with CXCL10 induction being cGAS-dependent. Knock out of the RNA sensing adaptor protein MAVS had no effect on induction of CXCL10 (Figure 1G). cGAS and MAVS knock out were confirmed by immunoblot (Figure S1C).

As expected, a lower dose of virus was required to see the effect of Vpr when the particles themselves activated sensing, and in this latter experiment, Vpr effects were clear at MOIs of 3 (Figure 1C, E). Moreover, single round titer of HIV-1 GFP was not affected by cGAS or MAVS knock out, confirming that sensing activation does not impact single round infectivity of HIV-1 GFP VSV-G pseudotypes in this assay consistent with HIV-1 vector not being particularly sensitive to IFN (Figure 1H, Figure S1B).

HIV-1 Vpr expression inhibits innate immune activation

We next tested whether Vpr expressed in isolation can suppress innate immune activation by cGAMP. Vpr from the primary founder HIV-1 clone SUMA (Fischer et al., 2010) was expressed in

THP-1 IFIT1-luc cells using an HIV-1 vector we called pCSVIG (Figure S2A, S2B). Vpr was expressed using MOIs of approximately 0.2-1. Forty hours after transduction, cells were treated with cGAMP (5µg/ml), and IFIT1-luc was measured 8 hours later. Prior expression of Vpr reduced IFIT1-luc responses in a dose-dependent manner whilst the highest dose of empty vector had no effect (Figure 2A). Vpr expression (MOI=1, Figure S2C) also suppressed cGAMP-mediated induction of endogenous ISG mRNA expression, measured by qRT-PCR for *MxA*, *CXCL10*, *IFIT2* and *viperin* (Figure 2B) and inhibited cGAMP induced CXCL10 secretion (Figure 2C; infection data in Figure S2D).

IFIT1-luc expression stimulated by transfection of herring testis (HT) DNA was also inhibited by Vpr expression, consistent with the notion that Vpr antagonizes DNA sensing (Figure 2D, Figure S2E). Strikingly, Vpr also reduced Sendai virus induced activation of IFIT1-luc, which is mediated by MDA5 and RIGI RNA sensing (Andrejeva et al., 2004; Rehwinkel et al., 2010) (Figure 2E, Figure S2G) and IFIT1-luc activation after stimulation with the TLR4 ligand LPS (Figure 2F, S2H). Thus, Vpr expression appeared to mediate a generalized suppression of innate immune activation.

Vpr inhibition of innate immune activation is dependent on DCAF1 but independent of cell cycle arrest

In order to separate innate immune antagonism from other Vpr functions, we used three Vpr mutants with distinct functional deficits. Vpr R80A, is defective in inducing cell cycle arrest (Laguette et al., 2014); Vpr Q65R fails to recruit DCAF1 and so cannot degrade target proteins (Laguette et al., 2014); and Vpr F34I/P35N fails to bind cyclophilin A and does not localize to the nuclear membrane (Vodicka et al., 1998; Zander et al., 2003).

All three mutant Vprs were efficiently incorporated into HIV-1 GFP particles (Figure 3A). When delivered by viral particles, Vpr R80A effectively suppressed IFIT1-luc induction by cGAMP in THP-1 cells, however Vpr Q65R and Vpr F34I/P35N had little if any suppressive effect (Figure 3B). In these experiments, cGAMP was added to the target cells directly after the virus. Suppression of IFIT1-luc induction by Vpr R80A suggested that cell cycle arrest was not required for innate immune antagonism. To further test this, we measured the effect of all three Vpr mutants on cell cycle progression. As reported, WT Vpr expression in THP-1 cells induced a significant increase of cells in G2/M phase of cell cycle and Vpr R80A had no effect (Figure 3C) (Laguette et al., 2014). Vpr F34I/P35N, which cannot effectively suppress cGAMP mediated IFIT1-luc/ISG expression (Figure 3B, 3G), also induced G1/M cell cycle arrest, albeit slightly less efficiently than wild type Vpr protein, as previously described (Vodicka et al., 1998) (Figure 3C). The DCAF1 Vpr binding mutant Q65R did not inhibit cell cycle, as reported (Figure 3C) (Laguette et al., 2014). These data genetically separate the effects of Vpr expression on cell cycle, and on inhibition of innate immune activation, suggesting that these functions depend on manipulation of different target proteins. It

is striking that amino acids at positions 34/35 and 80 are close in Vpr structures and distant from the UNG2 binding site, suggesting an additional target binding interface, as seen in the highly related Vpx protein (Figure S3B, C) (Morellet et al., 2003; Schwefel et al., 2014; Wu et al., 2016).

We next asked whether DCAF-1 was required for innate immune antagonism, as suggested by the Vpr Q65R mutant, which fails to recruit DCAF1, and cannot suppress cGAMP-induced IFIT1-luc expression (Figure 3B). Depletion of DCAF1 in THP-1 cells by shRNA prevented Vpr from inhibiting cGAMP induction of IFIT1-luc (Figure 3D). Neither DCAF1 depletion, nor cGAMP treatment reduced infectivity of HIV-1 GFP vector (Figure S3A). Vpr was active in cells expressing a non-targeting shRNA (shControl) and suppressed IFIT1-luc induction. Expression of empty (no Vpr) vector had no effect on IFIT1-luc induction (Figure 3D). Effective depletion of DCAF1 was evidenced by immunoblot (Figure 3E). Thus, Vpr inhibition of innate immune activation requires DCAF1.

Expressed Vpr had similar mutation sensitivity as Vpr delivered by HIV-1 particles (compare Figures 3F, G and 3B). Expression of wild type Vpr, or Vpr R80A, prevented cGAMP activation of the IFIT1-luc reporter (Figure 3F), and of endogenous *MxA* in THP-1 cells (Figure 3G, S3D). HT DNA transfection, but not lipofectamine alone, activated IFIT1-luc reporter expression, as expected, and this was also sensitive to wild type and VprR80A expression, but not expression of Vpr F34I/P35N (Figure S3E, S3F). Vpr Q65R had only a small inhibitory effect consistent with data in Figure 3B.

Wild Type Vpr, but not sensing antagonism inactive Vpr mutants, colocalize with nuclear pores

Having identified Vpr mutants defective for antagonism of innate immune sensing, we sought further clues about Vpr mechanism by examining wild type and mutant Vpr location within cells. Vpr expressed in isolation is found in the nucleus and associated with nuclear pores (Fouchier et al., 1998; Le Rouzic et al., 2002). Concordantly, we found FLAG-Vpr in the nucleus, and colocalized with antibody staining the nuclear pore complex, when expressed by transient transfection in HeLa cells (Figure 4A, B). As previously reported for the single mutant F34I (Jacquot et al., 2007; Vodicka et al., 1998), we found that the double Vpr mutant F34I/P35N, as well as Vpr Q65R, were mislocalized, as compared to wild type and R80A Vpr. Thus mutants which fail to inactivate innate immune sensing, fail to localize to the nuclear membrane. Defective Vpr mutants F34I/P35N and Q65R appeared qualitatively different inside the nucleus, and nuclear rim staining was less well defined, suggesting that they have lost interactions with a protein(s) that normally influences their position within the cell. Fluorescence intensity measurements along transverse sections of nuclei in single confocal images showed two distinct peaks of nuclear pore staining representing each edge of the nucleus. These peaks overlapped with WT and Vpr R80A

fluorescence but not with Vpr F34I/P35N or Vpr Q65R fluorescence, which was more diffuse and less well defined at the nuclear rim (Figure 4C). These data link Vpr nuclear membrane association with antagonism of innate immune sensing for the first time.

Vpr has been described to interact with cyclophilin A (CypA) and mutating Vpr residue P35 was reported to prevent this interaction (Zander et al., 2003). The nuclear pore complex has cyclophilin-like domains, which are structurally very similar to CypA, at the end of the Nup358 fibers that protrude into the cytoplasm (Schaller et al., 2011). To test whether Nup358 was required for Vpr association with the nuclear rim, we expressed FLAG-Vpr in Nup358-depleted HeLa cells (Schaller et al., 2011) and stained the Vpr FLAG tag (green) and NPC (red) (Figure S4A, B). Despite effective Nup358 depletion (Figure S4C), Vpr remained associated with the nuclear rim suggestig that Nup358 is not required for Vpr nuclear rim association (Figure S4A, B, D).

Vpr inhibits IRF3 nuclear translocation

cGAMP is produced by activated cGAS and is recruited by STING, which then forms an active kinase complex in which TBK1 phosphorylates STING, TBK1 itself, and the transcription factor IRF3 (Liu et al., 2015; Zhang et al., 2019). IRF3 phosphorylation promotes nuclear translocation and subsequent activation of gene expression including type 1 IFNs (Chen et al., 2008). As expected, transfection of THP-1 IFIT1-luc cells with HT DNA induced phosphorylation of STING, TBK1 and IRF3-S386 (Figure 5A). Measurement of IFIT1-luc expression, in the same samples, three hours after stimulation, indicated induction of IFIT1-luc by HT DNA but not after prior Vpr expression using a lentiviral vector (Figure 5B). Strikingly, Vpr expression for 48 hours did not impact STING, TBK1 or IRF3 protein levels, or their phosphorylation status 3 hours after DNA transfection, measuring IRF3 phosphorylation at S386 (Figure 5A). Empty vector expression had no detectable effect on protein levels or phosphorylation (Figure 5A). Actin was detected as a loading control and Vpr/empty vector were used at a vector MOI of about 1 (Figure S5A). A second example of this experiment is presented in Figure S5B-E. IRF3 is phosphorylated at multiple sites during activation including at IRF3 S-396. We therefore examined IRF3 S-396 phosphorylation using a phospho-IRF3-S396 specific antibody and flow cytometry because this antibody didn't work well by immunoblot. We found that in this case, Vpr delivery by VLP did reduce phosphorylation of IRF3-S-396 after stimulation by either cGAMP or HT DNA in THP-1 cells (Figure 5C).

Given that Vpr is associated with the nuclear rim, and Vpr mutations that break antagonism of innate sensing mislocalize Vpr, we hypothesized that rather than impacting levels of signaling proteins, Vpr may act at nuclear pores to influence nuclear transport of inflammatory transcription factors. This would be consistent with the broad innate immune antagonism that we have observed (Figure 2), and with previous reports of Vpr influencing nuclear transport, for example, of viral

nucleic acids (Heinzinger et al., 1994; Miyatake et al., 2016; Popov et al., 1998), and inhibiting sensing of HIV-1 (Trotard et al., 2016). We therefore investigated the effect of Vpr on cGAMP-induced IRF3 nuclear translocation. THP-1 were differentiated with 50ng/ml phorbol-12 myristate acetate (PMA) to attach them to glass for microscopy. In these experiments, VLP with or without Vpr are used to infect cells immediately after they are treated with innate immune stimulants. IRF3 translocation is measured three hours later by immunofluorescent labeling. VSV-G pseudotyped HIV-1 GFP bearing Vpr reduced cGAMP-stimulated IRF3 nuclear translocation in a dose-dependent way whilst HIV-1 lacking Vpr had no effect (Figure 5D, E, S5F). These data are consistent with a previous report in which Vpr suppressed nuclear transport of IRF3-GFP on HIV-1 infection of HeLa cells in which DNA sensing had been reconstituted by expression of STING (Trotard et al., 2016). Importantly, in our experiments in THP-1, suppression of IRF3 nuclear translocation by Vpr was sensitive to Vpr mutation, with the same specificity as before (Compare Figure 3, 4, 5F, S5G-J). HIV-1 GFP bearing Vpr F34I/P35N, or Vpr Q65R, failed to efficiently suppress IRF3 nuclear localization after cGAMP stimulation (Figure 5F, S5G) or after transfection of differentiated THP-1 with HT DNA (Figure 5G, S5H). Conversely, HIV-1 GFP bearing wild type Vpr, or Vpr R80A, effectively suppressed IRF3 nuclear localization after stimulation with cGAMP or HT DNA (Figure 5F, G S5G, H). Similar inhibition specificity by Vpr was also seen after activation of IRF3 nuclear translocation by transfection with the RNA mimic poly I:C (Figure S5I, J). Thus, suppression of IRF3 nuclear translocation correlates with the capacity of Vpr mutants to antagonize innate immune activation.

Vpr inhibits NF-κB p65 nuclear translocation and NF-κB sensitive plasmid expression

DNA sensing by cGAS is known to activate NF-κB as well as IRF3 (Fang et al., 2017). To test whether Vpr influenced NF-κB activation we repeated the experiment in Figure 1C-F but using THP-1 cells bearing an NF-κB -luciferase reporter (THP-1 NF-κB-luc) (Figure 6A-C). VSV-G pseudotyped HIV-1 GFP vector bearing Vpr minimally activated NF-κB-luc expression, whereas Vpr negative HIV-1 GFP activated NF-κB-luc expression effectively (Figure 6A). Activation was dependent on viral genome because similar doses of HIV-1 VLP, made without genome, did not induce NF-κB-luc expression (Figure 6A). Viral doses were equalized by measurement of RT activity (SGPERT) (Jolien Vermeire et al., 2012). Vpr bearing, and Vpr negative, HIV-1 GFP were equally infectious and genome-free VLP were not infectious, as expected (Figure 6B). VSV-G pseudotyped HIV-1 GFP bearing Vpr, but not virus lacking Vpr, suppressed cGAMP-mediated activation of the NF-κB-sensitive gene *IL6* (Figure 6C). We could not detect NF-κB nuclear localization in THP-1 after cGAMP treatment, perhaps due to timing, so we tested mutant Vpr specificity using poly I:C to stimulate NF-κB nuclear localization. Again, we transfected differentiated THP-1 cells, this time with Poly I:C and then immediately infected them with HIV-1 GFP bearing or lacking Vpr and fixed and stained for NF-κB localisation three hours later. We found Vpr inhibited NF-κB nuclear localisation with similar sensitivity to mutation as for IRF3: VLP

bearing wild type Vpr or Vpr R80A inhibited NF-κB nuclear localisation but VLP bearing Vpr F34I/P35N or Vpr Q65R did not (Figure 6D, S6B).

Previous work has shown that Vpr inhibits the activity of the human CMV major immediate early promoter (MIEP) (Liu et al., 2015). We hypothesized that this effect may be due to the dependence of this promoter on NF-κB (DeMeritt et al., 2004). As expected Flag-Vpr expression suppressed GFP expression from a co-transfected CMV MIEP – GFP construct (Figure 6E) as well as several other NF-κB sensitive constructs expressing luciferase (Figure S6A). Importantly, Vpr mutants F34I/P35N, and Vpr Q65R suppressed GFP expression much less effectively than WT Vpr, or Vpr R80A, consistent with this effect being due to inhibition of NF-κB nuclear entry (Figure 6E, S6D, E). To probe this further, we used two constructs lacking NF-κB binding sites in which GFP is driven from the Ubiquitin C (Ub) promoter (Matsuda & Cepko, 2004) or from the elongation factor 1 alpha (EF1α) promoter (Matsuda & Cepko, 2004). Expression of GFP from these constructs was minimally affected by Vpr co-transfection, but GFP expression from the CMV MIEP was reduced as before (Figure 6F). Importantly, CMV MIEP-GFP expression was induced by activation of NF-κB with exogenous tumour necrosis factor alpha (TNFα) whereas Ub-GFP and EF1α-GFP were not, providing further evidence that Vpr inhibition correlated with promoter sensitivity to NF-κB (Figure 6G, S6E-F). Thus, inhibition of NF-κB nuclear transport by Vpr likely explains the observation that Vpr suppresses expression from the CMV MIEP, but not promoters that are independent of NF-κB activity for expression. This is important because previous studies have used Vpr co-transfection with CMV MIEP driven promoters to address Vpr function (Su et al., 2019).

HIV-1 Vpr interacts with karyopherins and inhibits NF-κB (p65) and IRF3 recruitment

WT Vpr suppresses nuclear entry of IRF3 and NF-κB, but Vpr DCAF1 binding mutant Q65R does not (Figure 5, 6). This suggested that Vpr might degrade particular nuclear transport proteins to exert its effect. We therefore tested whether Vpr expression caused degradation of karyopherins KPNA1, KPNA2, KPNA3, KPNA4, KPNA5, KPNA6 or KPNB1. We infected cells with Vpr encoding HIV-1 vector, extracted total protein 48 hours after infection, and detected each protein using immunoblot (Figure 7A). However, we did not detect reduced levels of any of these karyopherins. It is possible that Vpr recruits karyopherins but does not degrade them. To test this, we sought interaction between Vpr and karyopherins KPNA1, KPNA2 and KPNA3 by co-immunoprecipitation. We found that immunoprecipitation of wild type HA-Vpr co-precipitated Flag-KPNA1, as has been reported previously (Miyatake et al., 2016; Nitahara-Kasahara et al., 2007; Vodicka et al., 1998) and to a lesser degree Flag-KPNA2 and Flag-KPNA3, but not Flag-tagged GFP (Figure 7B). In a second experiment we tested whether KPNA1-3 interacted with the inactive Vpr mutant F34I/P35N. WT Vpr interacted with KPNA1 as before, with less efficient interaction with KPNA2 and KPNA3 (Figure 7C). Importantly, KPNA1 interacted with the Vpr F34I/P35N only very weakly,

and much less than WT Vpr, consistent with the mutant's reduced activity in antagonizing innate immune sensing (Figure 7C). Given that Vpr expression did not cause KPNA1 degradation, we sought evidence for Vpr disturbing interactions between KPNA1 and IRF3 or NF-κB p65. HA-IRF3 immunoprecipitated with Flag-KPNA1 as expected and this interaction was reduced by expression of WT Vpr, but not inactive mutant Vpr F34I/P35N (Figure 7D). A competing immunoprecipitation experiment with KPNA1 and NF-κB p65 gave similar results. Immunoprecipitation of Flag-KPNA1 co-precipitated NF-κB p65 and this was reduced by co-expression of WT Vpr, but not Vpr F34I/P35N (Figure 7E). Thus, for the first time, we explain the interaction of Vpr with karyopherins, by demonstrating that it prevents them from efficiently recruiting and transporting transcription factors IRF3 and NF-κB into the nucleus after innate immune activation. This finding provides a mechanistic basis for the broad innate immune antagonism activity of Vpr and links manipulation of nuclear transport with antagonism of innate immunity rather than with infection itself.

Discussion

Despite many studies investigating Vpr function, a clear mechanism for how HIV-1 Vpr promotes replication in macrophages has not been forthcoming, partly because Vpr replication phenotypes have not been clearly mechanistically linked to manipulation of specific target proteins. Early work connected nuclear membrane association of Vpr with replication in macrophages but not T cells (Connor et al., 1995; Dedera et al., 1989; Fouchier et al., 1998; Hattori et al., 1990; Mashiba et al., 2014; Vodicka et al., 1998). Early work also separated the effect of Vpr on cell cycle from its association with the nuclear envelope using Vpr mutants, particularly Vpr F34I, which, as confirmed herein, suppressed cell cycle, but did not recruit to the nuclear membrane (Jacquot et al., 2007; Vodicka et al., 1998). Vpr mutants that did not localise to the nuclear membrane did not promote macrophage replication, leading the authors to reasonably conclude that Vpr contributed to nuclear transport of the virus itself. This observation was consistent with the notion that a Vpr role supporting nuclear entry is expected to be more important in non-dividing cells (macrophages), than rapidly dividing cells (activated T cells). Vpr is also not typically required for infection of cell lines, even if they are not dividing (Yamashita & Emerman, 2005). Vpr has been linked to nuclear transport through karyopherin binding, but again, this function has not been clearly linked to a mechanism of replication enhancement, other than the hypothetical connection between Vpr and nuclear transport of the virus itself (Jacquot et al., 2007; Nitahara-Kasahara et al., 2007a; Popov et al., 1998; Vodicka et al., 1998).

In complementary studies, Vpr has been associated with antagonism of innate immune sensing in macrophages (Harman et al., 2015), T cells (Vermeire et al., 2016), as well as in HeLa cells reconstituted for DNA sensing by STING expression (Trotard et al., 2016). Here we propose a model that unifies Vpr's role in manipulating nuclear entry with its antagonism of innate immune signalling. We propose that Vpr interacts with karyopherin KPNA1 (Figure 7) to inhibit nuclear

transport of activated IRF3 and NF-κB (Figure 5-7) and subsequent gene expression changes downstream of innate immune sensing (Figures 1-3). Thus, HIV-1 Vpr antagonizes the consequences of innate immune activation by HIV-derived, and non-HIV derived PAMPs alike, explaining its importance for maximal replication in macrophages because activated T cells, and most cell lines, respond to innate immune agonists poorly, particularly DNA based PAMPs (Figure 1) (Cingöz & Goff, 2019; de Queiroz et al., 2019; Heiber & Barber, 2012; Xia et al., 2016; Xia et al., 2016). We propose that previous demonstrations of Vpr dependent HIV-1 replication in macrophages, that depended on Vpr-NPC association, or nuclear transport factors, are explained by Vpr inhibition of innate immune sensing and subsequent antiviral responses (Jacquot et al., 2007; Vodicka et al., 1998). For example, induction of an innate response by HIV-1 lacking Vpr might be expected to suppress viral nuclear entry because MxB induction in macrophages by IFN causes inhibition of HIV-1 nuclear entry (Goujon et al., 2013; Kane et al., 2013). Indeed, we hypothesise that Vpr provides an *in vivo* replication advantage because activation of IRF3 and NF-κB induces expression of inflammatory cytokines, including type 1 IFNs, and subsequently restriction factors for which HIV-1 does not encode antagonists. For example, in addition to MxB, IFN induces IFITM1-3 (Foster et al., 2016) and TRIM5α (Jimenez-Guardeño et al., 2019) all of which can inhibit HIV-1. Concordantly, accidental infection of a lab worker with a Vpr-defective HIV-1 isolate resulted in delayed seroconversion, suppressed viremia and normal T-cell counts without need for anti-viral treatment (Ali et al., 2018).

In most of the experiments herein, and in previous studies of Vpr function in cell lines (Yamashita & Emerman, 2005), Vpr did not impact infection of single round VSV-G pseudotyped HIV-1 vectors encoding GFP. We propose that this is because if antiviral inflammatory responses, e.g. IFN, are triggered at around the time of infection, either by exogenous signals, or by HIV-1 itself, then the activated antiviral effectors are too slow to inhibit that infection, ie the expression of GFP from an integrated provirus. Thus, a requirement for Vpr is only revealed by spreading infection assays in innate competent cells such as macrophages, which can suppress replication of subsequent rounds of infection.

We find that Vpr can promote HIV-1 replication, even if the innate immune stimulation does not originate from an HIV-1 derived PAMP, here exemplified by cGAMP treatment (Figure 1). We found that Vpr also antagonised the effects of exposure to LPS, RNA and DNA ligands, as well as other viral infections, exemplified here by Sendai virus infection, which whilst not a human virus, potently activates RNA sensing and IFN production in human macrophages (Matikainen et al., 2000)(Figure 2). We hypothesise that Vpr has evolved a mechanism of broad specificity innate immune inhibition to allow suppression of signals connected indirectly to infection. For example, HIV seroconversion has been associated with a cytokine storm (Stacey et al., 2009) and this may be mitigated by particle associated Vpr. Association between escape from innate sensing and successful

transmission is suggested by evidence for generally low HIV transmission frequency (Shaw & Hunter, 2012), HIV founder clones being particularly resistant to IFN (Iyer et al., 2017) as well as the transmission associated cytokine cascade (Stacey et al., 2009). Concordantly, Vpu, Nef and Vif, and Vpr, antagonize innate immunity to enhance viral replication, reviewed in Sumner et al., 2019.

Vpr has been suggested to cause IRF3 degradation (Okumura et al., 2008) but we did not detect IRF3 degradation in THP-1 cells under conditions when gene expression and IRF3 nuclear transport were strongly suppressed (Figure 5). Furthermore, in addition to suppressing IRF3 nuclear transport, we found that Vpr reduced IRF3 phosphorylation at S396 but not at S386 (Figure 5). Previous studies have suggested that phosphorylation of IRF3 at S386 is necessary and sufficient for IRF3 activation (Lin et al., 1999; Mori et al., 2004; Schirrmacher, 2015; Servant et al., 2003; Suhara et al., 2000; Yoneyama et al., 1998). Thus our data are consistent with a more complex picture of IRF3 activation by phosphorylation. It is possible that phosphorylation at S396 occurs in a karyopherin or NPC-dependent way that is occluded by Vpr recruitment to karyopherin. Phosphorylation of IRF3 at S396 has been associated with enhanced association and multimerization with transcriptional coactivator CREB binding protein (CBP/p300) suggesting a later role than phosphorylation at S386 (Chen et al., 2008). It is possible that the lack of S396 IRF3 phosphorylation is a consequence of IRF3 dephosphorylation at S396 as nuclear entry is prevented.

Inhibition of IRF3 phosphorylation is also consistent with reported inhibition of TBK1 by Vpr although this study detected inhibition of TBK phosphorylation, whereas we did not (Harman et al., 2015). In that study, Vpr promoted infection in macrophages and dendritic cells, despite HIV induced formation of innate immune signalling complexes containing TBK1, IRF3 and TRAF3, visualised by immunofluorescence staining. Thus TBK1 inhibition by Vpr may occur in addition to Vpr activity on nuclear transport, because TBK1 is seen in the cytoplasm, not at the nuclear envelope, in these HIV infected cells (Harman et al., 2015). IRF3 degradation was not detected in this study and nor was HIV-1 induced IRF3 phosphorylation, although the impact of infection on IRF3 by wild type HIV-1 and HIV-1 deleted for Vpr were not compared.

The regulation of the nuclear import of NF-κB and IRF3 by multiple karyopherins is expected to be complex (Fagerlund et al., 2005, 2008; Kumar et al., 2002; Liang et al., 2013). Targeting karyopherins is a typical viral strategy for manipulation of cellular responses but the different ways viruses perform this function hints at the complexity required to inhibit innate responses whilst avoiding shutting down viral transcription. For example, Japanese encephalitis virus NS5 targets KPNA2, 3 and 4 to prevent IRF3 and NF-κB nuclear translocation (Ye et al., 2017). Hantaan virus nucleocapsid protein inhibits NF-κB p65 translocation by targeting KPNA1, -2, and -4 (Taylor et

al., 2009). Most recently, vaccinia virus protein A55 was shown to interact with KPNA2 to disturb its interaction with NF-κB (Pallett et al., 2019). Hepatitis C virus NS3/4A protein restricts IRF3 and NF-κB translocation by cleaving KPNB1 (importin-β) (Gagne et al., 2017). We propose that the different mechanisms of NF-κB/IRF3 manipulation by different viruses reflect their reliance on transcriptional activation while simultaneously depending on inhibition of the same transcription factors activated by defensive processes. We hypothesise that each virus has specifically adapted to manipulate nuclear transport of transcription factors to facilitate replication while dampening activation of inhibitory effectors. Cell type clearly also plays a role in Vpr function. For example, in monocyte derived dendritic cells, Vpr has been reported to activate NF-κB to drive viral transcription (Miller et al., 2017). A model incorporating context dependent NF-κB activation or inhibition, depending on life cycle stage and cell type, could explain apparently contradictory reports that Vpr both inhibits (Ayyavoo et al., 1997; Kogan et al., 2013), but also activates NF-κB (Liu et al., 2014; Liu et al., 2013; Vermeire et al., 2016). One possibility to explain specific inhibition of NF-κB by incoming particle associated Vpr, but not Vpr expressed in the context of infection, is that once the provirus is formed, and Gag is expressed, Gag recruits Vpr to viral particles to reduce further manipulation of NF-κB that is required for on-going viral transcription (Belzile et al., 2010).

Vpr has previously been shown to interact with a variety of mouse (Miyatake et al., 2016), yeast (Vodicka et al., 1998) and human karyopherin proteins including human KPNA1, 2 and 5 (Nitahara-Kasahara et al., 2007). Indeed, the structure of a C-terminal Vpr peptide (residues 85-96) has been solved in complex with mouse importin α2 (Miyatake et al., 2016) although this study did not shed light on mechanism of innate immune manipulation by Vpr because this Vpr peptide is distant from residues 34/35 shown to impact sensing (Figures 3, 5-7) and nuclear membrane localisation (Figure 4). Here we confirm an interaction with KPNA1 by co-immunoprecipitation and confirm that this interaction is reduced by Vpr mutation F34I/P35N (Figure 7). Critically, we demonstrate that wild type Vpr, but not Vpr F34I/P35N, inhibits recruitment of IRF3 and NF-κB explaining inhibition of transcription factor nuclear entry. Failure to degrade karyopherin proteins suggests that some KPNA1 nuclear import function may be left intact by the virus to facilitate a more subtle manipulation of host cell biology (Figure 7). A similar model of inhibition of KPNA target binding to manipulate nuclear import has been suggested by a crystal structure of Ebola Virus VP24 protein in complex with KPNA5. This study proposed that VP24 targets a KPNA5 NLS binding site to specifically inhibit nuclear import of phosphorylated STAT1 (Xu et al., 2014).

Our data also explain previous reports of the suppression of expression from co-transfected CMV MIEP-driven plasmids by Vpr (Liu et al., 2015). Vpr inhibition of NF-κB transport into the nucleus to activate the MIEP likely explains these data, but another possibility is that transcription factor bound to cytoplasmic plasmid DNA has a role in importing plasmid into the nucleus, and it is plasmid transport that is inhibited (Mesika et al., 2001). Vpr insensitivity of NF-κB-independent

ubiquitin and EF1α promoters (Figure 6) is consistent with this model, summarized in Figure S7. This is important because inhibition of transfected plasmid driven protein expression may explain the effect of cotransfected SIV Vpr on STING and cGAS signaling reported recently (Su et al., 2019). Note that STING expression was not affected by Vpr co-expression but STING was expressed from the Vpr and NF-κB-insensitive EF1α promoter (Figure 6), whereas cGAS, which was not measured by western blot, was expressed from a Vpr and NF-κB-sensitive (Figure 6) CMV driven plasmid VR1012 (Hartikka et al., 1996).

Importantly, our data are consistent with reports that manipulation of cell cycle by Vpr is independent of interaction with karyopherin proteins. The Vpr R80A mutant, which does not arrest cell cycle, or manipulate SLX4 complex (Gaynor & Chen, 2001; Laguette et al., 2014) was functional in inhibition of innate sensing (Figures 3, 5, 6). Mapping the residues of Vpr that are important for innate immune inhibition onto structures resolved by NMR and X-ray crystallography reveals a potentially distinct interface from that targeting UNG2 because residues Vpr 34/35 are distant from the UNG2 binding site (Figure S3B, S3C). Given that Vpr has been shown to bind FxFG motif in p6 of Gag during virion incorporation (Zhu et al., 2004), and FG motifs at the NPC (Fouchier et al., 1998) it is possible that interaction of Vpr with nuclear pore proteins via the FG motifs contribute to Vpr mediated inhibition of IRF3 and NF-κB nuclear import.

Our data are consistent with a model in which HIV-1 particle associated Vpr can suppress the consequences of sensing (Figures 1, 3B, 5C, 6A, B). Higher amounts of activation, caused by global activation of cells by externally derived PAMPs, simulated here by transfection of Poly:IC, DNA treatment with LPS, or infection with Sendai virus, can also be suppressed by Vpr bearing viral particles, here best evidenced by measurements of IRF3 and NF-κB nuclear localisation (Figures 5 and 6). Given that infection typically depends on exposing cells to more than one viral particle, requiring 10s of particles in even the most conservative estimates, it is likely that Vpr delivered by particles that do not eventually form a provirus, contributes to suppression of sensing. Certainly a lower MOI is required for Vpr activity when the stimulation comes from the Vpr bearing viral particles themselves, compare external stimulus (MOI 20 required, Figure 1B) and virus associated stimulus (MOI 3 required, Figure 1C)

We and others, have argued that the genome of wild type HIV-1 is not efficiently sensed by nucleic acid sensors, or degraded by cellular nucleases, because the capsid protects the HIV-1 genome, and regulates the process of reverse transcription, during transport across a hostile cytoplasmic environment, prior to uncoating at the NPC, or in the nucleus of infected cells (Bejarano et al., 2019; Burdick et al., 2017; Francis et al., 2016; Jacques et al., 2016; Rasaiyaah et al., 2013; Schaller et al., 2011; Sumner et al., 2019; N. Yan et al., 2010; Zila et al., 2019). Cingoz et al reported failure of VSV-G pseudotyped HIV-1 (ΔEnv, ΔNef, ΔVpr) to activate sensing in a variety

of cell lines (Cingöz & Goff, 2019). However, other studies have demonstrated sensing of wild type HIV-1 DNA by cGAS (Gao et al., 2013; Lahaye et al., 2013), and here we observed cGAS-dependent, Vpr-sensitive, induction of CXCL10 or NF-κB reporter by high dose (MOI 3) VSV-G pseudotyped single round HIV-1 GFP vector in THP-1 cells (Figure 1, 6). We assume that virus dose is the most important difference between studies. Cingoz used luciferase to measure infection and therefore MOIs are obscure. Note that herein, MOI calculated by GFP expression is included in supplementary data for most experiments. We propose that both capsid and Vpr have a role in preventing HIV-1 stimulating innate immune sensing but that Vpr can suppress stimulation from external sources.

In vitro, primary myeloid cells behave according to the stimuli they have received. Thus, inconsistent results between studies, for example the requirement here for cGAMP, but not in other studies, to cause Vpr dependent replication in macrophages (Figure 1), could be explained by differences in myeloid cell stimulation due to differences in cell purification and differentiation methods or reagents used. Methods of virus preparation, here viruses were purified by centrifugation through sucrose, may also be a source of target cell activation and experimental variation. We hypothesise that cGAMP induced Vpr dependence in MDM (Figure 1) because cells were not activated prior to cGAMP addition, whereas in other studies basal activation produced Vpr dependent replication. Replication in activated primary CD4+ T cells was, in our hands, independent of Vpr in the presence and absence of cGAMP, which was inhibitory, suggesting that Vpr cannot overcome signalling downstream of cGAMP in these cells. This implies that activated T-cells respond differently to cGAMP than macrophages, consistent observations that in T cell/macrophage mixed cultures, the negative effects of cGAMP on HIV-1 replication were principally mediated via macrophages (Xu et al., 2016). Vpr sensitive, cGAS dependent, IFN production from T cells has been reported suggesting that in the right circumstances, T cells can sense HIV-1 DNA, via cGAS, in T cells (Vermeire et al., 2016). Importantly, this study used integration inhibition to demonstrate provirus-dependent detection of HIV-1 suggesting that incoming HIV-1 DNA is not the cGAS target in this study. Certainly, further work is required to understand the different requirements for Vpr function in T cells and macrophages.

In summary our findings connect Vpr manipulation of nuclear transport with inhibition of innate immune sensing, rather than viral nuclear import. They highlight the crucial role of particle associated Vpr in inhibiting innate immune activation during the early stages of the viral life cycle and unify a series of studies explaining previously apparently unconnected observations. Given the complexity of NF-kB activation, and the different ways each virus manipulates defensive transcriptional responses, we propose that the further study of viral inhibition of PAMP-driven inflammatory responses will lead to a better understanding of the biology of the transcription factors involved and highlight novel, tractable targets for therapeutic anti-inflammatory development.

Acknowledgements

We thank Veit Hornung for providing THP-1-IFIT-1 cells wild type and knock outs, Geoffrey Smith for providing constructs encoding KPNA1-3 and Clare Jolly and Richard Sloan for providing NL4.3ΔVpr. This work was funded through an MRC PhD studentship (HK) an MRC Clinical Training Fellowship (CVT), a Wellcome Trust clinical training fellowship (DF), a Wellcome Trust Senior Biomedical Research Fellowship (GJT), the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC (grant HIVInnate 339223) (GJT), a Wellcome Trust Collaborative award (GJT) and was supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre.

Author contributions

HK, CPT, RPS, LZA, LT, DF and GJT conceived the study. HK, RPS, JR, CPT, MTRP, CVT and DS performed experiments. HK, RPS, RSBM and GJT analyzed the data. HK, RPS, RSBM and GJT wrote the manuscript.

Methods

Cells and reagents

HEK293T cells were maintained in DMEM (Gibco) supplemented with 10 % foetal calf serum (FCS, Labtech) and 100 U/ml penicillin and 100 µg/ml streptomycin (Pen/Strep; Gibco). THP-1 cells were maintained in RPMI (Gibco) supplemented with 10% FCS and Pen/Strep. THP-1-IFIT-1 luciferase reporter cells express Gaussia luciferase under the control of the endogenous IFIT1 promoter have been described (Mankan et al., 2014). THP-1 CRISPR control, *cGAS-/-* and *MAVS -/-* knock out cells have been described (Mankan et al., 2014). Nup358 depleted HeLa cells have been described (Schaller et al., 2011). Lipopolysaccharide, poly I:C and TNFα were obtained from PeproTech. Sendai virus was obtained from Charles River Laboratories. Herring-testis DNA was obtained from Sigma. cGAMP was obtained from Invivogen. NF-κB Lucia THP-1 reporter cells were obtained from Invivogen.

Cloning and plasmids

The Vpr gene from HIV-1 founder clone SUMA (Fischer et al., 2010) was codon optimised and synthesised by GeneArt. To generate the HIV-1 vector encoding Vpr (pCSVIG), the codon optimised SUMA Vpr gene was cloned into pSIN-BX-IRES-Em between BamHI and XhoI sites under the control of the SFFV LTR promoter. pSIN-BX-IRES-Em was obtained from Dr Yasuhiro Takeuchi. EF1α-GFP and UB-GFP were obtained from Addgene (Matsuda & Cepko, 2004). The CMV-GFP construct was pEGFPC1 (Clontech). HIV-1 bearing a Ba-L envelope gene has been described (Rasaiyaah et al., 2013). Flag- KPNA1-3 plasmids were obtained from Prof. Geoffrey

609 Smith. HIV-1ΔVpr was a gift from Richard Sloan and encoded an 17 nucleotide insertion (Vpr 64-
610 81) that destroys the Vpr coding sequence.
611
612 **Production of virus in HEK293T cells**
613 Replication competent HIV-1 and VSV-G pseudotyped HIV-1 GFP vectors were produced by
614 transfection of HEK293T cells in T150 flasks using Fugene 6 transfection reagent (Promega)
615 according to the manufacturer's instructions. Briefly, just-subconfluent T150 flasks were
616 transfected with 8.75 µg of HIV-1 YU2 or HIV-1 YU2 lacking Vpr (HIV-1 YU2 ΔVpr) and 30 µl
617 Fugene 6 in 500 µl Optimem (Thermofisher Scientific). To make VSV-G pseudotyped HIV-1 GFP,
618 each T150 flask was transfected with 2.5 µg of vesicular stomatitis virus-G glycoprotein encoding
619 plasmid (pMDG) (Genscript), 2.5 µg of packaging plasmid, p8.91 (encoding Gag-Pol, Tat and Rev)
620 or p8.2 (encoding Gag-Pol, Tat and Rev and Vif, Vpr, Vpu and Nef) (Zufferey et al., 1997), and
621 3.75 µg of GFP encoding genome plasmid (pCSGW) using 30 µl Fugene 6 in 500µl optimum. To
622 make Vpr encoding HIV-1 GFP, 3.75 µg pCSVIG was transfected with 2.5 µg of pMDG and 2.5 µg
623 of p8.91. To make HIV-1 GFP particles bearing Vpr, 1 µg of Vpr expressing pcDNA3.1 (wild type
624 SUMA Vpr or Vpr mutants) was transfected with 2.5 µg of pMDG and 2.5 µg of p8.91 in 30ul
625 Fugene-6 and 500µl Optimem. All virus supernatants were harvested at 48 and 72 h post-
626 transfection, replicate flasks were pooled, and supernatants subjected to ultracentrifugation
627 through a 20% sucrose cushion at 23000 rpm for 2 hours in a 30 ml swingout rotor (Sorvall)
628 (72000G). Viral particles were resuspended in RPMI supplemented with 10% FCS. HIV-GFP
629 produced with p8.91 or p8.2 used in Figure 1 were DNase treated for 2 hours at 37°C (DNaseI,
630 Sigma) prior to ultracentrifugation. Viruses were titrated by infecting THP-1 cells ($2x10^5$ cells/ml)
631 with dilutions of sucrose purified virus in the presence of polybrene (8 µg/ml, Sigma) and incubating
632 for 48 h. GFP-positive, infected cells were counted by flow cytometry using a BD Accuri C6
633 (BDBiosciences). HIV-1 vector encoding shRNA targeting DCAF1 has been described and was
634 prepared as above (Berger et al., 2015).
635
636 **SG-PERT**
637 Viral doses were determined by measuring reverse transcriptase activity of virus preparations by
638 qPCR using a SYBR Green-based product-enhanced PCR assay (SG-PERT) as described (Jolien
639 Vermeire et al., 2012).
640
641 **Isolation of primary monocyte-derived macrophages and CD4+ T cells from peripheral**
642 **blood**
643 Primary monocyte-derived macrophages (MDM) were prepared from fresh blood from healthy
644 volunteers. The study was approved by the joint University College London/University College
645 London Hospitals NHS Trust Human Research Ethics Committee. Primary CD4+ T cells were
646 obtained from leukocyte cones from healthy donors. Peripheral blood mononuclear cells (PBMCs)

were isolated by density gradient centrifugation using Lymphoprep (Stemcell Technologies). For MDM preparation, PBMCs were washed three times with PBS and plated to select for adherent cells. Non-adherent cells were washed away after 1.5 h and the remaining cells incubated in RPMI (Gibco) supplemented with 10 % heat-inactivated pooled human serum (Sigma) and 40 ng/ml macrophage colony stimulating factor (R&D systems). Cells were further washed after 3 days and the medium changed to RPMI supplemented with 10% heat-inactivated human serum (Sigma). MDM were then infected 3-4 days later at low multiplicity of infection. Spreading infection was detected by Gag staining and counting Gag positive cells as described (Rasaiyaah et al., 2013). For CD4+ T cells, untouched CD4+ T cells were purified from PBMCs with an indirect magnetic labeling system (MACS, Miltenyi Biotec), according to manufacturer's instructions. Cells were then cultured with 2 µg/ml of plate-bound anti-CD3 and anti-CD28 monoclonal antibodies (αCD3αCD28 stimulation) (mAbs) (eBioscience) and 25 U/ml of recombinant human interleukin-2 (IL-2; Roche Applied Science) at a concentration of 1.5-2 x 10^6 cells/ml in RPMI supplemented with 10% heat-inactivated Human Serum (HS) (SigmaAldrich). Cells were maintained at 37°C in 5% CO_2 in a humidified incubator for 72 h. CD4+ T cells were then assessed for spreading infection of CXCR4-tropic HIV-1 NL4.3 WT and ΔVPR at low multiplicity of infection (300 mU of HIV-1 RT Activity per 1x10^6 cells). Percentage of HIV-1-infected primary CD4+ T cells was determined by flow cytometry measuring p24Gag antigen employing the monoclonal antibody p24Gag-FITC (HIV-1 p24 (24-4), Santa Cruz Biotechnology).

Innate immune sensing assays

THP-1 cells were seeded in 96 well plates (5x10^5 cells/ml). For Vpr expression, cells were infected with an empty or Vpr expressing (pCSVIG) lentiviral vectors for 40 hours. For stimulation of cells with HT-DNA or poly I:C, 0.2 µl of lipofectamine and 25 µl of Optimem were incubated with HT-DNA or poly I:C (amounts stated in figure legends) for 20 minutes and added to cells. Lipopolysaccharide (1 µg/ml), TNFα (200 ng/ml), Sendai virus (200 HA U/ml) or cGAMP (5 µg/ml) were added directly to the media. For experiments with virion delivered/associated Vpr, cells were stimulated at the time of infection. Gaussia/Lucia luciferase activities were measured 8 hours post cell stimulation/infection by transferring 10 µl supernatant to a white 96 well assay plate, injecting 50 µl per well of coelenterazine substrate (Nanolight Technologies, 2 µg/ml) and analysing luminescence on a FLUOstar OPTIMA luminometer (Promega). Data were normalized to a mock-treated control to generate a fold induction.

ELISA

Cell supernatants were harvested for ELISA at 8 h post-stimulation and stored at -80 °C. CXCL-10 protein was measured using Duoset ELISA reagents (R&D Biosystems) according to the manufacturer's instructions.

ISG qPCR

RNA was extracted from THP-1 cells using a total RNA purification kit (Norgen) according to the manufacturer's protocol. Five hundred ng RNA was used to synthesise cDNA using Superscript III reverse transcriptase (Invitrogen), also according to the manufacturer's protocol. cDNA was diluted 1:5 in water and 2 µl was used as a template for real-time PCR using SYBR® Green PCR master mix (Applied Biosystems) and a 7900HT Real-Time PCR machine (Applied Biosystems). Expression of each gene was normalised to an internal control (*GAPDH*) and these values were then normalised to mock-treated control cells to yield a fold induction. The following primers were used:

GAPDH: Fwd 5'-GGGAAACTGTGGCGTGAT-3', Rev 5'-GGAGGAGTGGGTGTCGCTGTT-3'
CXCL-10: Fwd 5'-TGGCATTCAAGGAGTACCTC-3', Rev 5'-TTGTAGCAATGATCTCAACACG-3'
IFIT-2: Fwd 5'-CAGCTGAGAATTGCACTGCAA-3', Rev 5'-CGTAGGCTGCTCTCCAAGGA-3'
MxA: Fwd 5'-ATCCTGGGATTTTGGGGCTT-3', Rev 5'-CCGCTTGTCGCTGGTGTCG-3'
Viperin: Fwd 5'-CTGTCCGCTGGAAAGTG-3', Rev 5'-GCTTCTTCTACACCAACATCC-3'
IL-6: Fwd 5'- AAATTCGGTACATCCTCGACG-3', Rev 5'- GGAAGGTTCAGGTTGTTTTCT-3'

Immunofluorescence

For confocal microscopy, HeLa cells ($5x10^4$ cells/ml) were seeded into 24-well plates containing sterile glass coverslips. For nuclear translocation assays, we used THP-1 cells ($4x10^5$ cells/ml) adhered in an optical 96-well plate (PerkinElmer) with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Peprotech) for 48 hours. Where cells were infected and transfected (DNA, PolyI:C) or treated (cGAMP) with innate immune stimulants, the cells were treated or transfected first, and then viral supernatant added to the cultures. Cells were then fixed and stained three hours after this. For fixation, HeLa or adhered THP-1 cells were washed twice with ice-cold PBS and fixed in 4% (vol/vol) paraformaldehyde. Autofluorescence was quenched in 150 mM ammonium chloride, the cells permeabilized in 0.1% (vol/vol) Triton X-100 in PBS and blocked for 30 min in 5% (vol/vol) FCS in PBS. Cells were incubated with primary Ab for 1 hour followed by incubation with secondary Ab for 1 hour. Cells were washed with PBS three times between each step. The coverslips were placed on a slide prepared with a 30 µl drop of mounting medium (Vectashield, containing 4',6-diamidino-2-phenylindole (DAPI)) and allowed to set before storing at 4° C. Images were taken on a Leica TCS SPE confocal microscope and analyzed in ImageJ. For IRF3/NF-κB(p65) translocation, images were taken on Hermes WISCAN (IDEA Bio-Medical) and analyzed with Metamorph software (Molecular Devices). Metamorph calculated a translocation coefficient representing the proportion of staining in nuclear versus cytoplasmic compartments. A value of 1 represents "all staining in the nucleus", -1 is "exclusively in cytoplasm" and 0 is "equally distributed".

Primary antibodies were from the following sources: Mouse-anti-FXFG repeats containing

nucleoporins (Mab414) (Abcam), Rabbit-anti-flag (Sigma), Rabbit-anti-IRF3 (Santa Cruz Biotechnology) and Mouse-anti-NF-kB p65 (Santa Cruz Biotechnology). Primary antibodies were detected with Goat-anti-rabbit Alexa Fluor 488 IgG (Invitrogen) or Goat-anti-mouse Alexa Fluor 546 IgG (Invitrogen).

Immunoblotting

For immunoblotting of viral particles, sucrose purified (as described above) virions (1×10^{11} RT units) were boiled for 10 min in 6X Laemmli buffer (50 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 100 mM β-mercaptoethanol) before separating on 12 % polyacrylamide gel. Cells were lysed in lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1 % (v/v) Triton X100, 0.05 % (v/v) NP40 supplemented with protease inhibitors (Roche), clarified by centrifugation at 14,000 x *g* for 10 min and boiled in 6X Laemmli buffer for 10 min. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels. Proteins were transferred to a Hybond ECL membrane (Amersham biosciences) using a semi-dry transfer system (Biorad). Primary antibodies were from the following sources: Rabbit-anti-VSV-G (Sigma), Rabbit-anti-HIV-1 p24 (NIH AIDS reagent program), Rabbit-anti-STING (Cell signaling), Rabbit-anti-pSTING (Cell signaling), Rabbit-anti-TBK1 (Cell signaling), Rabbit-anti-pTBK1 (Cell signaling), Rabbit-anti-IRF3 (Cell signaling), Rabbit-anti-pIRF3-386 (Sigma), Mouse-anti-actin (Abcam), Rabbit-anti-cGAS (Cell Signaling Technology), Mouse-anti-MAVS (Cell Signaling Technology), Rabbit-anti-DCAF1 (Bethyl), Rabbit-anti-Nup358 (Abcam), Mouse-anti-flag (Sigma), Rabbit-anti-GFP (Abcam), KPNA1-6 (ABclonal), KPNB1 (ABclonal), Rabbit-anti-cypB (Abcam), Mouse-anti-FLAG (Sigma), Rabbit-anti-HA (Sigma) and Rabbit-anti-Vpr (NIH). Primary antibodies were detected with goat-anti-mouse/rabbit IRdye 800CW infrared dye secondary antibodies and membranes imaged using an Odyssey Infrared Imager (LI-COR Biosciences).

Cell cycle analysis

WT Vpr or Vpr mutants were expressed in THP-1 cells using pCSVIG at an MOI of 1. Cells were incubated for 48 hours and then washed with PBS and fixed in 1 ml cold 70% ethanol on ice for 30 minutes. To ensure efficient fixing and minimise clumping, ethanol was added dropwise while vortexing. Cell were pelleted in a microfuge and ethanol was removed followed by two wash steps with PBS. To remove RNA from the samples, RNase A (100 μg/ml) was added and the cells were stained with propidium iodide (PI) (50 μg/ml) to stain cellular DNA. Cells were incubated for 10 minutes at room temperature and DNA content analysed by flow cytometry on a BD FACSCalibur (BD Biosciences). The data were analysed with FlowJo.

Generation of Vpr mutants

Site directed mutagenesis was performed using Pfu Turbo DNA Polymerase (Agilent) according to the manufacturer's instructions with the following primers using either pCDNA3.1 or pCSVIG encoding SUMA Vpr as template.

VprF34I+P35N: Fwd 5'-GCCGTGCGGCACATCAACAGACCTTGGCTGCATAGC-3',

Rev 5'GCTATGCAGCCAAGGTCTGTTGATGTGCCGCACGGC-3'

VprQ65R: Fwd 5'-GCCATCATCAGAATCCTGCGGCAGCTGCTGTTCATC-3',

Rev 5'-GATGAACAGCAGCTGCCGCAGGATTCTGATGATGGC-3'

VprR80A: Fwd 5'-GGCTGCCGGCACAGCGCCATCGGCATCACCCCT-3',

Rev 5'-AGGGGTGATGCCGATGGCGCTGTGCCGGCAGCC-3'

Co-immunoprecipitation assays

HEK293T cells were grown in 10 cm dishes and co-transfected with plasmids expressing a FLAG-tagged protein (1 µg KPNA1, KPNA2, KPNA3, GFP or empty vector (EV)) and 1 µg of a plasmid expressing HA-tagged SUMA Vpr wild-type, or Vpr F34I/P35N mutant using 6 µl Fugene-6 (Promega). For KPNA-cargo IPs HEK293T cells were grown in 10 cm dishes and co-transfected with 1 µg of a plasmid expressing FLAG-tagged KPNA1, 1 µg of a plasmid expressing HA-tagged p65 or IRF3 and 1 µg of a plasmid expressing un-tagged Vpr, VprF34I+P35N or empty vector control. After 24 h cells were lysed in lysis buffer (0.5 (v/v) % NP-40 in PBS supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Roche), pre-cleared by centrifugation and incubated with 25 µl of mouse-anti-HA agarose beads (Millipore) or mouse-anti-FLAG M2 agarose affinity gel (Sigma) for 2-4 h. Immunoprecipitates were washed 3 times in 1 ml of lysis buffer and eluted from the beads by boiling in 20 µl of 2X sample buffer containing SDS and β-mercaptoethanol. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (NuPAGE 4-12 % Bis-Tris protein gels, Invitrogen) and detected by immunoblotting.

Statistical analyses

Data were analysed by statistical tests as indicated in the figure legends. * represent statistical significance: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

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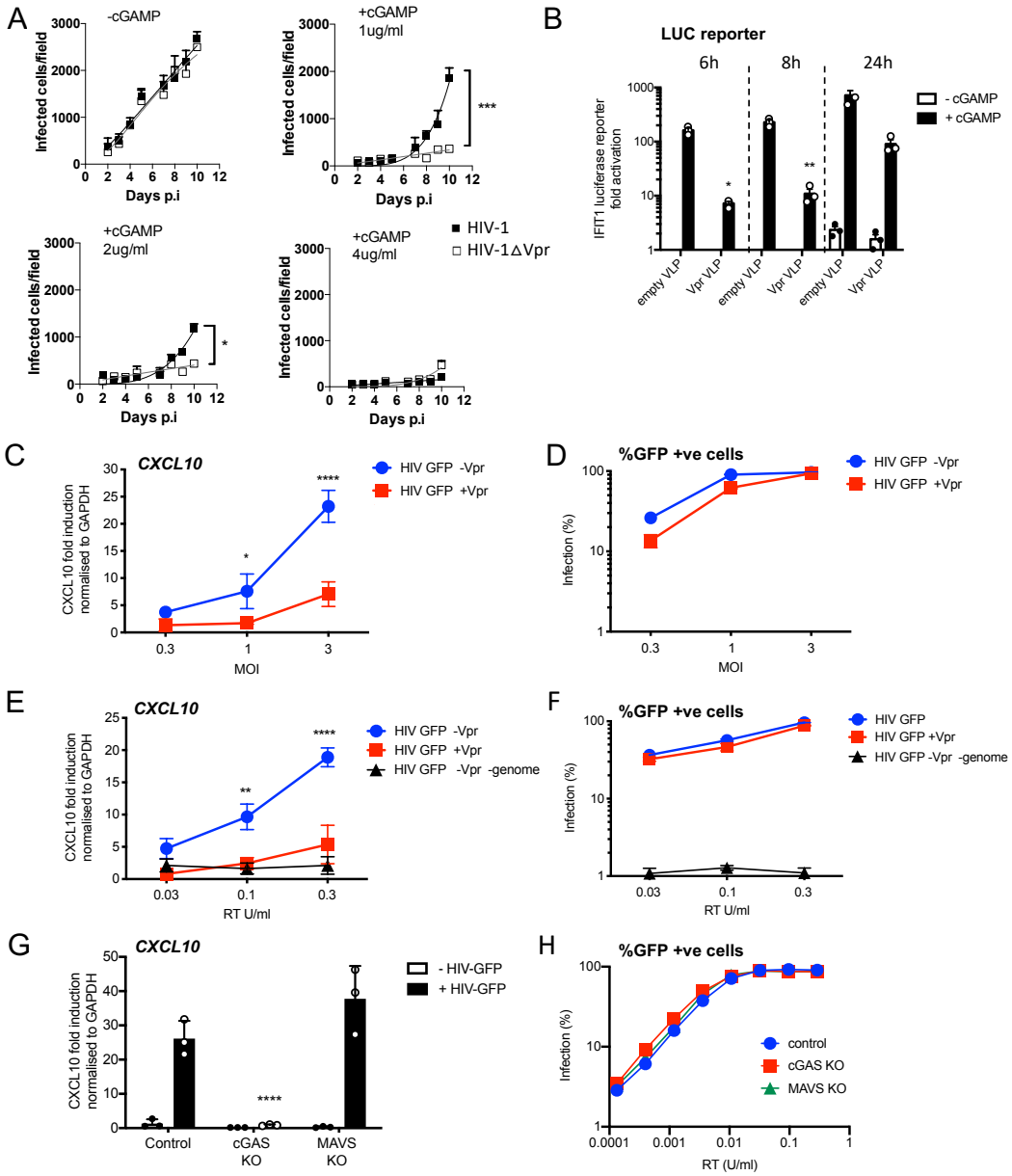

Figure 1 HIV-1 replication in cGAMP stimulated MDMs requires Vpr

(**A**) Replication of WT Yu2 HIV-1 or Yu2 HIV-1ΔVpr in MDMs stimulated with 1 µg/ml, 2 µg/ml or 4 µg/ml cGAMP or left unstimulated, infection measured by counting Gag positive cells stained with anti-p24. Mean+/-SEM n=3 1 and 2 µg/ml cGAMP; n=2 4 µg/ml cGAMP. *** = 2 way ANOVA p value <0.001, * = p<0.05. (**B**) Fold induction of IFIT1-Luc after activation of STING by cGAMP (5 µg/ml) and infection with HIV-1 virus like particles (VLP) lacking genome and bearing Vpr (+Vpr) or lacking Vpr (-Vpr) (1 RT U/ml) in IFIT1-Luc reporter THP-1 cells. cGAMP and virus were added to cells at the same time. (**C**) Fold induction of *CXCL10* after infection of THP-1 cells with HIV-GFP -Vpr or HIV-GFP +Vpr at the indicated MOI. (**D**) Percentage of THP-1 cells infected by HIV-GFP -Vpr or HIV-GFP +Vpr in (C). (**E**) Fold induction of *CXCL10* after infection of THP-1 cells with HIV-GFP -Vpr, HIV-GFP +Vpr or HIV-1 particles lacking Vpr and genome, at indicated doses measured by reverse transcriptase SG-PERT assay. (**F**) Percentage of THP-1 cells infected by HIV-GFP viruses in (E). (**G**) Fold induction of *CXCL10* after infection of unmodified control, cGAS-/- or MAVS-/- THP-1 knock out cells with HIV-GFP lacking Vpr (0.3 RT U/ml). (**H**) Percentage infection of control, cGAS-/- or MAVS-/- THP-1 knockout cells infected with HIV-GFP at indicated doses of RT (SG-PERT).

(B-H) Data are expressed as means ± SD (n = 3) with two-way ANOVA * (p<0.05), ** (p<0.01), *** (p<0.001), **** (p<0.0001) compared to virus without genome (B), HIV GFP+Vpr (C, E) and control (G).

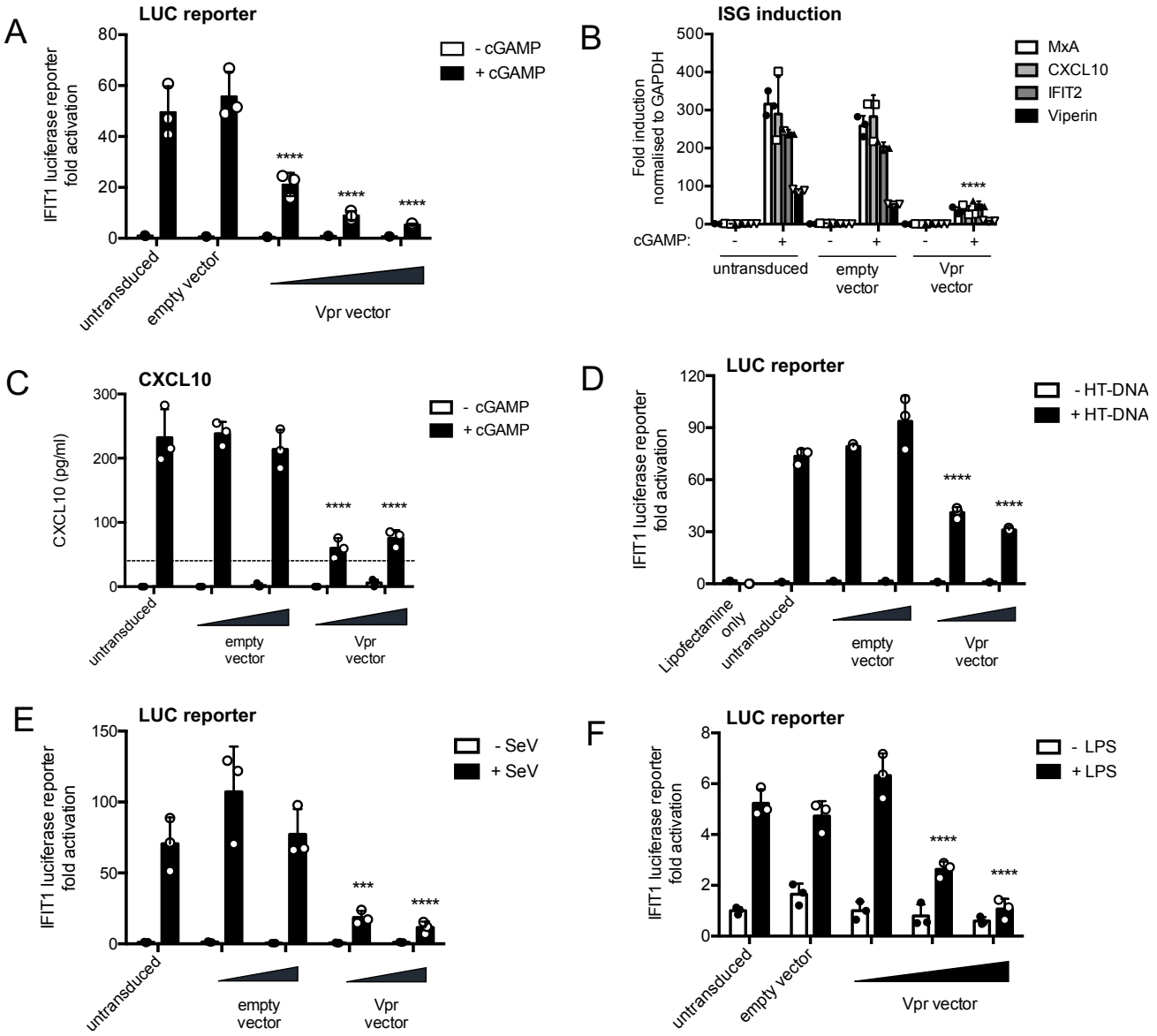

Figure 2 HIV-1 Vpr expression inhibits interferon stimulated gene expression after stimulation with various innate immune stimuli

(**A**) Fold induction of IFIT1-Luc, after activation of STING by cGAMP (5 µg/ml), in IFIT1-Luc reporter THP-1 cells expressing Vpr from a lentiviral vector delivered at MOIs of 0.25, 0.5, 1, or after empty vector transduction (MOI 1) or in untransduced cells. (**B**) Fold induction of ISGs *MxA*, *CXCL10*, *IFIT2* and *Viperin* after activation of STING by cGAMP (5 µg/ml) in cells expressing Vpr from a lentiviral vector (MOI 1), or after empty vector transduction (MOI 1) or in untransduced THP-1 cells. (**C**) Secreted CXCL10 (ELISA) after activation of STING by cGAMP (5 µg/ml) in cells expressing Vpr from a lentiviral vector (MOI 0.5, 1), or after transduction with empty vector (MOI 0.5, 1) or in untransduced THP-1 cells. Dotted line shows limit of detection. (**D**) Fold induction of IFIT1-Luc after HT-DNA transfection (5 µg/ml) of cells expressing Vpr from a lentiviral vector (MOI 0.5, 1), or empty vector (MOI 0.5, 1) or in untransduced IFIT1-Luc reporter THP-1 cells. (**E**) Fold induction of IFIT1-Luc, after Sendai virus infection, of cells expressing Vpr from a lentiviral vector (MOI 0.5, 1), or after transduction by empty vector (MOI 0.5, 1) or in untransduced IFIT1-Luc reporter THP-1 cells. (**F**) Fold induction of IFIT1-Luc, after LPS treatment (1 µg/ml), of cells expressing Vpr from a lentiviral vector (MOI 0.25, 0.5, 1), after transduction by empty vector (MOI 1) or in untransduced IFIT1-Luc reporter THP-1 cells.
Data are expressed as mean ± SD (n = 3) analysed using two-way ANOVA * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$) compared to data for empty vector. n= 3 (A, D-F) or 2 (B-C) independent experiments.

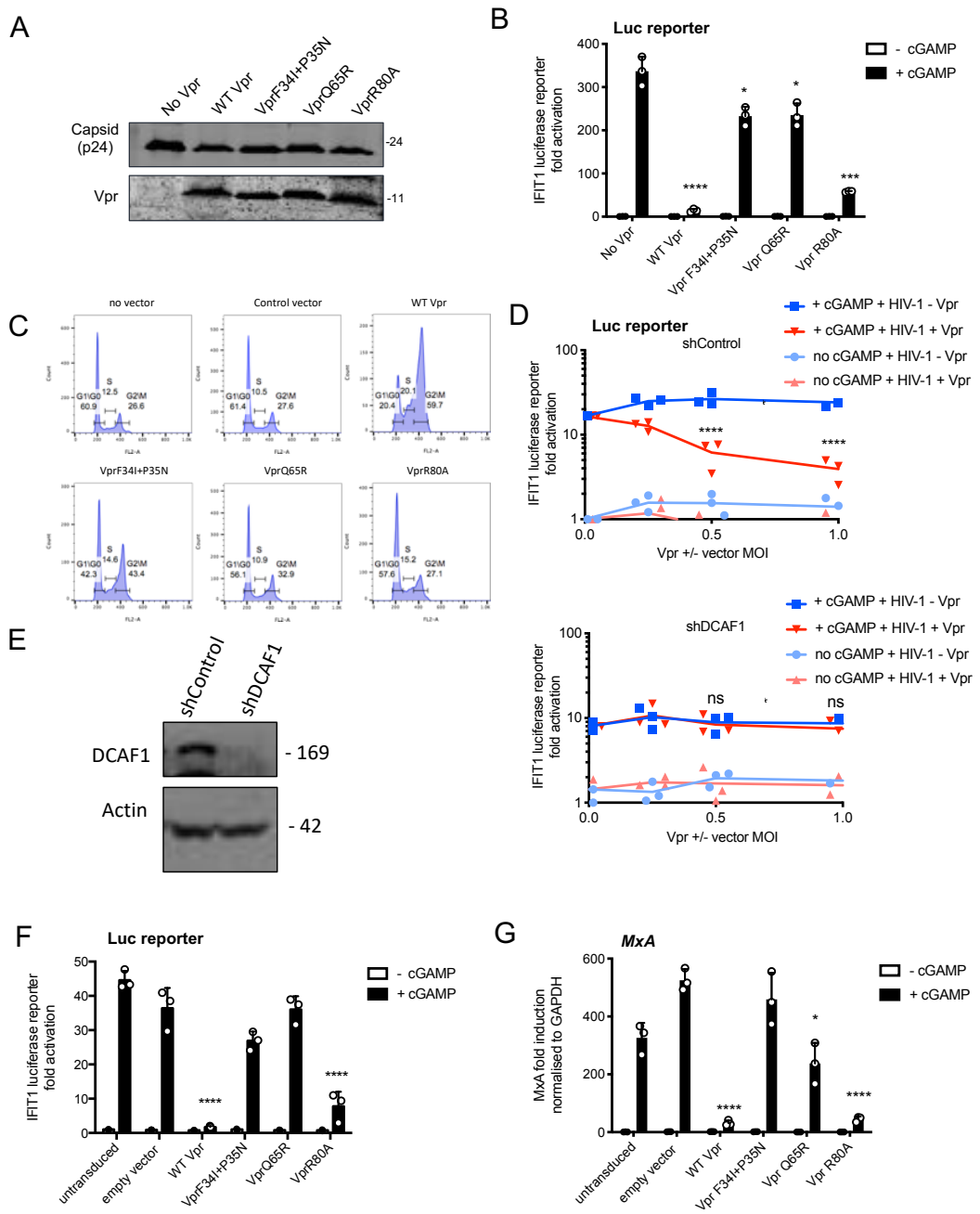

Figure 3 Vpr inhibition of innate immune activation is dependent on DCAF1 but independent of cell cycle arrest

(**A**) Immunoblot detecting p24 (capsid) or Vpr in pelleted VSV-G pseudotyped VLP lacking genome used in (B). (**B**) Fold induction of IFIT1-Luc after activation of STING by cGAMP (5 µg/ml) and infection with VLP bearing WT or mutant Vpr, or lacking Vpr (1 RT U/ml) in THP-1. Cells were infected at the same time as cGAMP treatment. (**C**) Flow cytometry showing cell cycle phases of THP-1 transduced with an empty vector, WT Vpr, or mutant Vpr, encoding vector (MOI 1) or left untransduced as a control, stained with propidium iodide to label DNA. Percentage cells in each cell cycle stage are shown. (**D**) Fold induction of IFIT1-Luc after activation of STING by cGAMP (5 µg/ml) in cells expressing Vpr from a lentiviral vector, or expressing empty vector, or in untransduced THP-1 expressing a control, or a DCAF1 targeting shRNA. Mean +/-SEM n=3 independent experiments. (**E**) Immunoblot detecting DCAF1, or actin as a loading control, from extracted THP-1 cells expressing a non-targeting, or DCAF1-targeting, shRNA. (**F**) Fold induction of IFIT1-Luc after activation of STING by cGAMP (5 µg/ml) in cells expressing WT, or mutant, Vpr from a lentiviral vector (MOI 1), or empty vector (MOI 1) or in untransduced THP-1. (**G**) Fold induction of *MxA* mRNA after activation of STING by cGAMP (5 µg/ml) in cells

expressing WT, or mutant, Vpr from a lentiviral vector (MOI 1), or after transduction by empty vector (MOI 1) or in untransduced THP-1. Data are mean ± SD (n = 3). Two-way ANOVA: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$) compared to no Vpr or empty vector controls. Data are representative of three (B-D, F) or two (A, E, G) independent experiments.

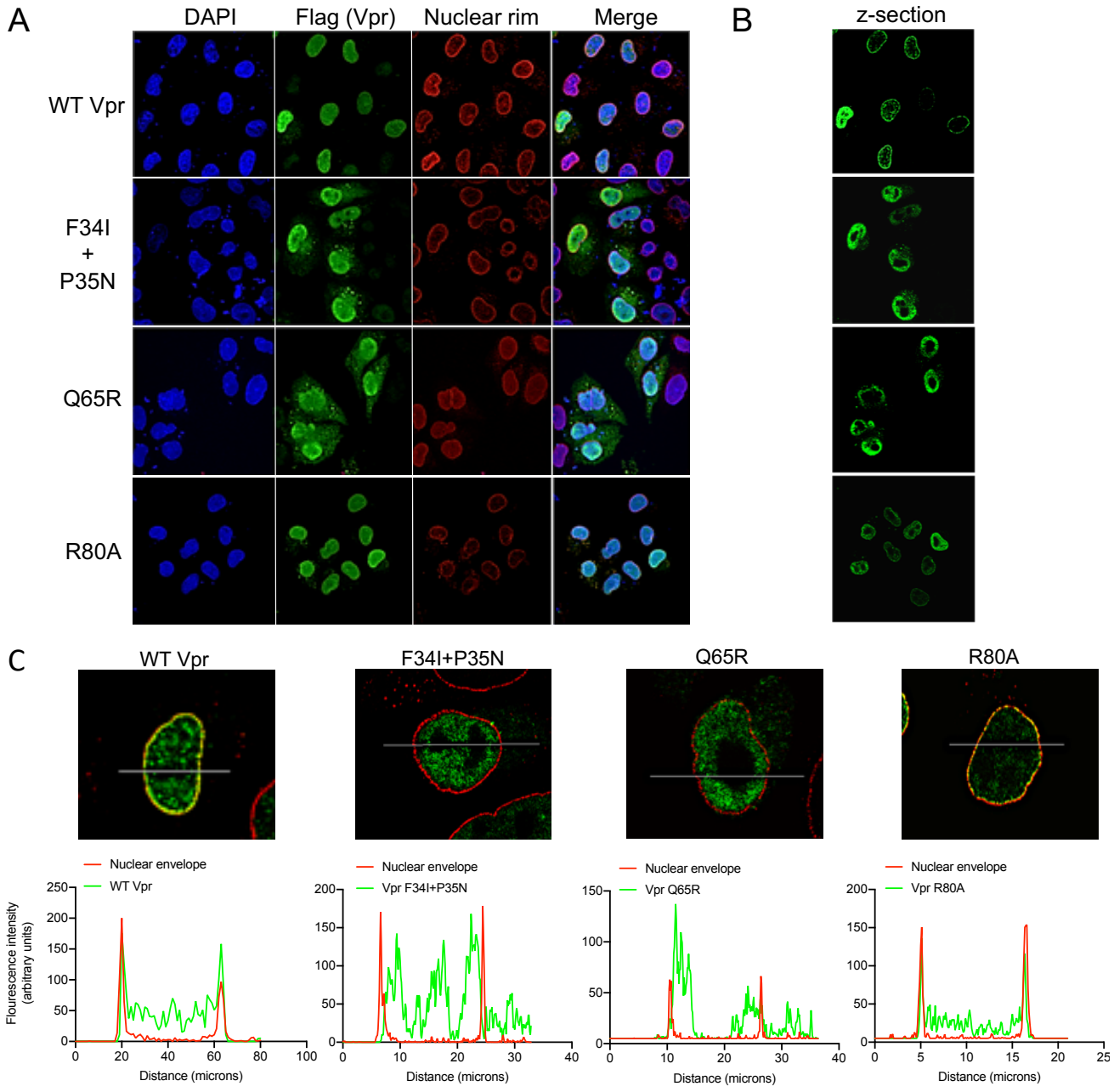

Figure 4 Wild Type Vpr, but not sensing antagonism inactive Vpr mutants, localise to nuclear pores

(**A**) Immunofluorescence confocal projections of HeLa cells transfected with Flag-tagged WT, or mutant, Vpr encoded by pcDNA3.1 plasmid (50 ng) and stained using antibodies detecting the Flag-tag (green) or nuclear pore complex (mab414) (red). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) stains nuclear DNA (Blue). (**B**) Selected confocal images (z-section) of cells in (A) showing effect of Vpr mutation on Vpr colocalization with mab414 nuclear pore staining. (**C**) Assessment of colocalization of Vpr with mab414 nuclear pore staining.

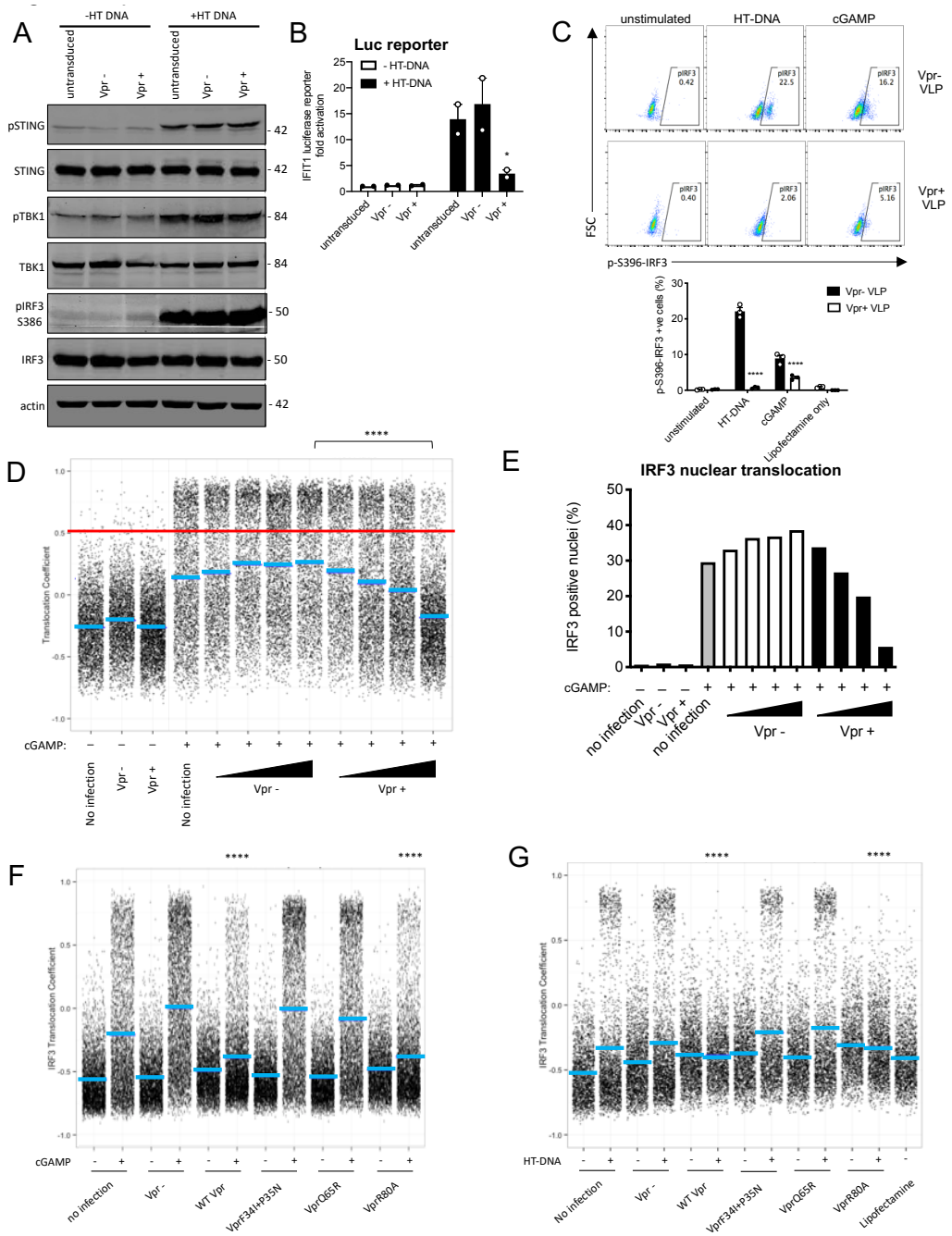

Figure 5 Vpr inhibits IRF3 nuclear translocation

(A) Immunoblot detecting Phospho-STING (Ser366), total STING, phospho-TBK1 (Ser172), total TBK1, phospho-IRF3 (Ser386), total IRF3, or actin as a loading control, from extracted THP-1 cells expressing Vpr from a lentiviral vector, or empty vector (MOI 1), or THP-1 left untransduced as a control and transfected with HT-DNA (5 µg/ml) or left untransfected as a control. Size markers are shown in kDa. (**B**) Mean fold induction of IFIT1-Luc in cells from Figure 5A and Figure S5B (**C**) Flow cytometry plot (forward scatter vs pIRF3-S396 fluorescence) of THP-1 cells stimulated with cGAMP (5 µg/ml) or HT-DNA transfection (5 µg/ml) and then immediately infected with Vpr bearing virus-like particles (VLP) lacking genome (1 RT U/ml), or Vpr free VLP and fixed three hours after infection. Lower panel shows the flow cytometry data as a bar graph, plotting pIRF3-S396 positive cells. (**D**) Single cell immunofluorescence measurement of IRF3 nuclear translocation in PMA differentiated THP-1 cells treated with cGAMP, or left untreated, and then immediately infected with HIV-1 GFP bearing Vpr, lacking Vpr or left untransduced. Cells were fixed and stained three hours after infection. Red line shows the translocation coefficient threshold. Blue lines represent mean translocation coefficient. (**E**) Percentage of cells in Figure 5D with IRF3 translocation coefficient greater than 0.5 (above red line). (**F**) Single cell

immunofluorescence measurement of IRF3 nuclear translocation in PMA differentiated THP-1 cells stimulated with cGAMP (5 µg/ml), or left unstimulated, and then immediately infected with HIV-1 GFP lacking Vpr or bearing WT Vpr or Vpr mutants as shown (1 RT U/ml) or left uninfected. (**G**) Single cell immunofluorescence measurement of IRF3 nuclear translocation in PMA differentiated THP-1 cells transfected with HT-DNA (5 µg/ml), or left untransfected, and immediately infected with HIV-1 GFP lacking Vpr, or bearing WT or mutant Vpr (1 RT U/ml) or left uninfected.

Data in B is expressed as means ± SEM (n = 2). Data is analysed using two-way ANOVA: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$) compared to data from infection with HIV-1 lacking Vpr. Data are representative of three (C–G) or two (A, B) independent experiments.

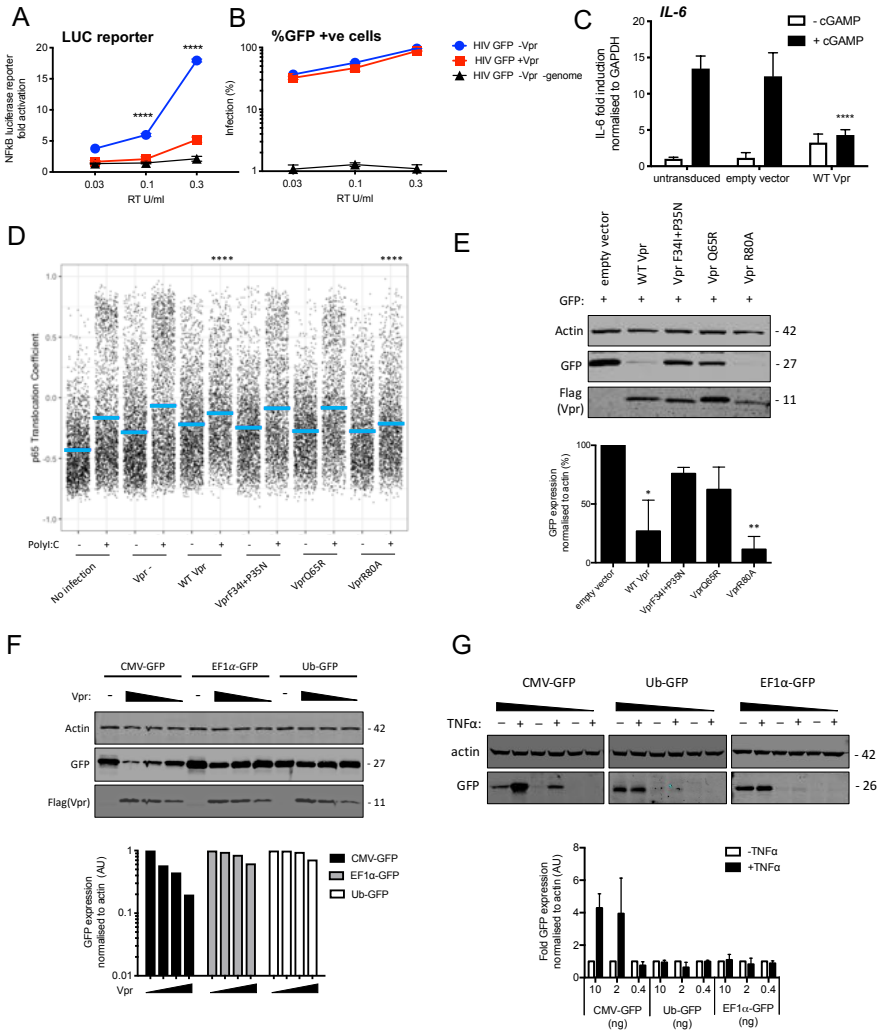

Figure 6 Vpr inhibits NF-κB p65 nuclear translocation and NF-κB sensitive plasmid expression

(**A**) Fold induction of NF-κB-Luc after infection of THP-1 cells with HIV-GFP lacking Vpr, HIV-GFP bearing Vpr, or HIV-GFP lacking Vpr and genome, at the indicated doses. (**B**) Percentage of THP-1 cells in (A). (**C**) Fold induction of *IL-6* after activation of STING by cGAMP (5 µg/ml) in cells expressing empty vector or Vpr encoding vector (MOI 1), or in untransduced THP-1 cells. (**D**) Single cell immunofluorescence measurement of NF-kB (p65) nuclear translocation in PMA differentiated THP-1 cells transfected with Poly I:C (50 ng/ml), or left untreated, and infected with HIV-1 GFP lacking Vpr, HIV-1 GFP bearing Vpr (1 RT U/ml) or left uninfected. Cells were stained three hours after transfection and infection. (**E**) Immunoblot detecting Flag-Vpr, GFP, or actin as a loading control, from HEK293T cells transfected with 50 ng of empty vector, Flag-tagged WT Vpr vector, or Flag-tagged mutant Vpr vector, and CMV-GFP vector (50 ng). Size markers are shown in kDa. GFP expression from two independent immunoblots was quantified by densitometry and is shown in the lower panel. (**F**) Immunoblot detecting Flag-Vpr, GFP, or actin as a loading control, from HEK293T cells transfected with empty vector (200 ng) or Vpr vector (50ng, 100ng, 200ng) and CMV-GFP, EF1α-GFP or Ub-GFP plasmids (50 ng). Size markers are shown in kDa. GFP expression quantified by densitometry is shown in the lower panel. (**G**) Immunoblot detecting GFP, or actin as a loading control, from HEK293T cells transfected with CMV-GFP, EF1α-GFP or Ub-GFP plasmids (10 ng, 2 ng, 0.4 ng) and stimulated with TNFα (200 ng/ml) or left unstimulated. Size markers are shown in kDa. GFP expression, from two independent immunoblots, quantified by densitometry, is shown in the lower panel.

Data in (A, B, C) is expressed as mean ± SD (n = 3). Data in (E, F, G) is expressed as mean ± SD (n=2). Two-way ANOVA: * (p<0.05), ** (p<0.01), *** (p<0.001), **** (p<0.0001) compared to empty vector or HIV GFP+Vpr.

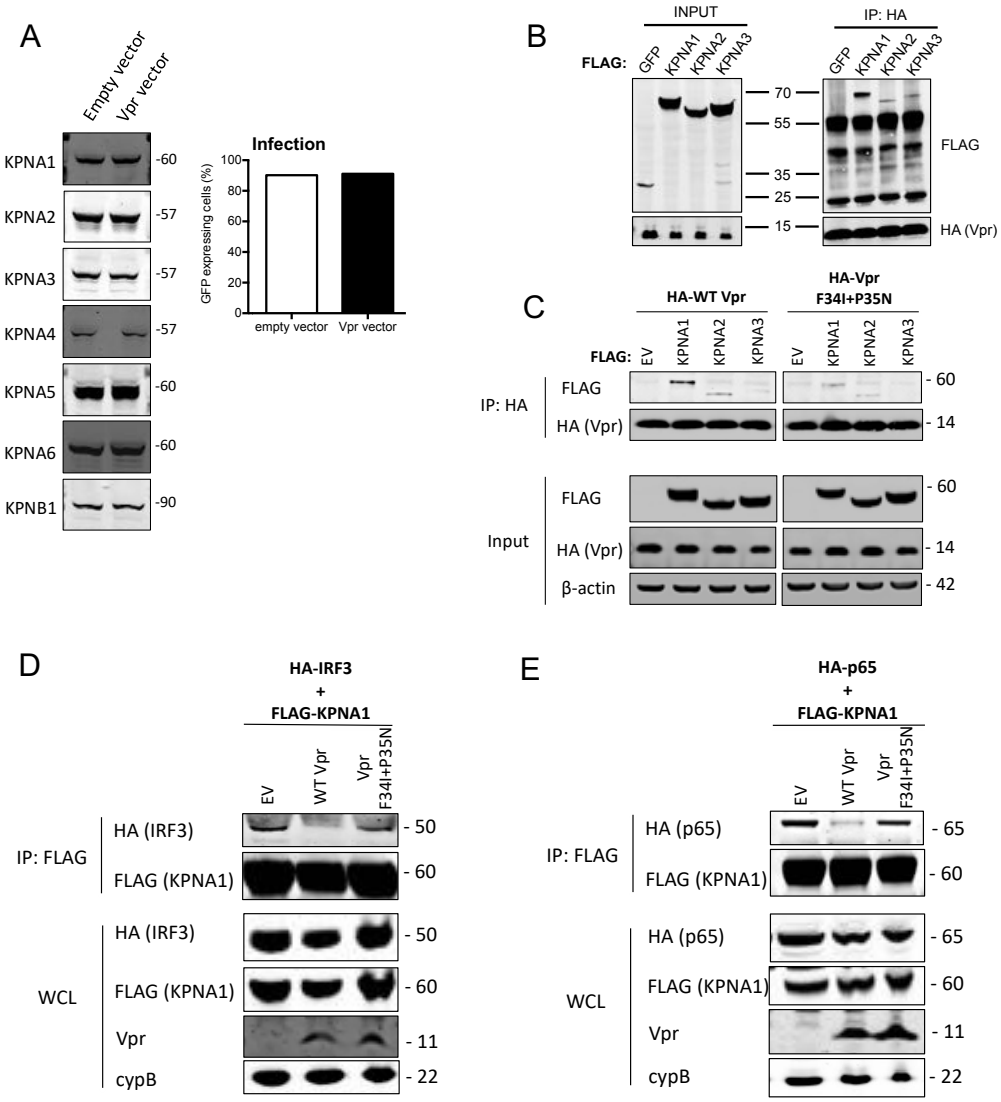

Figure 7 HIV-1 Vpr interacts with karyopherins and inhibits IRF3/NF-κB(p65) recruitment to KPNA1

(**A**) Immunoblot detecting KPNA1-6 or KPNB1 from extracted HEK293T cells infected with empty vector, or Vpr encoding vector at a dose of 0.05 RT U/ml (MOI=2). Size markers are shown in kDa. Percentage infection by HIV-1 GFP bearing Vpr encoding or empty vector is shown on the right. (**B**) Co-immunoprecipitation of Flag-KPNA1-3 and HA-Vpr. Input shows immunoblot detecting extracted HEK293T whole cell lysates expressing flag-KPNA1-3, flag-GFP and HA-Vpr before immunoprecipitation. Co-immunoprecipitation precipitates Vpr with HA-beads and detects Flag-KPNA1-3. (**C**) Co-immunoprecipitation of Flag-KPNA1-3 and WT HA-Vpr or HA-Vpr F34I+P35N. Input shows immunoblots detecting HA-Vpr or Flag-KPNA1-3 in extracted HEK293T whole cell lysates (WCL) before immunoprecipitation. β-Actin is detected as a loading control. Co-immunoprecipitation precipitates Vpr with HA-beads and detects Flag-KPNA1-3. (**D**) Co-immunoprecipitation of HA-IRF3 and Flag-KPNA1 in the presence and absence of WT Vpr or Vpr F34I+P35N to detect competition between Vpr and IRF3 for KPNA1. Input shows immunoblots detecting HA-IRF3 or Flag-KPNA1 or Vpr in extracted HEK293T whole cell lysates (WCL) before immunoprecipitation. CypB is detected as a loading control. Co-immunoprecipitation precipitates KPNA1 with Flag-beads and detects HA-IRF3 in the presence and absence of WT Vpr or inactive Vpr F34I+P35N. (**E**) Co-immunoprecipitation of HA-p65 and Flag-KPNA1 in the presence and absence of WT Vpr or Vpr F34I+P35N to detect competition between Vpr and p65 for KPNA1. Input shows immunoblots detecting HA-p65 or Flag-KPNA1 or Vpr in extracted HEK293T whole cell lysates (WCL) before immunoprecipitation. CypB is detected as a loading control. Co-immunoprecipitation precipitates KPNA1 with Flag-beads and detects HA-p65 in the presence and absence of WT Vpr or Vpr F34I+P35N.