

1 **Disrupting HIV-1 capsid formation causes cGAS sensing of viral DNA**

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19 **Summary (150 words)**

20 Detection of viral DNA by cyclic GMP-AMP synthase (cGAS) is a first line of defence leading

21 to the production of type-I interferon (IFN). As HIV-1 is not a strong inducer of IFN we have

22 hypothesised that its capsid cloaks viral DNA from cGAS. To test this we generated defective

23 viral particles by treatment with HIV-1 protease inhibitors or by genetic manipulation of *gag*.

24 These viruses had defective Gag cleavage, reduced infectivity and diminished capacity to

25 saturate TRIM5 $\alpha$ . Importantly, unlike wild-type HIV-1, infection with cleavage defective HIV-1

26 triggered an IFN response in THP-1 cells and primary human macrophages that was

27 dependent on viral DNA and cGAS. Infection in the presence of the capsid destabilising small

28 molecule PF-74 also induced a cGAS-dependent IFN response. These data demonstrate a

29 protective role for capsid and suggest that antiviral activity of capsid- and protease-targeting

30 antivirals may benefit from enhanced innate and adaptive immunity *in vivo*.

31

## 32 Introduction

33 The innate immune system provides the first line of defence against invading pathogens such  
34 as viruses. Cells are armed with pattern recognition receptors (PRRs) that recognise  
35 pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids, and lead to the  
36 activation of a potent antiviral response in the form of secreted interferons (IFNs),  
37 proinflammatory cytokines and chemokines, the expression of which is driven by the  
38 activation of key transcription factors such as IFN regulatory factor 3 (IRF3) and nuclear  
39 factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) (Chow, Franz et al., 2015). For  
40 HIV-1 a number of cytosolic PRRs have been demonstrated to contribute to the detection of  
41 the virus in infected cells including DNA sensors cyclic GMP-AMP synthase (cGAS) (Gao, Wu  
42 et al., 2013, Lahaye, Satoh et al., 2013, Rasaiyaah, Tan et al., 2013), IFI16 (Jakobsen, Bak et  
43 al., 2013, Jonsson, Laustsen et al., 2017) and PQBP1 (Yoh, Schneider et al., 2015) and RNA  
44 sensors DDX3 (Gringhuis, Hertoghs et al., 2017) and also MDA5, although only in the  
45 circumstance where the genome lacked 2'-O-methylation by 2'-O-methyltransferase FTSJ3  
46 (Ringear, Marchand et al., 2019). The best studied of these sensors is cGAS, which upon  
47 binding double-stranded DNA, such as HIV-1 reverse transcription (RT) products, produces  
48 second messenger 2'3'-cGAMP (Ablasser, Goldeck et al., 2013, Sun, Wu et al., 2013, Wu,  
49 Sun et al., 2013) that binds and induces phosphorylation of ER-resident adaptor protein  
50 STING and its translocation to perinuclear regions (Tanaka & Chen, 2012). Phosphorylation  
51 of STING provides a platform for the recruitment of TBK1 and IRF3 leading to IRF3  
52 phosphorylation and its subsequent translocation to the nucleus to drive expression of IFN  
53 and IFN stimulated genes (ISGs) (Liu, Cai et al., 2015). Activation of STING by 2'3'-cGAMP  
54 also activates IKK and the transcription of NF- $\kappa$ B-dependent genes (Ishikawa & Barber,  
55 2008).

56

57 Of course, detection of infection by sensing is not universal and viruses are expected to hide  
58 their PAMPs and typically have mechanisms to antagonise specific sensors and downstream  
59 restriction factors. Work from our lab (Rasaiyaah et al., 2013) and others (Cingoz & Goff,  
60 2019) has demonstrated that primary monocyte-derived macrophages (MDMs) can be  
61 infected by wild-type (WT) HIV-1 without significant innate immune induction. However, MDM

62 sense HIV-1 if, for example, mutations are made in the viral capsid to prevent the recruitment  
63 of cellular cofactors such as CPSF6 and cyclophilin A (Rasaiyaah et al., 2013) or after  
64 depletion of the cellular exonuclease TREX1 (Rasaiyaah et al., 2013, Yan, Regalado-Magdos  
65 et al., 2010). This sensing was found to be dependent on viral reverse transcription (RT) and  
66 the cellular DNA sensing machinery cGAS and STING. In addition to recruitment of cofactors,  
67 a variety of evidence suggests that capsid remains intact in the cytoplasm to protect the  
68 process of viral DNA synthesis, preventing degradation of RT products by cellular nucleases  
69 such as TREX1 and from detection by DNA sensors (Burdick, Delviks-Frankenberry et al.,  
70 2017, Francis & Melikyan, 2018).

71  
72 Here we have tested the hypothesis that an intact capsid is crucial for innate immune evasion  
73 by disrupting the process of viral particle maturation, either biochemically using protease  
74 inhibitors (PIs), or genetically, by mutating the cleavage site between the capsid protein and  
75 spacer peptide 1. The resulting viral particles had defective Gag cleavage, reduced infectivity  
76 and, unlike wild-type HIV-1, activated an IFN-dependent innate immune response in THP-1  
77 cells and primary human macrophages. This innate response was mostly dependent on viral  
78 DNA synthesis and the cellular sensors cGAS and STING. Defective viruses were less able  
79 to saturate restriction by TRIM5 $\alpha$  indicating a reduced ability to bind this restriction factor,  
80 likely due to aberrant particle formation. Finally, we show that the capsid binding small  
81 molecule inhibitor PF-74, which has been proposed to accelerate capsid opening (Marquez,  
82 Lau et al., 2018), also induces HIV-1 to activate an innate response in THP-1 cells, that is  
83 dependent on cGAS. Together these data support the hypothesis that the viral capsid plays a  
84 physical role in protecting viral DNA from the cGAS/STING sensing machinery in  
85 macrophages and that disruption of Gag cleavage and particle maturation leads to aberrant  
86 capsid formation and activation of an IFN response that may be harnessed therapeutically *in*  
87 *vivo* during PI treatment of HIV-1.

88

## 89 **Results**

### 90 **Protease inhibitor treatment of HIV-1 leads to innate immune induction in**

#### 91 **macrophages**

92 To test the hypothesis that intact viral capsids protect HIV-1 DNA from detection by DNA  
93 sensors we sought to generate defective viral particles by disrupting capsid maturation. The  
94 protease inhibitor (PI) class of anti-retrovirals block the enzymatic activity of the viral  
95 protease, preventing Gag cleavage and proper particle formation, as observed by electron  
96 microscopy (Muller, Anders et al., 2009, Schatzl, Gelderblom et al., 1991). By producing VSV-  
97 G-pseudotyped HIV-1 $\Delta$ Env.GFP (LAI strain (Peden, Emerman et al., 1991) with the Nef  
98 coding region replaced by GFP, herein called HIV-1 GFP) in the presence of increasing  
99 doses of the PI lopinavir (LPV, up to 100 nM) we were able to generate viral particles with  
100 partially defective Gag cleavage, as assessed by immunoblotting of extracted viral particles  
101 detecting HIV-1 CA protein (Fig. 1A). At the highest dose of LPV (100 nM) increased amounts  
102 of intermediate cleavage products corresponding to capsid and spacer peptide 1 (CA-SP1),  
103 matrix and CA (MA-CA), MA, CA, SP1 and nucleocapsid (MA-NC) were particularly evident  
104 along with increased amounts of full length uncleaved Gag (Fig. 1A, Suppl. Fig. 2A).  
105 Uncleaved CA-SP1 was also evident at 30 nM LPV. As expected, defects in Gag cleavage  
106 were accompanied by a reduction in HIV-1 GFP infectivity in both phorbol myristyl acetate  
107 (PMA)-treated THP-1 (Fig. 1B) and U87 cells (Fig. 1C). For the highest dose of LPV this  
108 corresponded to a 24- and 48-fold defect in infectivity in each cell type respectively. Viral  
109 titres were calculated according to the number of genomes, assessed by qPCR (see  
110 Methods), to account for small differences in viral production between conditions. These  
111 differences were no more than 2-fold from untreated virus.

112

113 To test the visibility of PI inhibited viruses to innate sensing responses we generated a THP-1  
114 cell line that was stably depleted for the HIV restriction factor SAMHD1 (Suppl. Fig. 1A).  
115 Monocytic THP-1 cells can be differentiated into macrophage-like adherent cells by treatment  
116 with PMA, yielding a cell line that is highly competent for innate immune sensing, including  
117 DNA sensing. Differentiation of THP-1 normally leads to SAMHD1 activation by  
118 dephosphorylation and potent restriction of HIV-1 infection (Cribier, Descours et al., 2013).  
119 SAMHD1 depletion effectively relieved this restriction and allowed HIV-1 GFP infection  
120 (Suppl. Fig. 1A, B). SAMHD1-depleted THP-1 cells (herein referred to as THP-1 shSAMHD1  
121 cells) remained fully competent for innate immune sensing and produced interferon-

122 stimulated genes (ISGs) and inflammatory chemokines including *CXCL-10*, *IFIT-2* (also  
123 known as *ISG54*) and *CXCL-2* in response to a range of stimuli, including transfection of  
124 herring-testis DNA (HT-DNA), exposure to 2'3'-cGAMP and infection by Sendai virus (Suppl.  
125 Fig. 1C-E).

126

127 Infection of PMA-treated THP-1 shSAMHD1 cells with HIV-1 GFP that had been produced in  
128 the presence of increasing doses of LPV led to a virus and LPV dose-dependent increase in  
129 the expression of ISGs *CXCL-10*, *IFIT-2* and *MxA* at the mRNA level (Fig. 1D-F), and *CXCL-*  
130 *10* protein secretion (Fig. 1G). In agreement with previous reports in primary macrophages  
131 (Cingoz & Goff, 2019), HIV-1 GFP produced in the absence of LPV induced very little, or no  
132 ISG expression in THP-1 cells at the doses tested, consistent with the hypothesis that HIV-1  
133 shields its PAMPs from cellular PRRs (see Fig 1D-G, 0 nM drug dose). Virus dose in these  
134 experiments was normalised according to RT activity, as measured by SG-PERT (see  
135 Methods), which differed no more than 5-fold in the LPV-treated versus untreated virus.  
136 Infection levels in differentiated THP-1 cells were approximately equivalent between the  
137 various LPV doses tested (Suppl. Fig. 2B) because HIV-1 GFP infection of THP-1 is maximal  
138 at about 70 % GFP positivity (Pizzato, McCauley et al., 2015). Similar results were obtained  
139 with the PI darunavir (DRV); treatment of HIV-1 GFP with increasing doses of DRV (up to 50  
140 nM) led to defects in Gag cleavage (Suppl. Fig. 3A), decreased infectivity (Suppl. Fig. 3B, F)  
141 and at 12.5 and 25 nM DRV activated an ISG response in PMA-treated THP-1 shSAMHD1  
142 cells (Suppl. Fig. 3D-E).

143

144 To test whether LPV-treated HIV-1-induced ISG expression in THP-1 cells depended on IFN  
145 production or direct activation of ISGs, infections were repeated in the presence of the  
146 JAK1/2 inhibitor ruxolitinib (Quintas-Cardama, Vaddi et al., 2010). Activation of STAT  
147 transcription factors downstream of IFN receptor engagement requires phosphorylation by  
148 JAKs and hence ruxolitinib inhibits IFN signalling (Fig. 1H). Induction of *MxA* (Fig. 1H) and  
149 *CXCL-10* (Suppl. Fig. 2C) expression by LPV-treated HIV-1 GFP was severely reduced in the  
150 presence of ruxolitinib, indicating that induction of ISG expression in these experiments  
151 requires an infection-driven type I IFN response. Treatment of cells with type I IFN provided a

152 positive control for ruxolitinib activity (Fig. 1H, Suppl. Fig. 2C). Importantly, viral DNA  
153 production, measured by qPCR in infected PMA-treated THP-1 shSAMHD1 cells, was not  
154 changed by increasing LPV dose suggesting that PI inhibited HIV-1 makes normal levels of  
155 DNA but fails to protect PAMPs from innate immune sensors (Fig. 1I).

156

157 To test whether PI inhibition of HIV-1 caused similar innate immune activation in primary  
158 human cell infection we turned to HIV-1 R9 (Ba-L Env) infection of primary human  
159 macrophages. Production of R9 (BaL-Env) in HEK293T cells in the presence of 10-100 nM  
160 LPV induced the expected defects in Gag cleavage (Fig. 1J) and infectivity (Suppl. Fig. 2D  
161 and E) as observed with VSV-G-pseudotyped HIV-1 GFP (Fig. 1A-C). Furthermore, virus  
162 produced in the presence of 30 and 100 nM LPV induced the expression of *CXCL-10* on  
163 infection of primary MDM, whereas virus grown in the absence of LPV, or at low LPV  
164 concentrations (10 nM), induced very little *CXCL10* expression (Fig. 1K). Increasing  
165 concentrations of LPV during HIV-1 production led to a decrease in MDM infection, read out  
166 by p24 positivity, in these experiments (Fig. 1L). Together, these data demonstrate that  
167 infection by PI-treated HIV-1 induces an IFN-dependent innate immune response in PMA-  
168 treated THP-1 cells and primary human MDM that is not observed on infection with untreated  
169 virus.

170

### 171 **HIV-1 bearing Gag cleavage mutations also induces innate immune activation**

172 Producing virus in the presence of PI suppresses Gag cleavage at multiple sites. Previous  
173 work suggested that inhibition of the CA-SP1 cleavage site was particularly toxic to infectivity  
174 and particles were defective with irregular partial polyhedral structures (Mattei, Tan et al.,  
175 2018, Muller et al., 2009). Concordantly our data show a defect in cleavage at the CA-SP1  
176 site in the presence of LPV (Fig. 1A, J) or DRV (Suppl. Fig. 3A). Importantly, the presence of  
177 even small proportions of CA-SP1 cleavage mutant exerted trans-dominant negative effects  
178 on HIV-1 particle maturation (Muller et al., 2009). To test whether a CA-SP1 cleavage defect  
179 can cause HIV-1 to trigger innate sensing we prepared chimeric VSV-G pseudotyped HIV-1  
180 GFP viruses by transfecting 293T cells with varying ratios of WT HIV-1 GFP and HIV-1 GFP  
181 with CA-SP1 Gag mutant L363I M367I (Checkley, Luttge et al., 2010, Wieggers, Rutter et al.,

182 1998). Increasing the proportion of the  $\Delta$ CA-SP1 mutant increased the presence of uncleaved  
183 CA-SP1 detected by immunoblot (Fig. 2A). Defective cleavage was accompanied by a  
184 modest decrease in infectivity on U87 cells (Fig. 2B).

185

186 As with HIV-1 GFP produced in the presence of PIs, infection of PMA-treated THP-1  
187 shSAMHD1 cells with the HIV-1 GFP  $\Delta$ CA-SP1 mutants led to a  $\Delta$ CA-SP1 dose-dependent  
188 increase in the expression of *CXCL-10* (Fig. 2D) and *MxA* mRNA (Fig. 2E), and CXCL-10 at  
189 the protein level (Fig. 2F). Induction was not explained by differences in the amount of viral  
190 DNA in infected cells and similar levels of viral DNA (Fig. 2C) and infection (Suppl. Fig. 4A)  
191 were observed at the viral doses tested. Virus dose in these experiments was normalised  
192 according to RT activity, which differed no more than 5-fold between viruses. Cleavage  
193 defective viruses, and not wild type virus, also induced dose-dependent luciferase expression  
194 from an undifferentiated THP-1 cell line that had been modified to express Gaussia luciferase  
195 under the control of the *IFIT-1* (also known as *ISG56*) promoter, herein called IFIT1-luc  
196 (Mankan, Schmidt et al., 2014) (Fig. 2G, Suppl. Fig. 4B). IFIT1-luc is both IRF-3- and IFN-  
197 sensitive (Mankan et al., 2014). HIV-1 bearing  $\Delta$ CA-SP1 mutant also induced a type I IFN  
198 response, evidenced by suppression of IFIT1-luc by ruxolitinib (Fig. 2H). In the IFIT1-luc cells  
199  $\Delta$ CA-SP1 mutation did not impact infection levels (Suppl. Fig. 4A-C) and neither did ruxolitinib  
200 treatment (Suppl. Fig. 4C). We propose that during single round infection the virus has  
201 already integrated by the time IFN is produced, thus explaining why ruxolitinib has no impact  
202 on the percentage of GFP positive cells. Together these data support our hypothesis that  
203 disruption of Gag maturation yields viral particles that fail to shield PAMP from innate  
204 sensors.

205

206 **Maximal innate immune activation by maturation defective viruses is dependent on**  
207 **viral DNA synthesis**

208 To determine whether viral DNA synthesis is required for HIV-1 bearing  $\Delta$ CA-SP1 to trigger  
209 sensing we infected THP-1 IFIT1-luc cells with HIV-1 75%  $\Delta$ CA-SP1 in the presence of  
210 reverse transcriptase inhibitor nevirapine and assessed sensing by measuring IFIT1-luc  
211 expression and CXCL10 secretion. As expected, infectivity was severely diminished by 5  $\mu$ M

212 nevirapine (Suppl. Fig. 5A) and both luciferase (Fig. 3A) and CXCL-10 (Fig. 3B) secretion was  
213 completely inhibited suggesting that viral DNA synthesis is required to activate sensing.  
214 Concordantly, expression of ISGs *IFIT-2* (Fig. 3C, Suppl. Fig. 5B) and *MxA* (Fig. 3D, Suppl.  
215 Fig. 5B) induced by HIV-1 75%  $\Delta$ CA-SP1 was also abolished in the presence of nevirapine. A  
216 small, but statistically significant reduction in luciferase (Fig. 3A) and CXCL-10 (Fig. 3B)  
217 secretion was observed in the presence of the integrase inhibitor raltegravir, although this  
218 was not observed in every experiment (Fig 3C-D). We conclude that viral DNA is the active  
219 PAMP and this notion was also supported by the observation that mutation D185E in the RT  
220 active site (HIV-1  $\Delta$ CA-SP1 RT D185E) also reduced activation of IFIT-1 luc expression (Fig.  
221 3E) and CXCL10 secretion (Fig. 3F) on infection of the THP-1 IFIT-1 reporter cells. Mutation  
222 D116N of the viral integrase (HIV-1  $\Delta$ CA-SP1 INT D116N) impacted neither luciferase  
223 induction (Fig. 3E) or CXCL-10 (Fig. 3F) secretion.

224

225 Surprisingly neither treatment with 10  $\mu$ M raltegravir (Suppl. Fig. 5A, B) or infection with HIV-1  
226  $\Delta$ CA-SP1 INT D116N (Suppl. 5C) led to a reduction in GFP positivity in monocytic THP-1  
227 cells. Importantly GFP expression was lost in parallel infection of PMA-treated THP-1 cells  
228 (Suppl. Fig. 5D, E) confirming that integration was indeed suppressed by 10  $\mu$ M raltegravir or  
229 D116N integrase mutation. We propose that the GFP positivity observed in monocytic THP-1  
230 cells in the presence of raltegravir, or by  $\Delta$ CA-SP1 INT D116N, is due to expression from 2'-  
231 LTR circles that has been observed in other cell types (Bonczkowski, De Scheerder et al.,  
232 2016, Van Loock, Hombrouck et al., 2013).

233

#### 234 **Viral DNA of maturation defective HIV-1 is sensed by cGAS and STING**

235 To investigate which innate sensors were involved in detecting cleavage defective HIV-1, we  
236 infected cells that had been genetically manipulated by CRISPR/Cas 9 technology to lack the  
237 DNA sensing component proteins cGAS (Invivogen) or STING (Tie, Fernandes et al., 2018),  
238 or the RNA sensing component MAVS (Tie et al., 2018). As expected, STING<sup>-/-</sup> cells did not  
239 respond to transfected Herring Testis (HT)-DNA but ISG induction was maintained in  
240 response to the RNA mimic poly I:C (Fig. 4A). MAVS<sup>-/-</sup> cells showed the opposite  
241 phenotype, responding to poly I:C, but not HT-DNA (Fig. 4A). As expected, Dual IRF reporter



242 THP-1 cells, knocked out for cGAS (Invivogen), responded normally to poly I:C, LPS and  
243 cGAMP but not transfected HT-DNA (Fig. 4B). Induction of IFIT1-luc activity in PMA-treated  
244 IFIT1-luc shSAMHD1 THP-1 cells by HIV-1 GFP bearing 75%  $\Delta$ CA-SP1 was completely  
245 absent in STING knock out cells, but maintained in the MAVS knock out cells, consistent with  
246 DNA being the predominant viral PAMP detected (Fig. 4A). Confirming these findings, no IRF  
247 reporter activity (Fig. 4C) or CXCL-10 production (Fig. 4D) was observed in PMA-treated  
248 THP-1 Dual shSAMHD1 cGAS<sup>-/-</sup> cells infected with HIV-1 75%  $\Delta$ CA-SP1. Similar findings  
249 were also observed for DRV-treated wild type HIV-1 GFP, where induction of IFIT1-luc  
250 reporter activity was dependent on STING (Fig. 4E) and cGAS (Fig. 4G), but not MAVS  
251 expression (Fig. 4E). Interestingly, whilst CXCL-10 production in these experiments was  
252 severely diminished in STING<sup>-/-</sup> (Fig. 4F) and cGAS<sup>-/-</sup> (Fig. 4H) cells, levels were also  
253 reduced in MAVS<sup>-/-</sup> cells (Fig. 4F) suggesting a contribution by HIV-1 RNA sensing in the  
254 production of this inflammatory cytokine. In all experiments, no significant difference in  
255 infection levels between the Ctrl and knockout cell lines was observed (Suppl. Fig. 6A-D).

256

257 To corroborate data obtained in the CRISPR cell lines, infection assays were also repeated in  
258 THP-1 Dual reporter cells in the presence of the recently available STING inhibitor H151  
259 (Haag, Gulen et al., 2018). ISG induction by 12.5 nM DRV-treated or HIV-1 GFP bearing 90%  
260  $\Delta$ CA-SP1 was greatly reduced by the presence of H151 (Fig. 4I), further supporting a role for  
261 DNA sensing in the detection of maturation defective HIV-1. As expected, IRF reporter activity  
262 was also suppressed by ruxolitinib (Fig. 4I). Neither H151 nor ruxolitinib affected infection  
263 levels in these experiments (Suppl. Fig. 6E).

264

### 265 **Maturation defective viruses fail to saturate TRIM5 $\alpha$ in an abrogation-of-restriction** 266 **assay**

267 If maturation defective viruses consist of defective particles that have a reduced ability to  
268 protect viral DNA from cGAS, we hypothesised that these particles may also have a reduced  
269 capacity to bind the restriction factor TRIM5 $\alpha$ . Rhesus monkey TRIM5 $\alpha$  binds HIV-1 capsid  
270 and forms hexameric cage-like structures around the intact HIV capsid lattice (Ganser-  
271 Pornillos, Chandrasekaran et al., 2011, Li, Chandrasekaran et al., 2016). TRIM5 $\alpha$  binding to

272 capsid leads to proteasome recruitment, disassembly of the virus and activation of an innate  
273 response (Fletcher, Christensen et al., 2015, Fletcher, Vaysburd et al., 2018, Pertel,  
274 Hausmann et al., 2011). Viral restriction can be overcome by co-infection with high doses of a  
275 saturating virus in an abrogation-of-restriction assay and this has been suggested to be  
276 dependent on the stability of the incoming viral capsid (Jacques, McEwan et al., 2016, Shi &  
277 Aiken, 2006).

278

279 As a measure of HIV-1 core integrity we tested the ability of the maturation defective viruses  
280 to saturate restriction by rhesus macaque TRIM5 $\alpha$ . Rhesus FRhK cells were co-infected with  
281 a fixed dose of HIV-1 GFP and increasing doses of either wild type untreated HIV-1 luc, LPV-  
282 treated HIV-1 luc or HIV-1 luc bearing  $\Delta$ CA-SP1. Rescue of HIV-1 GFP infectivity from  
283 TRIM5 $\alpha$  was assessed by flow cytometry measuring GFP positive cells. Viruses that induced  
284 a strong innate response, i.e. virus bearing 75 %  $\Delta$ CA-SP1 mutant (Fig. 5A, Suppl. Fig. 7A) or  
285 wild type HIV-1 treated with 30 or 100 nM LPV (Fig. 5B, Suppl. Fig. 7B) showed a reduced  
286 ability, or failed to saturate TRIM5 $\alpha$  restriction. These data are consistent with cleavage  
287 defective HIV-1 particles failing to form the authentic hexameric lattice required for  
288 recruitment of TRIM5 $\alpha$  (Ganser-Pornillos & Pornillos, 2019, Li et al., 2016) and protection of  
289 genome.

290

### 291 **Treatment with capsid binding small molecule PF-74 induces HIV-1 to trigger a DNA-** 292 **sensing dependent ISG response**

293 Recent single molecule analysis of capsid uncoating demonstrated that the capsid binding  
294 small molecule inhibitor of HIV, PF-74, accelerates capsid opening (Marquez et al., 2018).  
295 We therefore hypothesised that PF-74 treated HIV-1 may activate a DNA-sensing dependent  
296 innate immune response. To test this, we infected THP-1 IFIT-1 reporter cells with increasing  
297 doses of HIV-1 GFP (0.1-3 U/ml RT) in the presence or absence of 10  $\mu$ M PF-74. This dose  
298 was sufficient to inhibit infection up to 1 U/ml RT HIV-1 GFP, indicating PF-74 is an effective  
299 inhibitor of HIV-1, although its potency could be improved (Fig. 6B). Consistent with our  
300 hypothesis, at high dose HIV-1 infection (3 U/ml RT) luciferase reporter induction was  
301 observed in the presence of PF-74 but not in the DMSO control (Fig. 6A). ISG induction in the

302 presence of 10  $\mu$ M PF-74 was further confirmed in a second experiment by measuring  
303 endogenous *CXCL-10* (Fig. 6C) and *MxA* (Fig. 6D) mRNA expression by qPCR and secreted  
304 CXCL-10 by ELISA in the IFIT1-luc reporter cells (Fig. 6E). PF-74 treatment of HIV-1 GFP  
305 was further shown to induce a type I IFN response in these cells as IFIT1-luc reporter activity  
306 was diminished in the presence of ruxolitinib (Fig. 6F). As expected there was partial  
307 inhibition of infection with PF-74 and no difference in infection levels in the presence of  
308 ruxolitinib (Suppl. Fig. 8A). Finally, we were able to demonstrate that innate sensing of PF-74-  
309 treated HIV-1 was dependent on cGAS as luciferase secretion by PF-74 treated HIV-1 GFP  
310 was lost in cGAS<sup>-/-</sup> cells (Fig. 6G), but maintained in MAVS<sup>-/-</sup> cells (Fig. 6H). As previously  
311 observed, the loss of cGAS (Suppl. Fig. 8B) or MAVS (Suppl. Fig. 8C) had no impact on HIV-  
312 1 infectivity suggesting sensing does not contribute to the inhibitory effect of PF74 in these  
313 single round infections.

314

## 315 **Discussion**

316 Effective evasion of innate immune responses is expected to be crucial for successful  
317 infection and all viruses have evolved countermeasures to hide PAMPs and/or directly reduce  
318 activation of the IFN response (Schulz & Mossman, 2016). Given the small coding capacity of  
319 HIV-1 and the general lack of innate activation observed with this virus *in vitro* (Cingoz & Goff,  
320 2019, Lahaye et al., 2013, Rasaiyaah et al., 2013) we had hypothesised that HIV-1 uses its  
321 capsid to physically protect nucleic acid PAMPs from innate sensors such as cGAS. In this  
322 study we used three approaches to demonstrate that the HIV-1 capsid plays a protective role  
323 in preventing IFN induction by viral DNA. By treating HIV-1 with PIs LPV (Fig. 1) or DRV  
324 (Suppl. Fig. 3), or mutating the cleavage site between CA and SP1 (Fig. 2) we were able to  
325 generate aberrant particles by interfering with capsid maturation. In all cases the resulting  
326 viruses had perturbations in Gag cleavage, reduced infectivity (Fig. 1, Fig. 2, Suppl. Fig. 3)  
327 and had reduced capacity to saturate the restriction factor TRIM5 $\alpha$  in an abrogation-of-  
328 restriction assay, indicative of altered stability/capsid integrity (Fig. 5). Importantly, when  
329 these viruses were used to infect macrophages they induced a potent IFN response that was  
330 not observed on infection with untreated or WT HIV-1 (Fig. 1, Fig. 2, Suppl. Fig. 3). Innate

331 immune responses were almost entirely dependent on viral reverse transcription (Fig. 3) and  
332 the cellular DNA sensing machinery comprising cGAS and STING (Fig. 4), consistent with  
333 viral DNA being the most important PAMP in these experiments. As a third approach our  
334 results were corroborated using the capsid targeting small molecule inhibitor PF-74, which  
335 has been proposed to accelerate capsid opening (Marquez et al., 2018). Treatment of HIV-1  
336 with PF-74 also caused a DNA-sensing dependent IFN response (Fig. 6). Together these  
337 data support a model in which the WT HIV-1 core remains intact as it traverses the  
338 cytoplasm, thus protecting viral DNA from detection by cGAS. Conversely, disruption of  
339 capsid maturation or integrity, either chemically or genetically, yields particles that fail to  
340 conceal viral DNA and thus activate a cGAS-dependent type 1 IFN response (Suppl. Fig. 9).

341

342 We hypothesise that HIV-1 has evolved to cloak viral DNA synthesis within an intact  
343 capsid (Jacques et al., 2016, Rasaiyaah et al., 2013). However, a series of studies have  
344 reported innate immune activation by WT HIV-1 in macrophages or dendritic cells, but these  
345 have required suppression of SAMHD1 by co-transduction with Vpx-containing VLPs (Gao et  
346 al., 2013, Johnson, Lucas et al., 2018, Manel, Hogstad et al., 2010, Yoh et al., 2015), high  
347 doses of virus (Gao et al., 2013), unpurified viral stocks (Manel et al., 2010, Yan et al., 2010) or  
348 manipulation of nuclease TREX1 (Yan et al., 2010). A technical complication in testing  
349 whether HIV-1, or any other virus, triggers innate immune sensing is controlling for viral dose  
350 effects. We and others have found that at very high dose, HIV-1 activates innate immune  
351 pathways and this is influenced particularly by whether the viral supernatant is purified. In the  
352 experiments presented here, all viruses were DNase treated and purified by centrifugation  
353 through sucrose and experiments were designed to control dose between variables. For  
354 example, viral dose was normalised by measuring RT activity (SG-PERT) or the number of  
355 viral genomes (qPCR) in viral preparations to account for differences in virus production, see  
356 legends. Critically, mutating the CA-SP1 cleavage site does not impact RT activity and  
357 treatment with protease inhibitors only inhibited supernatant RT activity at the highest dose  
358 used, and only by a few fold. We propose that small ISG responses to the doses of WT HIV-1  
359 used here are likely due to low frequency uncoating events because the minimal ISG  
360 response to WT HIV-1 was also dependent on cGAS (Fig. 4C, Fig. 6G).

361

362 Further support for the important role of capsid in innate immune evasion comes from data in  
363 dendritic cells demonstrating that unlike wild type HIV-1, wild type HIV-2 activates a strong  
364 RT- and cGAS-dependent IFN response. This difference in innate activation mapped to  
365 capsid (Lahaye et al., 2013). Why the HIV-2 capsid, unlike the capsid of HIV-1, fails to protect  
366 RT products from innate sensors is the subject of ongoing investigation, but given that HIV-2  
367 does not replicate in dendritic cells and macrophages (Chauveau, Puigdomenech et al., 2015,  
368 Duvall, Lore et al., 2007) these observations suggest that evasion of sensing by cGAS is a  
369 necessary requirement for replication in myeloid cells.

370

371 Interestingly, we discovered here that single round infection triggering of IFN did not lead to  
372 reduction in viral infectivity. This was particularly apparent in experiments using the JAK1/2  
373 inhibitor ruxolitinib, which potently reduced the ISG response to viruses with defective  
374 capsids, but had no impact on GFP positivity of the cells (Suppl. Fig. 4C, Suppl. Fig. 6E,  
375 Suppl. Fig. 8A). Similarly, cGAS knockout severely blunted ISG responses, but did not lead to  
376 a corresponding increase in GFP positivity (Suppl. Fig. 6B, D, Suppl. Fig. 8B). We propose  
377 that during single round infections the virus has already integrated by the time IFN is  
378 produced and GFP expression is not particularly sensitive to its anti-viral effects. Indeed, the  
379 IFITM proteins (OhAinle, Helms et al., 2018, Petrillo, Thorne et al., 2018, Yu & Liu, 2018),  
380 TRIM5 $\alpha$  (OhAinle et al., 2018, Pertel et al., 2011) and MxB (Goujon, Moncorge et al., 2013,  
381 Kane, Yadav et al., 2013, OhAinle et al., 2018) are the major IFN-induced inhibitors of HIV-1  
382 in THP-1 cells and are not expected to impact GFP expression.

383

384 Another interesting finding that warrants further investigation is the observation that MAVS  
385 contributed to CXCL-10 production in response to infection with DRV-treated virus (Fig. 4F),  
386 but did not contribute to the corresponding IFIT-1 reporter activity (Fig. 4E). MAVS-dependent  
387 pathways are known to activate transcription factors other than IRF-3, such as NF- $\kappa$ B (Seth,  
388 Sun et al., 2005), which also contributes to the production of CXCL-10 (Yeruva, Ramadori et  
389 al., 2008), but not activation of the IFIT-1 reporter (Grandvaux, Servant et al., 2002). It is

390 therefore possible that activation of MAVS by HIV-1 contributes to NF- $\kappa$ B activation in these  
391 cells but not an IRF-3 response.

392

393 In summary these findings highlight the crucial role of the HIV-1 capsid in masking viral  
394 nucleic acids from innate immune sensors, particularly in protecting viral DNA from detection  
395 by cGAS/STING. As such, disrupting capsid integrity through mutation, treatment with  
396 protease inhibitors, or the capsid targeting small molecule PF-74 yields viral particles that fail  
397 to shield their PAMPs and thus activate a potent IFN response that is not observed with the  
398 WT virus. Together these data suggest that the therapeutic activity of capsid or protease -  
399 targeting therapeutics, for example the recently described HIV-1 capsid inhibitor from Gilead  
400 Sciences(Yant, Mulato et al., 2019), may be enhanced by induction of local antiviral IFN  
401 responses *in vivo* that could contribute to viral clearance by the innate and adaptive immune  
402 system. Furthermore these findings encourage the design of therapeutics targeting capsids or  
403 structural proteins generally, which may also benefit from unmasking viral PAMPs and  
404 induction of innate immune responses.

405

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412

#### 413 **Author Contributions**

414 RPS and GJT conceived the study. RPS, LH, TP, ET, MS and LZA performed the  
415 experiments. RPS, LH, TP and GJT analysed the data. RPS and GJT wrote the manuscript.

416

#### 417 **Declaration of Interests**

418 The authors declare no competing interests.

419

## 420 **Methods**

### 421 **Cells and reagents**

422 HEK293T and U87 cells were maintained in DMEM (Gibco) supplemented with 10 % foetal  
423 bovine serum (FBS, Labtech) and 100 U/ml penicillin plus 100 µg/ml streptomycin (Pen/Strep;  
424 Gibco). THP-1 cells were maintained in RPMI (Gibco) supplemented with 10 % FBS and  
425 Pen/Strep. THP-1-IFIT-1 cells that had been modified to express Gaussia luciferase under  
426 the control of the *IFIT-1* promoter were described previously (Mankan et al., 2014). THP-1  
427 Dual Control and cGAS<sup>-/-</sup> cells were obtained from Invivogen. Lopinavir (LPV), darunavir  
428 (DRV), nevirapine (NVP), raltegravir, zidovudine (AZT) and tenofovir (TDF) were obtained  
429 from AIDS reagents. STING inhibitor H151 was obtained from Invivogen. JAK inhibitor  
430 ruxolitinib was obtained from CELL guidance systems. PF-74 was obtained from Sigma.  
431 Lipopolysaccharide, IFNβ and poly I:C were obtained from Peprtech. Sendai virus was  
432 obtained from Charles River Laboratories. Herring-testis DNA was obtained from Sigma.  
433 cGAMP was obtained from Invivogen. For stimulation of cells by transfection, transfection  
434 mixes were prepared using lipofectamine 2000 according to the manufacturer's instructions  
435 (Invitrogen).

436

### 437 **Generation of ΔCA-SP1, RT D185E and INT D116N viruses**

438 pLAIΔEnv GFP/Luc ΔCA-SP1 (Gag mutant L363I M367I) was generated by two rounds of  
439 site-directed mutagenesis (using Pfu Turbo DNA polymerase, Agilent) using primers:  
440 LAI\_Gag\_L363I fwd: 5' CCGGCCATAAGGCAAGAGTTATCGCTGAAGCAATG 3'  
441 LAI\_Gag\_L363I rev: 5' GTTACTTGGCTCATTGCTTCAGCGATAACTCTTGC 3'  
442 LAI\_Gag\_M367I fwd: 5' GCAAGAGTTATCGCTGAAGCAATCAGCCAAGTAAC 3'  
443 LAI\_Gag\_M367I rev: 5' GTAGCTGAATTTGTTACTTGGCTGATTGCTTCAGC 3'  
444 pLAIΔEnv GFP and pLAIΔEnv GFP ΔCA-SP1 RT D185E and INT D116N were generated by  
445 site-directed mutagenesis using the following primers:  
446 LAI\_RT D185E fwd: 5' ATAGTTATCTATCAATACATGGAAGATTTGTATG 3'  
447 LAI\_RT D185E rev: 5' AAGTCAGATCCTACATACAAATCTTCCATGTATTG 3'  
448 LAI\_INT D116N fwd: 5' GGCCAGTAAAACAATACATACAAACAATGGCAGC 3'  
449 LAI\_INT D116N rev: 5' ACTGGTGAATTGCTGCCATTGTTTGTATGTATTG 3'

450 In all cases mutated sequences were confirmed by sequencing, excised by restriction  
451 digestion and cloned back into the original plasmid.

452

### 453 **Isolation of primary monocyte-derived macrophages**

454 Primary monocyte-derived macrophages (MDM) were prepared from fresh blood from healthy  
455 volunteers. The study was approved by the joint University College London/University College  
456 London Hospitals NHS Trust Human Research Ethics Committee and written informed  
457 consent was obtained from all participants. Peripheral blood mononuclear cells (PBMCs)  
458 were isolated by density gradient centrifugation using Lymphoprep (Stemcell Technologies).  
459 PBMCs were washed three times with PBS and plated to select for adherent cells. Non-  
460 adherent cells were washed away after 1.5 h and the remaining cells incubated in RPMI  
461 (Gibco) supplemented with 10 % heat-inactivated pooled human serum (Sigma) and 40 ng/ml  
462 macrophage colony stimulating factor (R&D systems). Cells were further washed after 3 days  
463 and the medium changed to RPMI supplemented with 10 % heat-inactivated FBS. MDM were  
464 then infected 3-4 days later. Replicate experiments were performed with cells derived from  
465 different donors.

466

### 467 **Editing of cells by CRISPR/Cas 9**

468 THP-1 IFIT-1 shSAMHD1 STING<sup>-/-</sup> and MAVS<sup>-/-</sup> cells were previously described (Tie et al.,  
469 2018). Briefly, lentiparticles to generate CRISPR/Cas9-edited cell lines were produced by  
470 transfecting 10 cm dishes of HEK293T cells with 1.5 µg of plentiCRISPRv2 encoding gene  
471 specific guide RNAs (Addgene plasmid #52961), 1 µg of p8.91 packaging plasmid (Zufferey,  
472 Nagy et al., 1997), and 1 µg of vesicular stomatitis virus-G glycoprotein expressing plasmid  
473 pMDG (Genscript) using Fugene 6 transfection reagent (Promega) according to the  
474 manufacturer's instructions. Virus supernatants were harvested at 48 and 72 h post-  
475 transfection, pooled and used to transduce THP-1 IFIT-1 shSAMHD1 cells by spinoculation  
476 (1000 xg, 1 h, room temperature). Transduced cells were selected using puromycin (1 µg/ml,  
477 Merck Millipore) and single clones isolated by limiting dilution in 96 well plates. Clones were  
478 screened for successful gene knock out by luciferase assay and immunoblotting.

479 gRNA sequences:



480 STING: TCCATCCATCCCGTGTCCCAGGG

481 MAVS: CAGGGAACCGGGACACCCTC

482 Non-targeting control: ACGGAGGCTAAGCGTCGCAA

483

#### 484 **Production of virus in 293T cells**

485 HIV-1 and lentiviral particles were produced by transfection of HEK293T cells in T150 flasks

486 using Fugene 6 transfection reagent (Promega) according to the manufacturer's instructions.

487 For full length HIV-1 with a BaL envelope cells were transfected with 8.75 µg pR9.BaL per

488 flask. For HIV-1 GFP/Luc each flask was transfected with 2.5 µg of vesicular stomatitis virus-

489 G glycoprotein expressing plasmid pMDG (Genscript) and 6.25 µg pLAIΔEnv GFP/Luc. Virus

490 supernatants were harvested at 48 and 72 h post-transfection, pooled, DNase treated (2 h at

491 37 °C, DNaseI, Sigma) and subjected to ultracentrifugation over a 20 % sucrose cushion.

492 Viral particles were finally resuspended in RPMI supplemented with 10 % FBS. For

493 production of viruses in the presence of lopinavir or darunavir, the inhibitors were added at 24

494 h post-transfection and replaced after harvest at 48 h. Lentiparticles for SAMHD1 depletion

495 were generated as previously described (Georgana, Sumner et al., 2018). Viruses were

496 titrated by infecting U87 cells ( $10^5$  cells/ml) or PMA-treated THP-1 cells ( $2 \times 10^5$  cells/ml) with

497 dilutions of sucrose purified virus in the presence of polybrene (8 µg/ml, Sigma) for 48 h and

498 enumerating GFP-positive cells by flow cytometry using the FACS Calibur (BD) and analysing

499 with FlowJo software.

500

#### 501 **SG-PERT**

502 Reverse transcriptase activity of virus preparations was quantified by qPCR using a SYBR

503 Green-based product-enhanced RT (SG-PERT) assay as described (Vermeire, Naessens et

504 al., 2012).

505

#### 506 **Genome copy/RT products measurements**

507 For viral genome copy measurements RNA was extracted from 2 µl sucrose purified virus

508 using the RNeasy mini kit (QIAGEN). The RNA was then treated with TURBO DNase (Thermo

509 Fisher Scientific) and subjected to reverse transcription using Superscript III reverse

510 transcriptase and random hexamers according to the manufacturer's protocol (Invitrogen).  
511 Genome copies were then measured by Taqman qPCR using primers against GFP (see  
512 below). For RT product measurements DNA was extracted from  $5 \times 10^5$  infected cells using the  
513 DNeasy Blood & Tissue kit (QIAGEN) according to the manufacturer's protocol. DNA  
514 concentration was quantified using a Nanodrop for normalisation. RT products were  
515 quantified by Taqman qPCR using TaqMan Gene Expression Master Mix (ThermoFisher) and  
516 primers and probe specific to GFP. A dilution series of plasmid encoding GFP was measured  
517 in parallel to generate a standard curve to calculate the number of GFP copies.

518 *GFP* fwd: 5'- CAACAGCCACAACGTCTATATCAT -3'

519 *GFP* rev: 5'- ATGTTGTGGCGGATCTTGAAG -3'

520 *GFP* probe: 5'- FAM-CCGACAAGCAGAAGAACGGCATCAA-TAMRA -3'

521

## 522 **Infection assays**

523 THP-1 cells were infected at a density of  $2 \times 10^5$  cells/ml. For differentiation THP-1 cells were  
524 treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Peprotech) for 48 h. Luciferase  
525 reporter assays were performed in 24 well plates and qPCR and ELISA in 12 well plates.  
526 Infection levels were assessed at 48 h post-infection through enumeration of GFP positive  
527 cells by flow cytometry. Infections in THP-1 cells were performed in the presence of  
528 polybrene (8  $\mu$ g/ml, Sigma). Input dose of virus was normalised either by RT activity  
529 (measured by SG-PERT) or genome copies (measured by qPCR) as indicated.

530

## 531 **Luciferase reporter assays**

532 Gaussia/Lucia luciferase activity was measured by transferring 10  $\mu$ l supernatant to a white  
533 96 well assay plate, injecting 50  $\mu$ l per well of coelenterazine substrate (Nanolight  
534 Technologies, 2  $\mu$ g/ml) and analysing luminescence on a FLUOstar OPTIMA luminometer  
535 (Promega). Data were normalised to a mock-treated control to generate a fold induction.

536

## 537 **ISG qPCR**

538 RNA was extracted from THP-1/primary MDM using a total RNA purification kit (Norgen)  
539 according to the manufacturer's protocol. Five hundred ng RNA was used to synthesise

540 cDNA using Superscript III reverse transcriptase (Invitrogen), also according to the  
541 manufacturer's protocol. cDNA was diluted 1:5 in water and 2 µl was used as a template for  
542 real-time PCR using SYBR® Green PCR master mix (Applied Biosystems) and a Quant  
543 Studio 5 real-time PCR machine (Applied Biosystems). Expression of each gene was  
544 normalised to an internal control (*GAPDH*) and these values were then normalised to mock-  
545 treated control cells to yield a fold induction. The following primers were used:

546 *GAPDH*: Fwd 5'-GGGAACTGTGGCGTGAT-3', Rev 5'-GGAGGAGTGGGTGTCGCTGTT-3'

547 *CXCL-10*: Fwd 5'-TGGCATTCAAGGAGTACCTC-3', Rev 5'-TTGTAGCAATGATCTCAACACG-3'

548 *IFIT-2*: Fwd 5'-CAGCTGAGAATTGCACTGCAA-3', Rev 5'-CGTAGGCTGCTCTCCAAGGA-3'

549 *MxA*: Fwd 5'-ATCCTGGGATTTGGGGCTT-3', Rev 5'-CCGCTTGTGCTGGTGTGCG-3'

550 *CXCL-2*: Fwd 5'-GGGCAGAAAGCTTGTCTCAA-3', Rev 5'-GCTTCCTCCTTCTTCTGGT-3'

551

## 552 **ELISA**

553 Cell supernatants were harvested for ELISA at 24 h post-infection/stimulation and stored at -  
554 80 °C. CXCL-10 protein was measured using Duoset ELISA reagents (R&D Biosystems)  
555 according to the manufacturer's instructions.

556

## 557 **Immunoblotting**

558 For immunoblotting of viral particles  $2 \times 10^{11}$  genome copies of virus were boiled for 10 min in  
559 6X Laemmli buffer (50 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v)  
560 bromophenol blue, 100 mM β-mercaptoethanol) before separating on 4-12 % Bis-Tris  
561 polyacrylamide gradient gel (Invitrogen). For immunoblot analysis of THP-1 cells,  $3 \times 10^6$  cells  
562 were lysed in a cell lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 10%  
563 (v/v) glycerol, 1 % (v/v) Triton X100, 0.05 % (v/v) NP40 supplemented with protease inhibitors  
564 (Roche), clarified by centrifugation at 14,000 x g for 10 min and boiled in 6X Laemmli buffer  
565 for 5 min. Proteins were separated by SDS-PAGE on 12 % polyacrylamide gels. After PAGE,  
566 proteins were transferred to a Hybond ECL membrane (Amersham biosciences) using a  
567 semi-dry transfer system (Biorad). Primary antibodies were from the following sources:  
568 mouse anti-β-actin (Abcam), rabbit-anti-SAMHD1 (Proteintech) and mouse-anti-HIV-1capsid  
569 p24 (183-H12-5C, AIDS Reagents). Primary antibodies were detected with goat-anti-  
570 mouse/rabbit IRdye 800CW infrared dye secondary antibodies and membranes imaged using

571 an Odyssey Infrared Imager (LI-COR Biosciences).

572

### 573 **Abrogation of restriction assay**

574 FRhK cells were plated in 48 well plates at  $5 \times 10^4$  cells/ml. The following day cells were co-  
575 transduced in the presence of polybrene (8  $\mu\text{g/ml}$ , Sigma) with a fixed dose of HIV-1 GFP  
576 ( $5 \times 10^7$  genome copies/ml) and increasing doses of HIV-LUC  $\Delta\text{CA-SP1}$  mutants or LPV-  
577 treated HIV-LUC viruses ( $1.7 \times 10^6$  -  $3.8 \times 10^9$  genome copies/ml). Rescue of GFP infectivity  
578 was assessed 48 h later by flow cytometry using the FACS Calibur (BD) and analysing with  
579 FlowJo software.

580

### 581 **Statistical analyses**

582 Statistical analyses were performed using an unpaired Student's t-test (with Welch's  
583 correction where variances were unequal) or a 2-way ANOVA with multiple comparisons, as  
584 indicated. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

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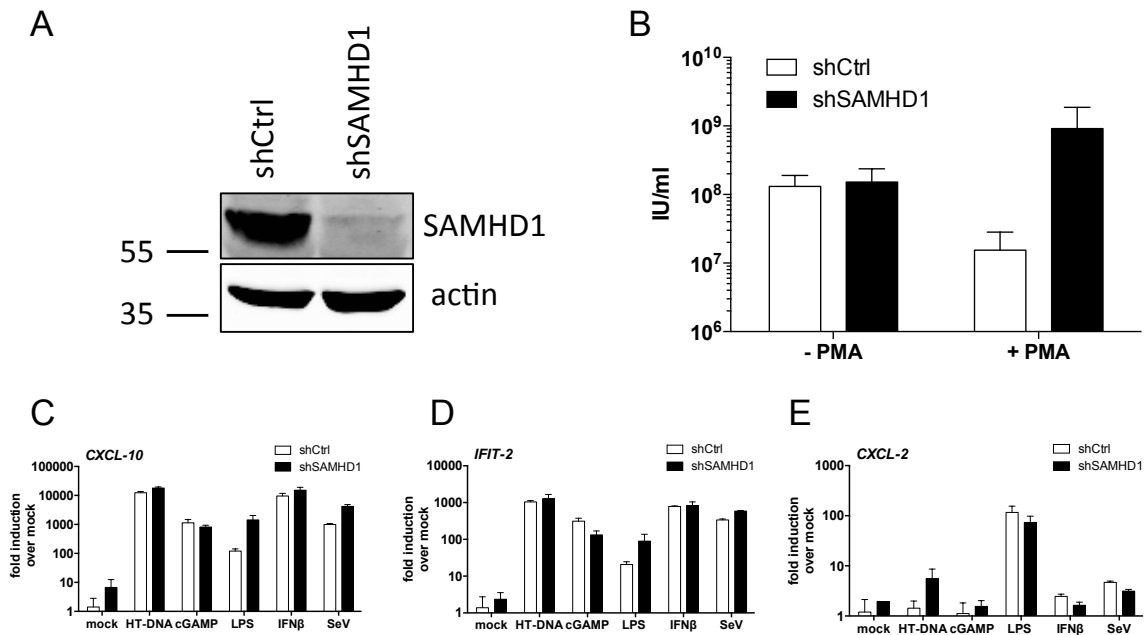
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767

**Suppl. Fig 1: Generation of THP-1 cell lines depleted for SAMHD1**



768

769 **Suppl. Fig. 1.** Generation of THP-1 cell lines depleted for SAMHD1. (A) Immunoblot of THP-  
 770 1 cell line with stable depletion of SAMHD1 (shSAMHD1) or a corresponding control shRNA  
 771 (shCtrl) (see Methods). Blots were probed with anti-SAMHD1 and anti-actin antibodies.

772 Molecular mass markers (in kDa) are indicated on the left. (B) Titration of HIV-1 GFP on THP-  
 773 1 shCtrl and THP-1 shSAMHD1 cells that had been treated or not with PMA (50 ng/ml) for 48  
 774 h. Infectious units per ml (IU/ml) were calculated by enumeration of GFP-positive cells by flow  
 775 cytometry. Data are presented as mean ± SD from three separate titrations performed in

776 singlet. (C-E) ISG qPCR from PMA-treated (50 ng/ml, 48 h) THP-1 shCtrl and THP-1

777 shSAMHD1 cells that had been stimulated for 8 h with HT-DNA (20 ng/ml, transfected),

778 cGAMP (1 µg/ml), LPS (1 µg/ml), IFNβ (10 ng/ml) or Sendai virus (SeV) (0.2 HA U/ml). Cells

779 were lysed for RNA extraction, cDNA was synthesised and then used for qPCR analysis.

780 Expression of *CXCL-10* (C), *IFIT-2* (D) and *CXCL-2* (E) was normalised to an internal control  
 781 (*GAPDH*) and these values were then normalised to those for the non-stimulated mock cells,

782 yielding the fold induction over mock. Data are presented as mean ± SD of duplicate data

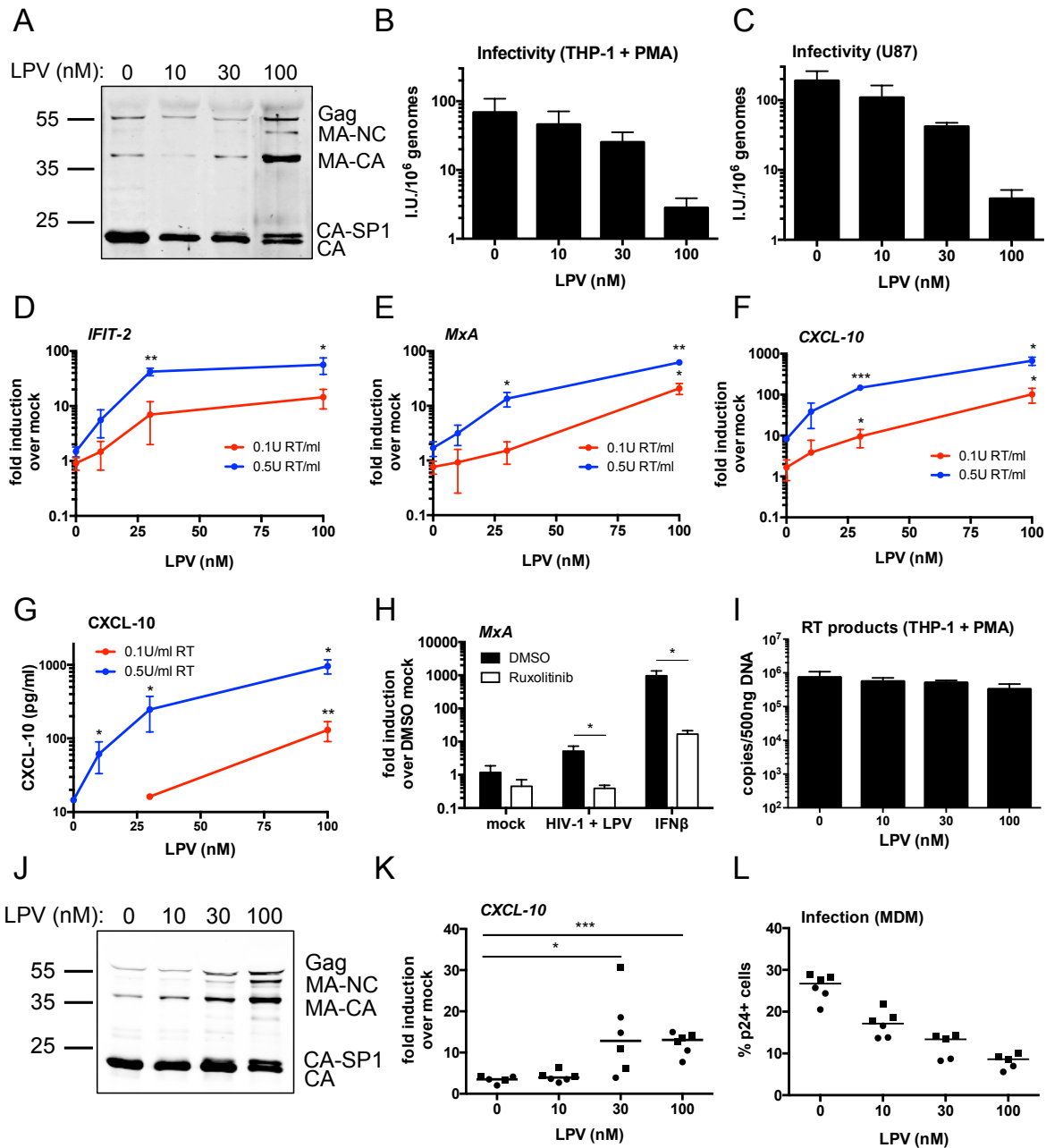
783 repeated at least twice.

784

785



**Fig 1:** PI treatment induces HIV-1 to trigger an ISG response in macrophages



786

787

788 **Fig. 1.** PI treatment induces HIV-1 to trigger an ISG response in macrophages. (A)

789 Immunoblot of HIV-1 GFP virus particles ( $2 \times 10^{11}$  genomes) produced in HEK293T cells in the  
790 presence of increasing doses of protease inhibitor lopinavir (LPV, 0-100 nM) and then purified  
791 through a 20 % sucrose cushion. The blot was probed with an anti-HIV-1 p24 antibody.

792 Molecular mass markers (in kDa) are indicated on the left and Gag cleavage products on the

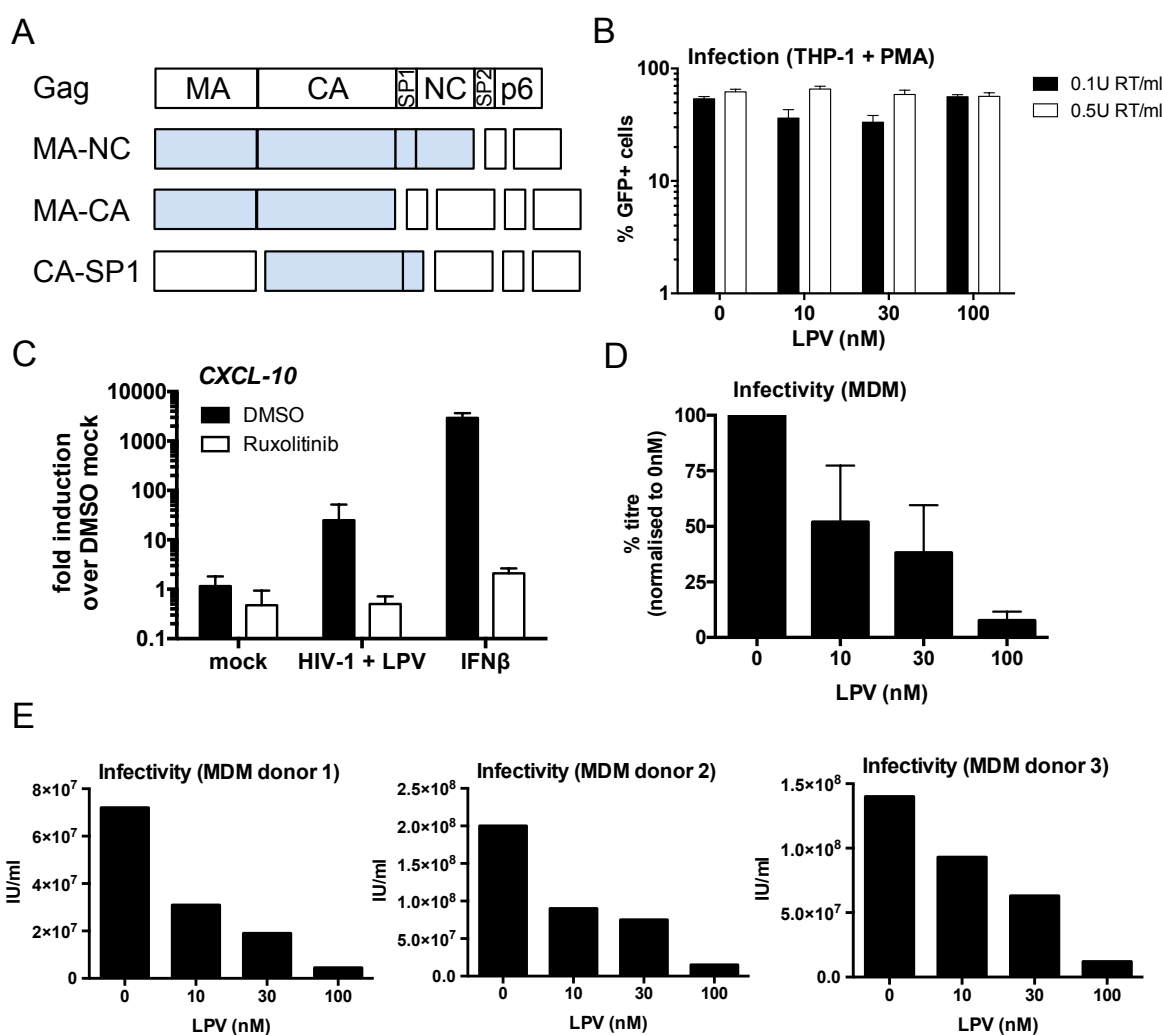
793 right. (B-C) Titration of LPV-treated HIV-1 GFP viruses on PMA-treated (50 ng/ml, 48 h) THP-

794 1 shSAMHD1 (B) or U87 (C) cells. Infectious units per ml (IU/ml) were calculated by

795 enumeration of GFP-positive cells by flow cytometry at 48 h post-transduction. These data  
796 were then normalised to genomes/ml data calculated by qPCR to account for variations in  
797 viral production. Data are presented as mean  $\pm$  SD from three separate titrations performed in  
798 singlet. (D-F) ISG qPCR from PMA-treated (50 ng/ml, 48 h) THP-1 shSAMHD1 cells that had  
799 been transduced for 24 h with LPV-treated HIV-1 GFP viruses (0.1 U RT/ml red line, 0.5 U  
800 RT/ml blue line). Expression of *IFIT-2* (D), *MxA* (E) and *CXCL-10* (F) was normalised to an  
801 internal control (*GAPDH*) and these values were then normalised to those for the non-  
802 transduced mock cells, yielding the fold induction over mock. (G) Level of *CXCL-10* protein in  
803 the cell supernatants from D-F was measured by ELISA. Data for D-G are presented as mean  
804  $\pm$  SD of triplicate data repeated at least three times. (H) ISG qPCR from PMA-treated (50  
805 ng/ml, 48 h) THP-1 shSAMHD1 cells that had been transduced for 24 h with 0.2 U RT/ml HIV-  
806 1 GFP that had been treated with 30 nM LPV, or stimulated with 1 ng/ml IFN $\beta$  as a control, in  
807 the presence or absence (DMSO control) of 2  $\mu$ M ruxolitinib. Expression of *MxA* was  
808 normalised to an internal control (*GAPDH*) and these values were then normalised to those  
809 for the non-transduced, DMSO-treated mock cells, yielding the fold induction over DMSO  
810 mock. Data are presented as mean  $\pm$  SD of triplicate data repeated at least twice. (I) RT  
811 products from PMA-treated (50 ng/ml, 48 h) THP-1 shSAMHD1 cells that had been  
812 transduced for 24 h with 0.3 U RT/ml LPV-treated HIV-1 GFP viruses. Cells were lysed for  
813 DNA extraction and then used for qPCR analysis using primers for GFP. Data are presented  
814 as mean  $\pm$  SD of triplicate data repeated at least twice. (J) Immunoblot of R9 BaL virus  
815 particles ( $2 \times 10^{11}$  genomes) that were produced in HEK293T cells in the presence of  
816 increasing doses of LPV (0-100 nM) and then purified through a 20 % sucrose cushion. The  
817 blot was probed with an anti-HIV-1 p24 antibody. Molecular mass markers (in kDa) are  
818 indicated on the left and Gag cleavage products on the right. (K) ISG qPCR from primary  
819 monocyte-derived macrophages (MDM) that had been infected for 24 h with LPV-treated R9  
820 BaL viruses (0.2 U RT/ml). Expression of *CXCL-10* (K) was normalised to an internal control  
821 (*GAPDH*) and these values were then normalised to those for the non-infected mock cells,  
822 yielding the fold induction over mock. (L) infection levels of cells from (K). Cells were stained  
823 with a FITC-labelled anti-p24 antibody and analysed by flow cytometry. Data presented for  
824 (K) and (L) are collated from two donors (represented by circles and squares), performed in

825 triplicate. Horizontal line represents the median. Statistical analyses were performed using  
 826 the Student's t-test, with Welch's correction where appropriate and comparing to the 0 nM  
 827 LPV condition. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . See also Suppl. Fig. 2 and 3.  
 828

**Suppl Fig 2: PI treatment induces HIV-1 to trigger an ISG response in macrophages**



829

830 **Suppl. Fig. 2.** PI treatment induces HIV-1 to trigger an ISG response in macrophages. (A)

831 Schematic of intermediate Gag cleavage products. MA: matrix, CA: capsid, SP1: spacer

832 peptide 1, NC: nucleocapsid, SP2: spacer peptide 2. (B) Infection levels of cells from Fig. 1D-

833 G. PMA-treated (50 ng/ml, 48 h) THP-1 shSAMHD1 cells were transduced for 48 h with LPV-

834 treated HIV-1 GFP viruses (0.1 U RT/ml or 0.5 U RT/ml). Cells were analysed for GFP

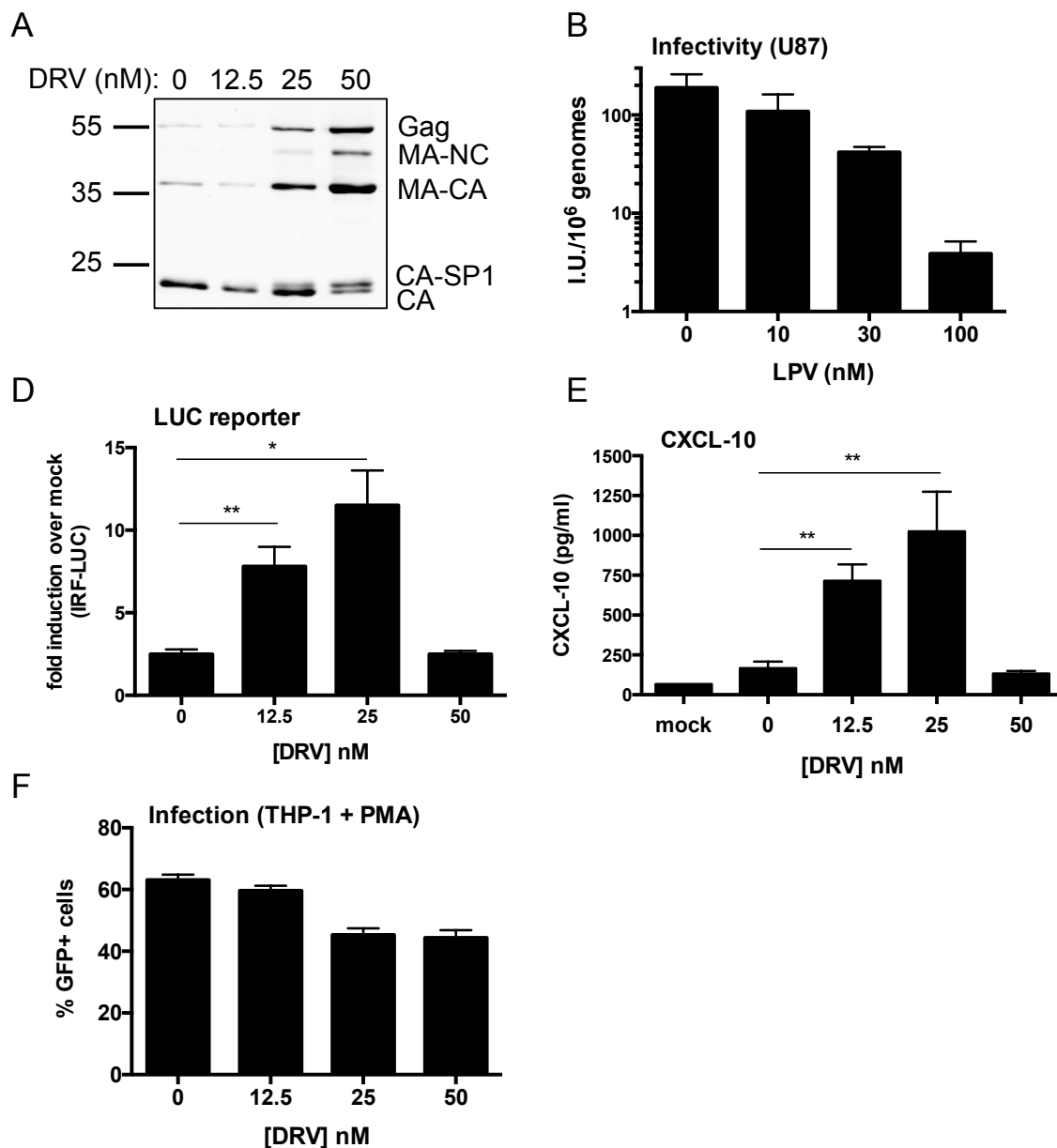
835 positivity by flow cytometry. Data are presented as mean  $\pm$  SD of triplicate data repeated at

836 least three times. (C) ISG qPCR from PMA-treated (50 ng/ml, 48 h) THP-1 shSAMHD1 cells

837 that had been transduced for 24 h with 0.2 U RT/ml HIV-1 GFP that had been treated with 30

838 nM LPV, or stimulated with 1 ng/ml IFN $\beta$  as a control, in the presence or absence (DMSO  
839 control) or 2  $\mu$ M ruxolitinib. Expression of *CXCL-10* was normalised to an internal control  
840 (*GAPDH*) and these values were then normalised to those for the non-transduced, DMSO-  
841 treated mock cells, yielding the fold induction over DMSO mock. Data are presented as mean  
842  $\pm$  SD of triplicate data repeated at least twice. (D-E) Titration of LPV-treated R9 BaL viruses  
843 on primary monocyte-derived macrophages. Infectious units per ml (IU/ml) were calculated by  
844 staining cells with a FITC-labelled anti-p24 antibody and analysing by flow cytometry. Data  
845 from individual donors are represented in E and collated in D, represented as percentage titre  
846 normalised to R9 BaL produced in the absence of LPV (0 nM). Data are shown as mean  $\pm$   
847 SD.  
848

**Suppl Fig 3: Treatment of HIV-1 with darunavir also induces an ISG response**



849

850 **Suppl. Fig. 3.** Treatment of HIV-1 with darunavir also triggers an ISG response. (A)

851 Immunoblot of HIV-1 GFP virus particles ( $2 \times 10^{11}$  genomes) produced in HEK293T cells in the

852 presence of increasing doses of protease inhibitor darunavir (DRV, 0-50 nM) and then

853 purified through a 20 % sucrose cushion. The blot was probed with an anti-HIV-1 p24

854 antibody. Molecular mass markers (in kDa) are indicated on the left and Gag cleavage

855 products on the right. (B) Titration of DRV-treated HIV-1 GFP viruses on U87 cells. Infectious

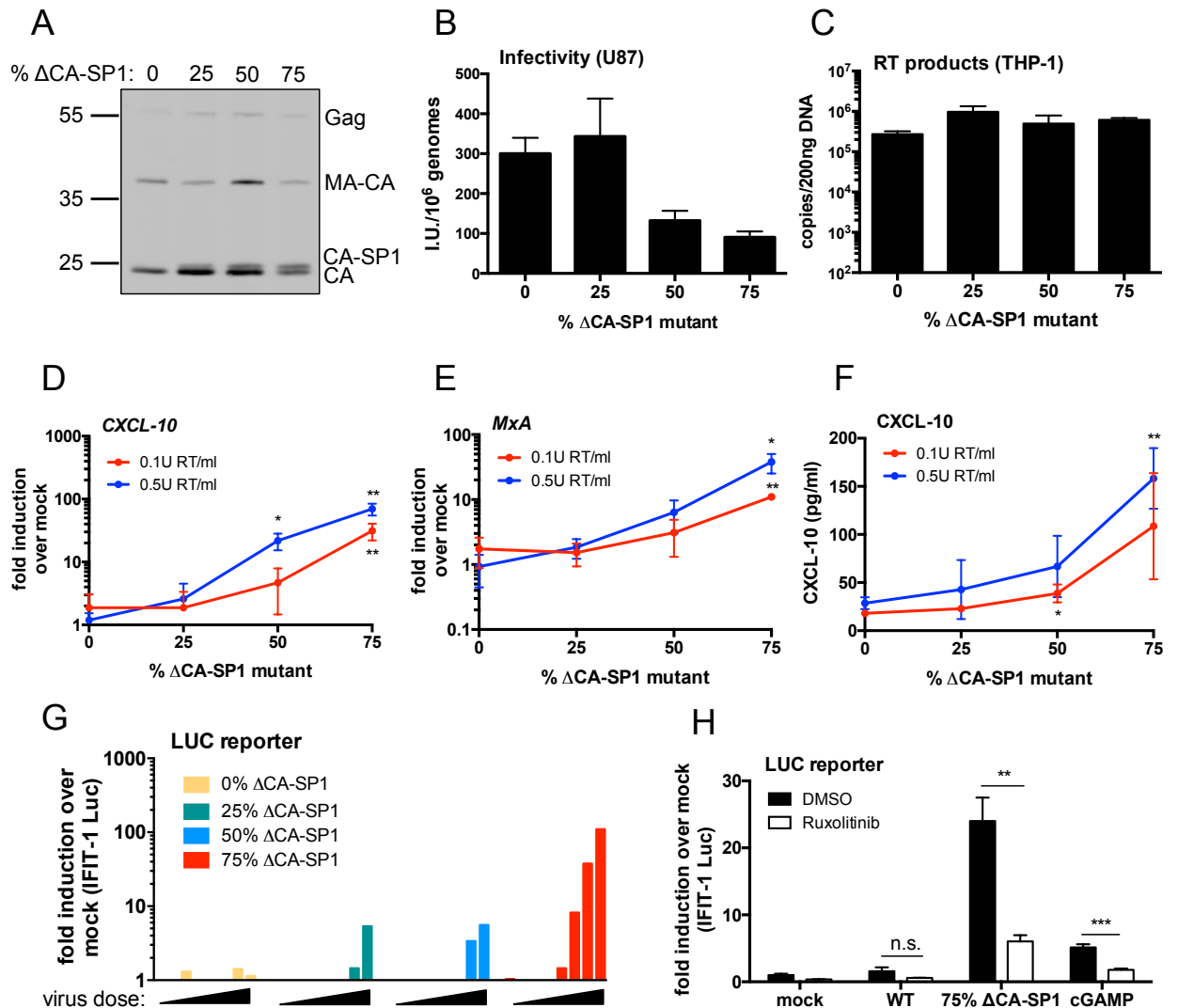
856 units per ml (IU/ml) were calculated by enumeration of GFP-positive cells by flow cytometry.

857 These data were then normalised to genomes/ml data calculated by qPCR to account for

858 variations in viral production. Data are presented as mean  $\pm$  SD from three separate titrations

859 performed in singlet. (D) IRF reporter activity from PMA-treated (50 ng/ml, 48 h) THP-1 Dual  
 860 shSAMHD1 cells transduced for 24 h with DRV-treated HIV-1 GFP ( $1 \times 10^{10}$  genomes/ml).  
 861 Gaussia luciferase activity in the supernatant was measured and normalised to mock  
 862 transduced cells, yielding a fold induction. (E) Level of CXCL-10 protein in the cell  
 863 supernatant from D was measured by ELISA. (F) Infection levels of cells from D-E. Cells were  
 864 analysed for GFP positivity by flow cytometry at 48 h post-transduction. Data for D-F are  
 865 presented as mean  $\pm$  SD of triplicate data repeated at least twice. Statistical analyses were  
 866 performed using the Student's t-test, with Welch's correction where appropriate and  
 867 comparing to the 0 nM DRV condition. \*  $P < 0.05$ , \*\*  $P < 0.01$ .  
 868

**Fig 2: HIV-1 with Gag protease cleavage mutation induces ISGs in THP-1 cells**



869

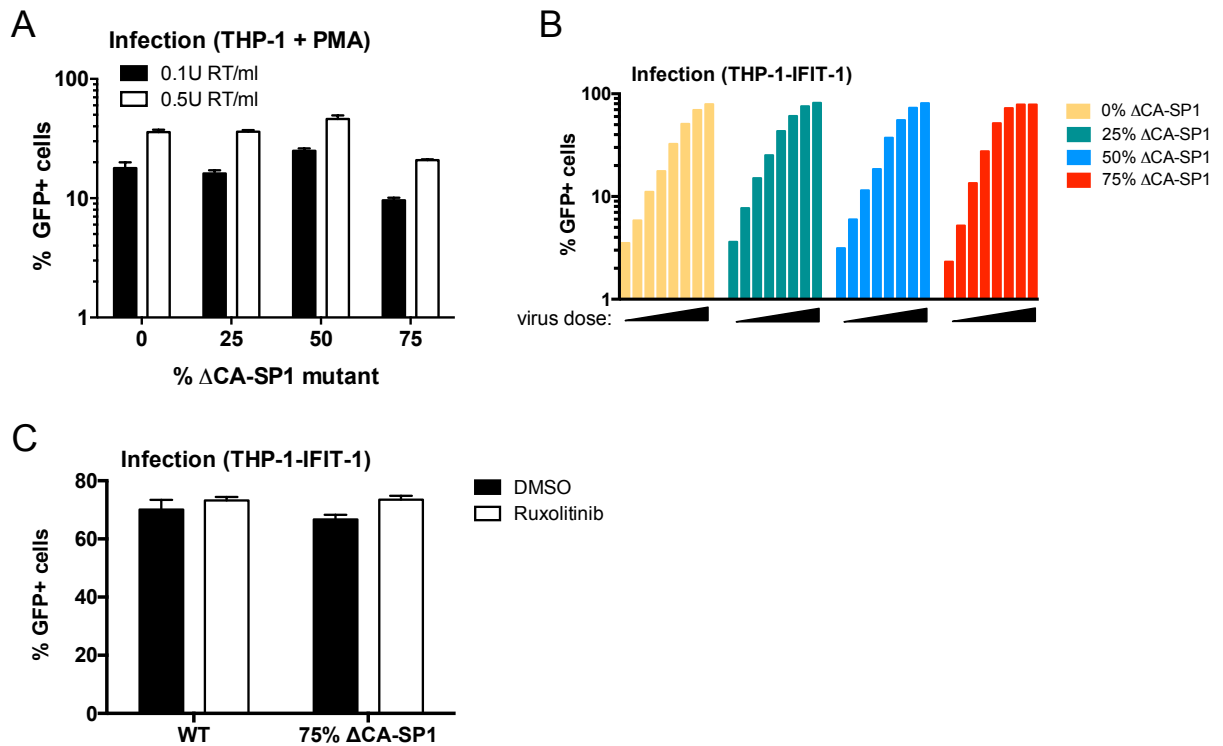
870 **Fig 2:** HIV-1 with Gag protease cleavage mutation induces ISGs in THP-1 cells. (A)  
871 Immunoblot of HIV-1 GFP virus particles ( $2 \times 10^{11}$  genomes) produced in HEK293T cells with  
872 varying proportions of  $\Delta$ CA-SP1 protease cleavage mutation and then purified through a 20  
873 % sucrose cushion. The blot was probed with an anti-HIV-1 p24 antibody. Molecular mass  
874 markers (in kDa) are indicated on the left and Gag cleavage products on the right. (B)  
875 Titration of HIV-1 GFP  $\Delta$ CA-SP1 viruses on U87 cells. Infectious units per ml (IU/ml) were  
876 calculated by enumeration of GFP-positive cells by flow cytometry. These data were then  
877 normalised to genomes/ml data calculated by qPCR to account for variations in viral  
878 production. Data are presented as mean  $\pm$  SD from three separate titrations performed in  
879 singlet. (C) RT products from THP-1 cells that had been transduced for 24 h with  $6 \times 10^9$   
880 genomes/ml (approx. 0.5 U RT/ml) HIV-1 GFP  $\Delta$ CA-SP1 viruses. Cells were lysed for DNA  
881 extraction and then used for qPCR analysis using primers for GFP. Data are presented as  
882 mean  $\pm$  SD of triplicate data repeated at least twice. (D-E) ISG qPCR from PMA-treated (50  
883 ng/ml, 48 h) THP-1 shSAMHD1 cells that had been transduced for 24 h with HIV-1 GFP  $\Delta$ CA-  
884 SP1 viruses (0.1 U RT/ml red line, 0.5 U RT/ml blue line). Expression of *CXCL-10* (D) and  
885 *MxA* (E) was normalised to an internal control (*GAPDH*) and these values were then  
886 normalised to those for the non-transduced mock cells, yielding the fold induction over mock.  
887 (F) Level of *CXCL-10* protein in the cell supernatants from D-E was measured by ELISA.  
888 Data for D-F are presented as mean  $\pm$  SD of triplicate data repeated at least three times. (G)  
889 IFIT-1 reporter activity from monocytic THP-1-IFIT-1 cells transduced for 24 h with varying  
890 doses of HIV-1 GFP  $\Delta$ CA-SP1 viruses (0.016 – 0.2 U RT/ml). Gaussia luciferase activity in  
891 the supernatant was measured and normalised to mock transduced cells to generate a fold  
892 induction. Data are shown as individual measurements and are representative of at least two  
893 repeats. (H) IFIT-1 reporter activity from monocytic THP-1-IFIT-1 cells transduced with HIV-1  
894 GFP containing either 0 % (WT) or 75 %  $\Delta$ CA-SP1 mutant, or stimulated with 4  $\mu$ g/ml cGAMP  
895 as a control, in the presence or absence (DMSO control) or 2  $\mu$ M ruxolitinib. Gaussia  
896 luciferase activity in the supernatant was measured and normalised to DMSO-treated mock  
897 cells, yielding the fold induction over DMSO mock. Data are presented as mean  $\pm$  SD of  
898 triplicate data repeated at least three times. Statistical analyses were performed using the  
899 Student's t-test, with Welch's correction where appropriate and comparing to the 0 %  $\Delta$ CA-

900 SP1 virus (D-F) or the DMSO control (H). \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . See also Suppl.

901 Fig. 4.

902

**Suppl Fig 4: HIV-1 with Gag protease cleavage mutation induces ISGs in THP-1 cells**



903

904 **Suppl. Fig. 4.** HIV-1 with Gag protease cleavage mutation induces ISGs in THP-1 cells. (A)

905 Infection levels of cells from Fig. 2D-F. PMA-treated (50 ng/ml, 48 h) THP-1 shSAMHD1 cells

906 were transduced for 48 h with HIV-1 GFP  $\Delta$ CA-SP1 viruses (0.1 U RT/ml or 0.5 U RT/ml).

907 Cells were analysed for GFP positivity by flow cytometry. (B) Infection levels of cells from Fig.

908 2G. THP-1-IFIT-1 cells were transduced for 48 h with HIV-1 GFP  $\Delta$ CA-SP1 viruses (0.016 –

909 0.2 U RT/ml). Cells were analysed for GFP positivity by flow cytometry. (C) Infection levels of

910 cells from Fig. 2H. THP-1-IFIT-1 cells were transduced for 48 h with HIV-1 GFP containing

911 either 0 % (WT) or 75 %  $\Delta$ CA-SP1 mutant in the presence or absence (DMSO control) of 2

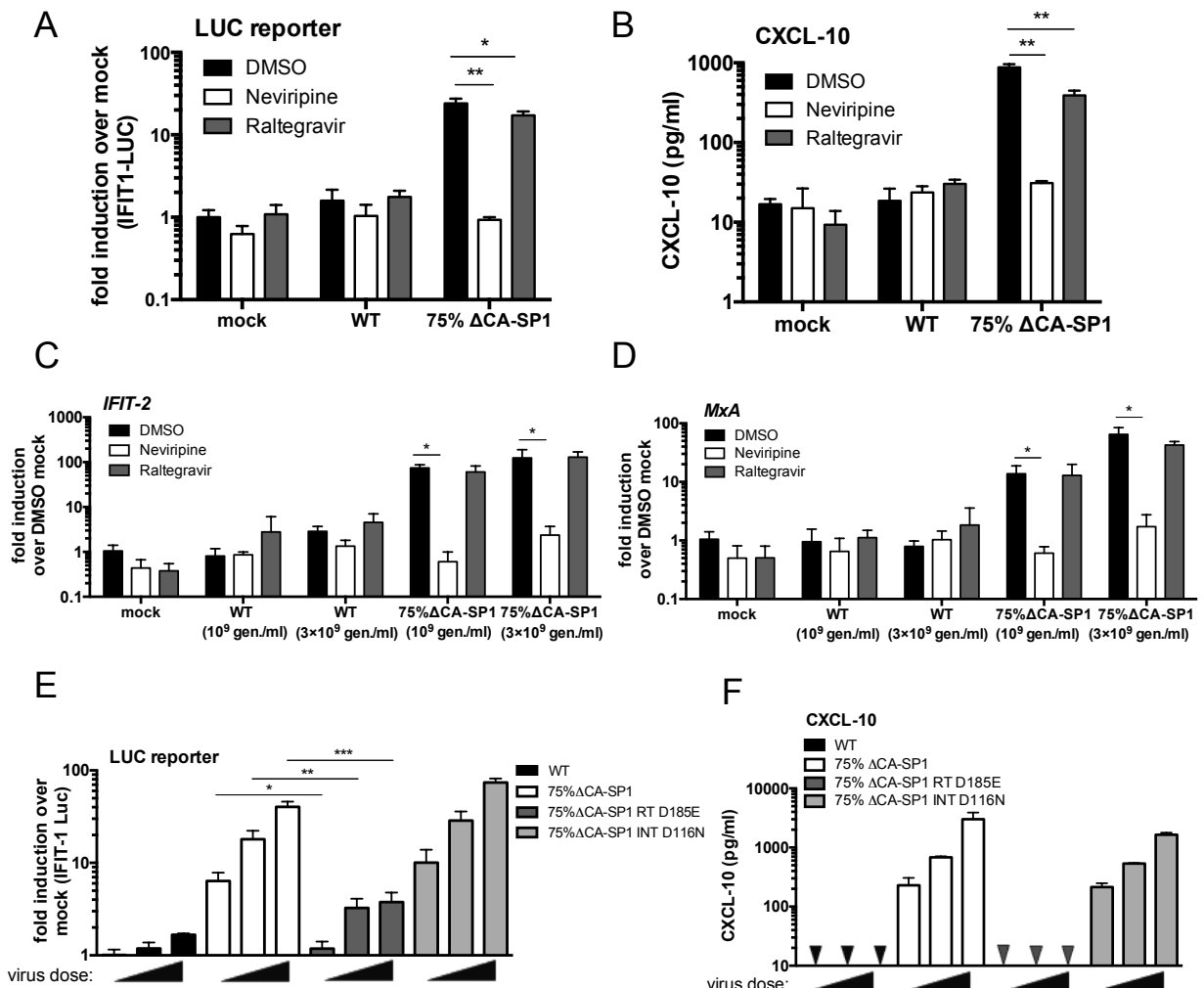
912  $\mu$ M ruxolitinib. Cells were analysed for GFP positivity by flow cytometry. Data are shown as

913 individual measurements (B) or mean  $\pm$  SD from triplicate data (A, C) repeated at least three

914 times.



**Fig 3: Innate immune activation is RT-dependent**

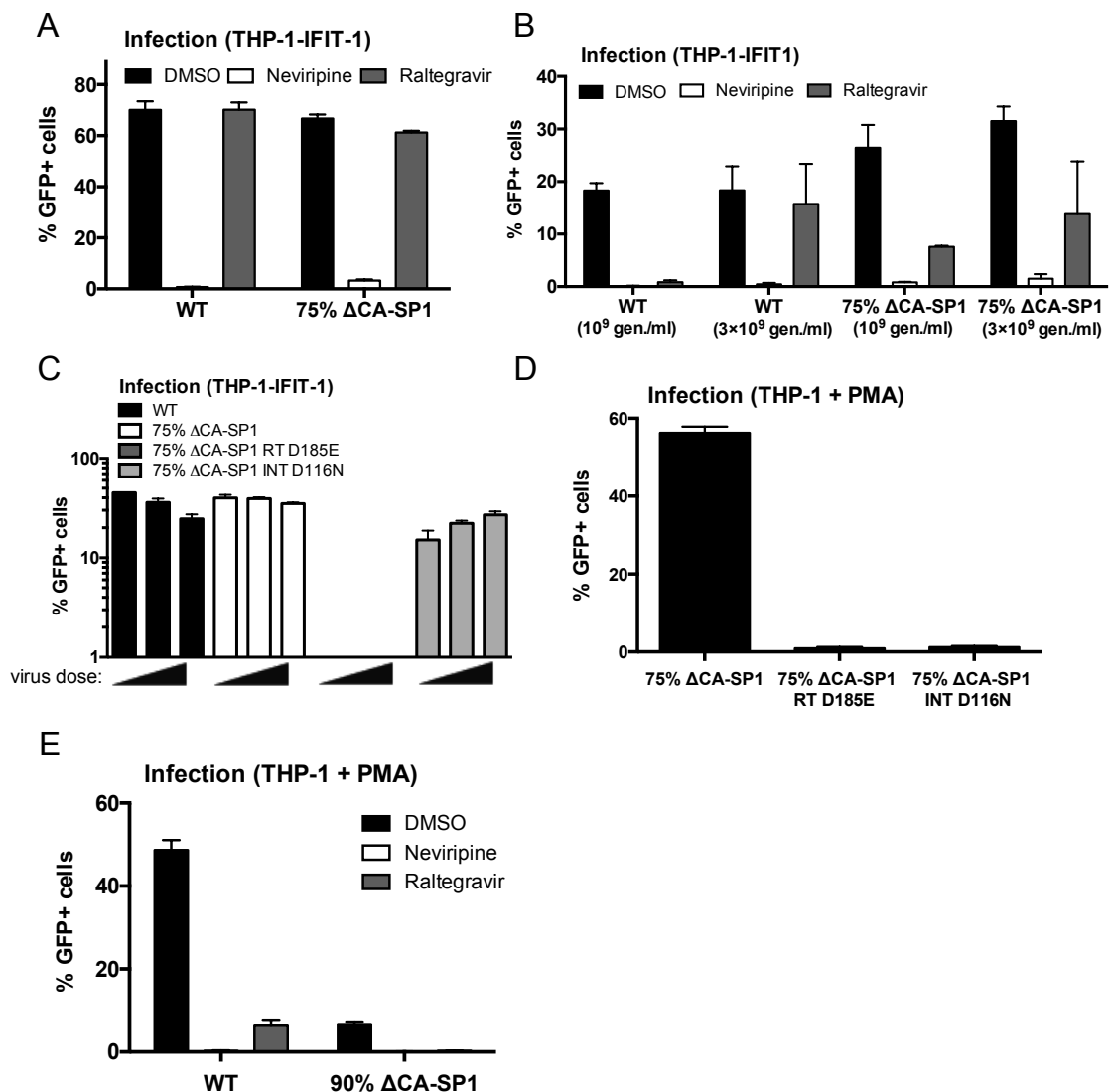


915

916 **Fig. 3.** Innate immune activation is RT-dependent. (A) IFIT-1 reporter activity from THP-1-  
 917 IFIT-1 cells transduced for 24 h with HIV-1 GFP containing 0 % (WT) or 75 % ΔCA-SP1  
 918 mutant (1 U RT/ml) in the presence or absence (DMSO control) of 5 μM neviripine or 10 μM  
 919 raltegravir. Gaussia luciferase activity in the supernatant was measured and normalised to  
 920 mock transduced cells to generate a fold induction. (B) Level of CXCL-10 protein in the cell  
 921 supernatants from (A) was measured by ELISA. (C-D) ISG qPCR from THP-1-IFIT-1 cells  
 922 that had been transduced for 24 h with increasing doses of 0 % (WT) or 75 % ΔCA-SP1  
 923 mutant (10<sup>9</sup> and 3×10<sup>9</sup> genomes/ml) in the presence or absence (DMSO control) of 5 μM  
 924 neviripine or 10 μM raltegravir. Expression of *IFIT-2* (C) and *MxA* (D) was normalised to an  
 925 internal control (*GAPDH*) and these values were then normalised to those for the non-  
 926 transduced mock cells, yielding the fold induction over mock. (E) IFIT-1 reporter activity from  
 927 THP-1-IFIT-1 cells transduced for 24 h with increasing doses of HIV-1 GFP containing 0 %

928  $\Delta$ CA-SP1 (WT), 75 %  $\Delta$ CA-SP1, 75 %  $\Delta$ CA-SP1 carrying a mutation in reverse transcriptase  
 929 (75 %  $\Delta$ CA-SP1 RT D185E) or 75 %  $\Delta$ CA-SP1 carrying a mutation in integrase (75 %  $\Delta$ CA-  
 930 SP1 INT D116N) ( $3.75 \times 10^9$ ,  $7.5 \times 10^9$  and  $1.5 \times 10^{10}$  genomes/ml). (F) Level of CXCL-10  
 931 protein in the cell supernatants from B was measured by ELISA. Triangles indicate CXCL-10  
 932 not detected. Data are presented as mean  $\pm$  SD of triplicate data repeated 2-3 times.  
 933 Statistical analyses were performed using the Student's t-test, with Welch's correction where  
 934 appropriate. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . See also Suppl. Fig. 5.  
 935

**Suppl Fig 5: Innate immune activation is RT-dependent.**

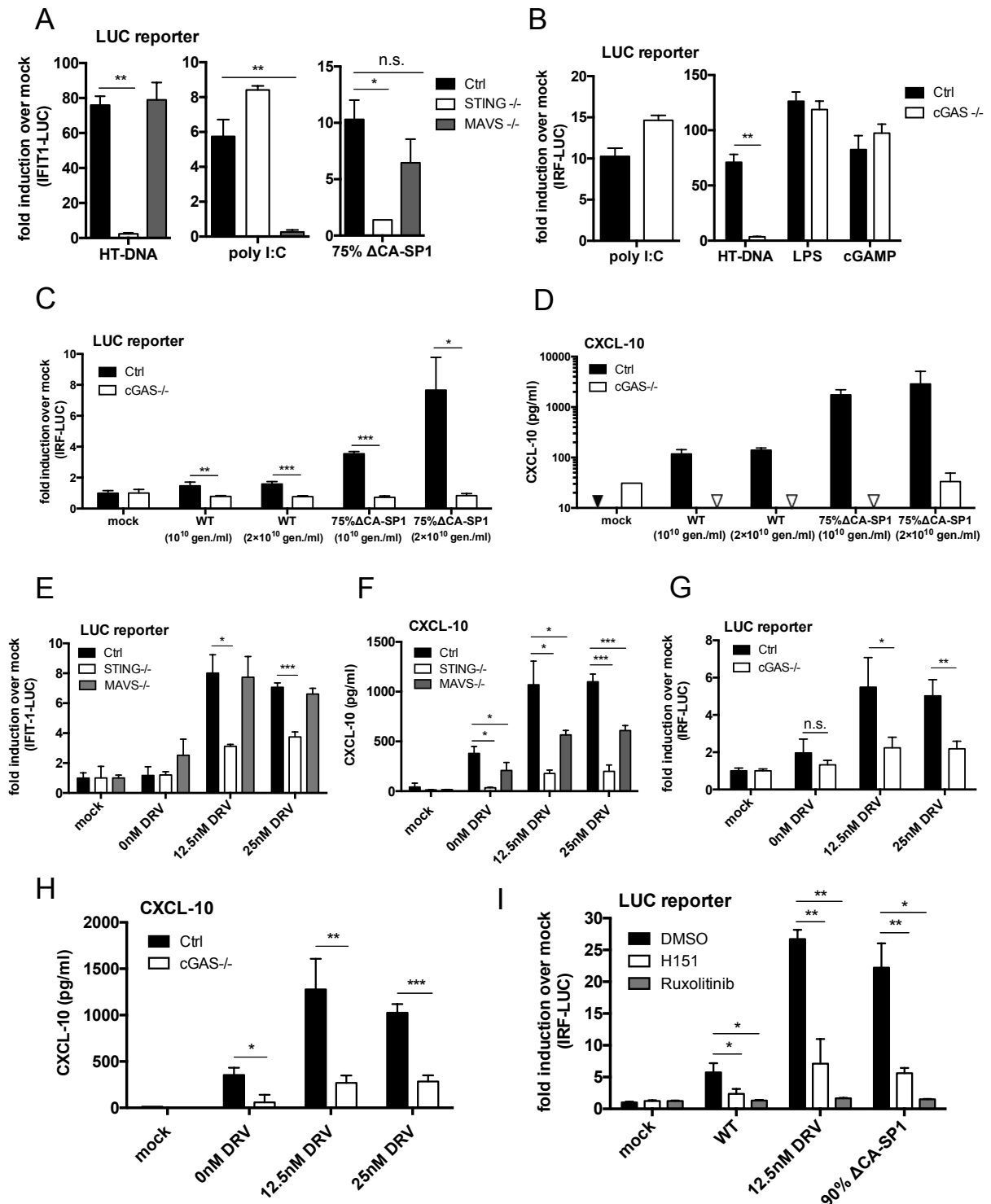


936

937 **Suppl. Fig. 5.** Innate immune activation is RT-dependent. (A) Infection levels from Figures 3A  
 938 and B. THP-1-IFIT-1 cells were transduced for 48 h with HIV-1 GFP containing 0 % (WT) or

939 75 %  $\Delta$ CA-SP1 mutant (1 U RT/ml) in the presence or absence (DMSO control) of 5  $\mu$ M  
940 neviripine or 10  $\mu$ M raltegravir. Cells were analysed for GFP positivity by flow cytometry. (B)  
941 Infection levels for Figures 3C and D. THP-1-IFIT-1 cells were transduced for 48 h with  
942 increasing doses of 0 % (WT) or 75 %  $\Delta$ CA-SP1 mutant ( $10^9$  and  $3 \times 10^9$  genomes/ml) in the  
943 presence or absence (DMSO control) of 5  $\mu$ M neviripine or 10  $\mu$ M raltegravir. Cells were  
944 analysed for GFP positivity by flow cytometry. (C) Infection levels for Figures 3E and F. THP-  
945 1-IFIT-1 cells were transduced for 48 h with with increasing doses of HIV-1 GFP containing 0  
946 %  $\Delta$ CA-SP1 (WT), 75 %  $\Delta$ CA-SP1, 75 %  $\Delta$ CA-SP1 carrying a mutation in reverse  
947 transcriptase (75 %  $\Delta$ CA-SP1 RT D185E) or 75 %  $\Delta$ CA-SP1 carrying a mutation in integrase  
948 (75 %  $\Delta$ CA-SP1 INT D116N) ( $3.75 \times 10^9$ ,  $7.5 \times 10^9$  and  $1.5 \times 10^{10}$  genomes/ml). Cells were  
949 analysed for GFP positivity by flow cytometry. (D) PMA-treated (50 ng/ml, 48 h) THP-1 Dual  
950 shSAMHD1 cells were transduced for 48 h with 75 %  $\Delta$ CA-SP1, 75 %  $\Delta$ CA-SP1 carrying a  
951 mutation in reverse transcriptase (75 %  $\Delta$ CA-SP1 RT D185E) or 75 %  $\Delta$ CA-SP1 carrying a  
952 mutation in integrase (75 %  $\Delta$ CA-SP1 INT D116N) ( $3 \times 10^9$  genomes/ml). Cells were analysed  
953 for GFP positivity by flow cytometry. (E) PMA-treated (50 ng/ml, 48 h) THP-1 Dual  
954 shSAMHD1 control cells were transduced for 48 h with WT HIV-1 GFP or 90 %  $\Delta$ CA-SP1  
955 mutant ( $1 \times 10^{10}$  genomes/ml) in the presence or absence (DMSO control) of 5  $\mu$ M neviripine  
956 or 10  $\mu$ M raltegravir. Data are presented as mean  $\pm$  SD of triplicate data repeated 2-3 times.

**Fig 4: Innate immune activation is DNA sensing-dependent**

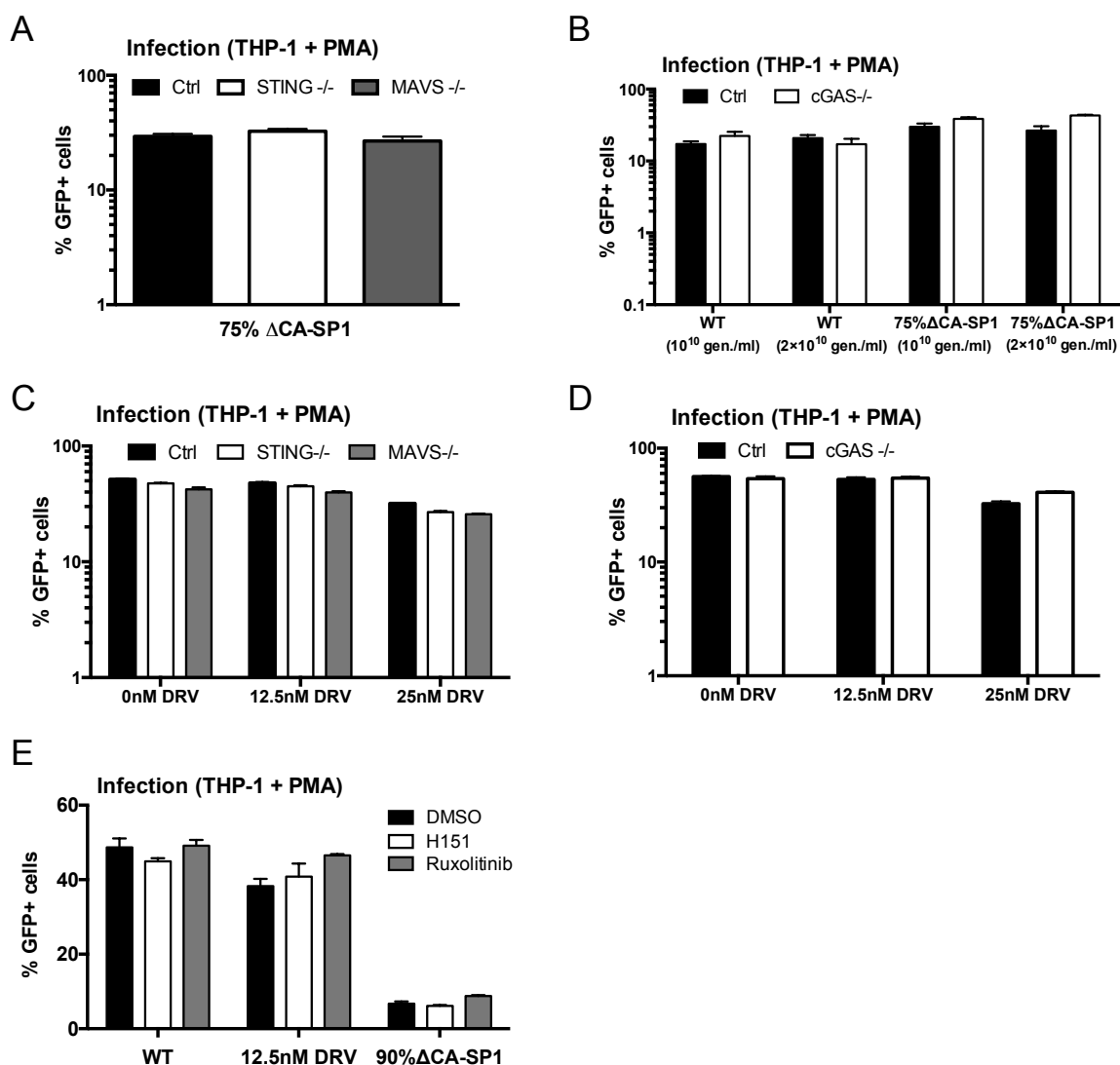


957

958 **Fig. 4.** Innate immune activation is DNA sensing-dependent. (A) IFIT-1 reporter activity from  
 959 PMA-treated (50 ng/ml, 48 h) THP-1-IFIT-1 shSAMHD1 cells lacking STING or MAVS, or a  
 960 gRNA control (Ctrl) cell line that were transduced for 24 h with HIV-1 GFP 75 % ΔCA-SP1

961 (0.4 U RT/ml) or stimulated by transfection with either HT-DNA (0.1 µg/ml) or poly I:C (0.5  
962 µg/ml). Gaussia luciferase activity in the supernatant was measured and normalised to mock  
963 transduced cells to generate a fold induction. (B-C) IRF reporter activity from PMA-treated (50  
964 ng/ml, 48 h) THP-1 Dual shSAMHD1 cells lacking cGAS or a matching control (Ctrl) cell line  
965 that were stimulated for 24 h with poly I:C (transfection, 0.5 µg/ml), HT-DNA (transfection, 0.1  
966 µg/ml), LPS (50 ng/ml) or cGAMP (transfection, 0.5 µg/ml) (B) or transduced for 24 h with  
967 increasing doses of HIV-1 GFP containing either 0 % (WT) or 75 % ΔCA-SP1 ( $1 \times 10^{10}$  and  
968  $2 \times 10^{10}$  genomes/ml) (C). (D) Level of CXCL-10 protein in the cell supernatants from (C) was  
969 measured by ELISA. Triangles indicate CXCL-10 not detected. (E) IFIT-1 reporter activity  
970 from PMA-treated (50 ng/ml, 48 h) THP-1-IFIT-1 shSAMHD1 cells lacking STING, MAVS or  
971 matching gRNA control (Ctrl) cell line that were transduced for 24 h with DRV-treated HIV-1  
972 GFP as indicated ( $1 \times 10^{10}$  genomes/ml). (F) Level of CXCL-10 protein in the cell supernatants  
973 from (E) was measured by ELISA. (G) IRF reporter activity from PMA-treated (50 ng/ml, 48 h)  
974 THP-1 Dual shSAMHD1 cells lacking cGAS or matching control (Ctrl) cell lines that were  
975 transduced for 24 h with DRV-treated HIV-1 GFP as indicated ( $1 \times 10^{10}$  genomes/ml). (H) Level  
976 of CXCL-10 protein in the cell supernatants from (G) was measured by ELISA. (I) IRF  
977 reporter activity from PMA-treated (50 ng/ml, 48 h) THP-1 Dual shSAMHD1 control cells  
978 transduced for 48 h with WT, DRV-treated (DRV, 12.5 nM) or HIV-1 GFP containing 90 %  
979 ΔCA-SP1 ( $1 \times 10^{10}$  genomes/ml) in the presence or absence (DMSO control) of 2 µM  
980 ruxolitinib or 0.5 µg/ml H151. Data are presented as mean ± SD of triplicate data repeated 2-4  
981 times. Statistical analyses were performed using the Student's t-test, with Welch's correction  
982 where appropriate. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . See also Suppl. Fig. 6.

**Suppl Fig 6: Innate immune activation is DNA sensing-dependent.**

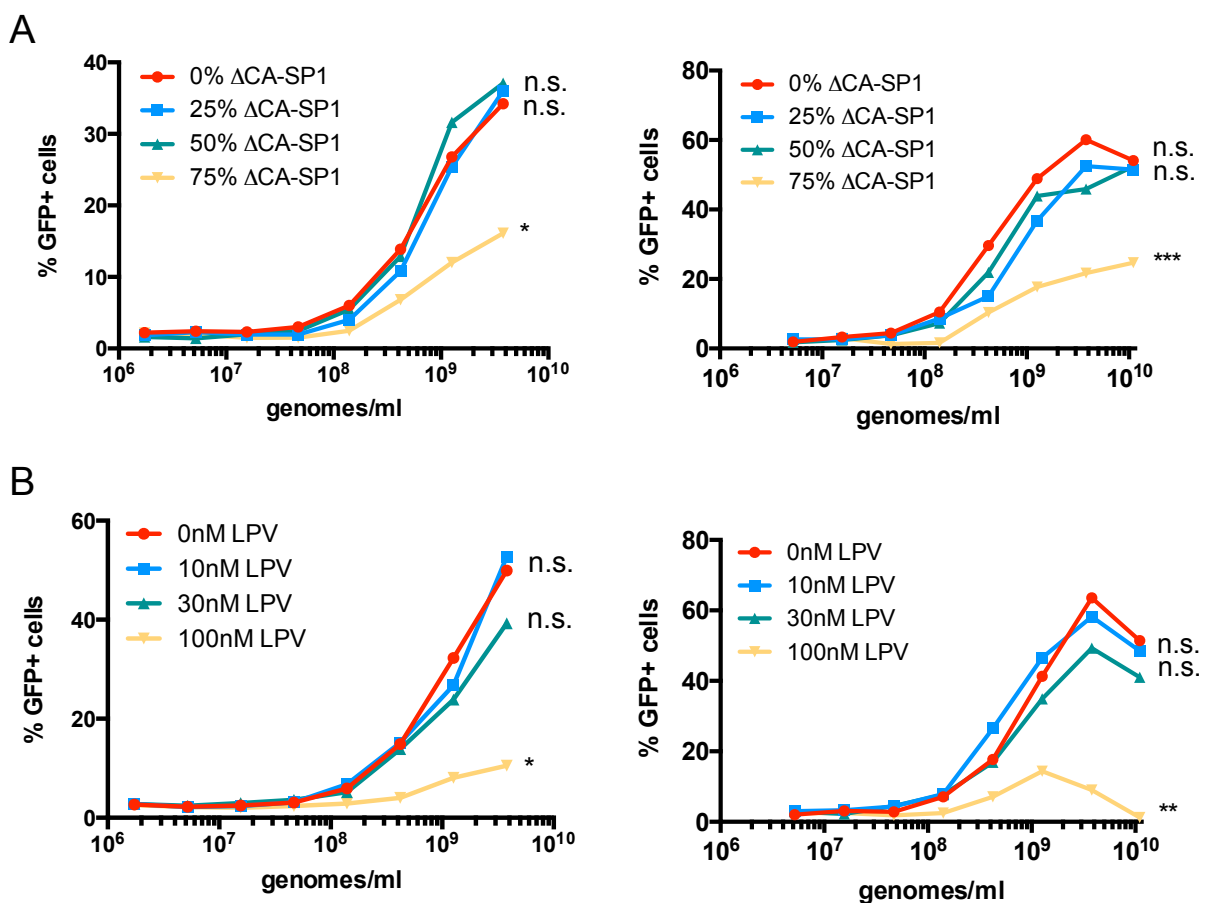


983

984 **Suppl. Fig. 6.** Innate immune activation is DNA sensing-dependent. (A) Infection levels of  
 985 cells from Fig. 4A. PMA-treated (50 ng/ml, 48 h) THP-1-IFIT-1 shSAMHD1 cells lacking  
 986 STING or MAVS, or a gRNA control (Ctrl) cell line that were transduced for 48 h with HIV-1  
 987 GFP 75 % ΔCA-SP1 (0.4 U RT/ml). (B) Infection levels of cells from Fig. 4 C-D. PMA-treated  
 988 (50 ng/ml, 48 h) THP-1 Dual shSAMHD1 cells lacking cGAS or a matching control (Ctrl) cell  
 989 line were transduced for 48 h with increasing doses of HIV-1 GFP containing either 0 % (WT)  
 990 or 75 % ΔCA-SP1 (1×10<sup>10</sup> and 2×10<sup>10</sup> genomes/ml). (C) Infection levels of cells from Fig. 4E-  
 991 F. PMA-treated (50 ng/ml, 48 h) THP-1-IFIT-1 shSAMHD1 cells lacking STING, MAVS or  
 992 matching gRNA control (Ctrl) cell lines were transduced for 48 h with DRV-treated HIV-1 GFP  
 993 as indicated (1×10<sup>10</sup> genomes/ml). (D) Infection levels of cells from Fig. 4G-H. PMA-treated

994 (50 ng/ml, 48 h) THP-1 Dual shSAMHD1 cells lacking cGAS or matching control (Ctrl) cell  
 995 lines were transduced for 48 h with DRV-treated HIV-1 GFP as indicated ( $1 \times 10^{10}$   
 996 genomes/ml). (E) Infection levels of cells from Fig. 4I. PMA-treated (50 ng/ml, 48 h) THP-1  
 997 Dual shSAMHD1 control cells were transduced for 48 h with WT, DRV-treated (DRV, 12.5  
 998 nM) or HIV-1 GFP 90 %  $\Delta$ CA-SP1 ( $1 \times 10^{10}$  genomes/ml) in the presence or absence (DMSO  
 999 control) of 2  $\mu$ M ruxolitinib or 0.5  $\mu$ g/ml H151. Cells were analysed for GFP positivity by flow  
 1000 cytometry. Data are presented as mean  $\pm$  SD of triplicate data repeated 2-4 times.

**Fig 5: Gag-defective HIV-1 particles are less able to saturate restriction factor TRIM5**

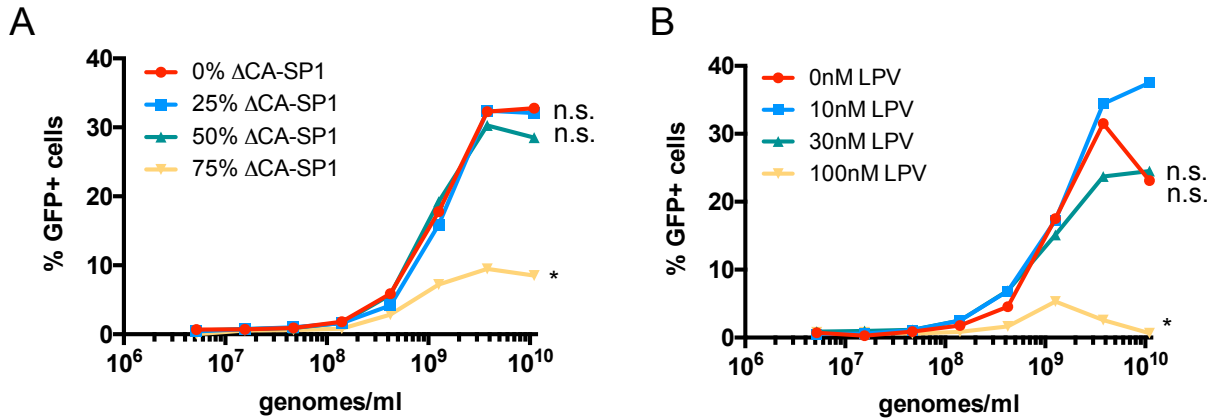


1001

1002 **Fig. 5.** Gag-defective HIV-1 particles are less able to saturate restriction factor TRIM5. (A-B)  
 1003 Abrogation-of-restriction assay in FRhK cells expressing restrictive rhesus TRIM5. FRhK cells  
 1004 were co-transduced with a fixed dose of HIV-1 GFP ( $5 \times 10^7$  genomes/ml) and increasing  
 1005 doses of HIV-LUC  $\Delta$ CA-SP1 mutants (A) or LPV-treated HIV-LUC viruses (B) as indicated  
 1006 ( $1.7 \times 10^6$  -  $3.8 \times 10^9$  genomes/ml). Rescue of GFP infectivity was assessed by flow cytometry.  
 1007 Data are presented as singlet % GFP values and two repeats of the experiment are shown.

1008 See also Suppl. Fig. 7. Statistical analyses were performed using 2-way ANOVA with multiple  
1009 comparisons. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

**Suppl. Fig 7: Gag-defective HIV-1 particles are less able to saturate restriction factor TRIM5**



1010

1011 **Suppl. Fig 7.** Gag-defective HIV-1 particles are less able to saturate restriction factor TRIM5.

1012 Abrogation-of-restriction assay in FRhK cells expressing restrictive rhesus TRIM5. (A) Repeat

1013 assay of data presented in Fig. 5A. FRhK cells were co-transduced with a fixed dose of HIV-1

1014 GFP ( $5 \times 10^7$  genomes/ml) and increasing doses of HIV-LUC  $\Delta$ CA-SP1 mutants as indicated

1015 ( $5.2 \times 10^6$  -  $1.1 \times 10^{10}$  genomes/ml). (B) Repeat assay of data presented in Fig. 5B. FRhK cells

1016 were co-transduced with a fixed dose of HIV-1 GFP ( $5 \times 10^7$  genomes/ml) and increasing

1017 doses of LPV-treated HIV-LUC viruses as indicated ( $5.2 \times 10^6$  -  $1.1 \times 10^{10}$  genomes/ml).

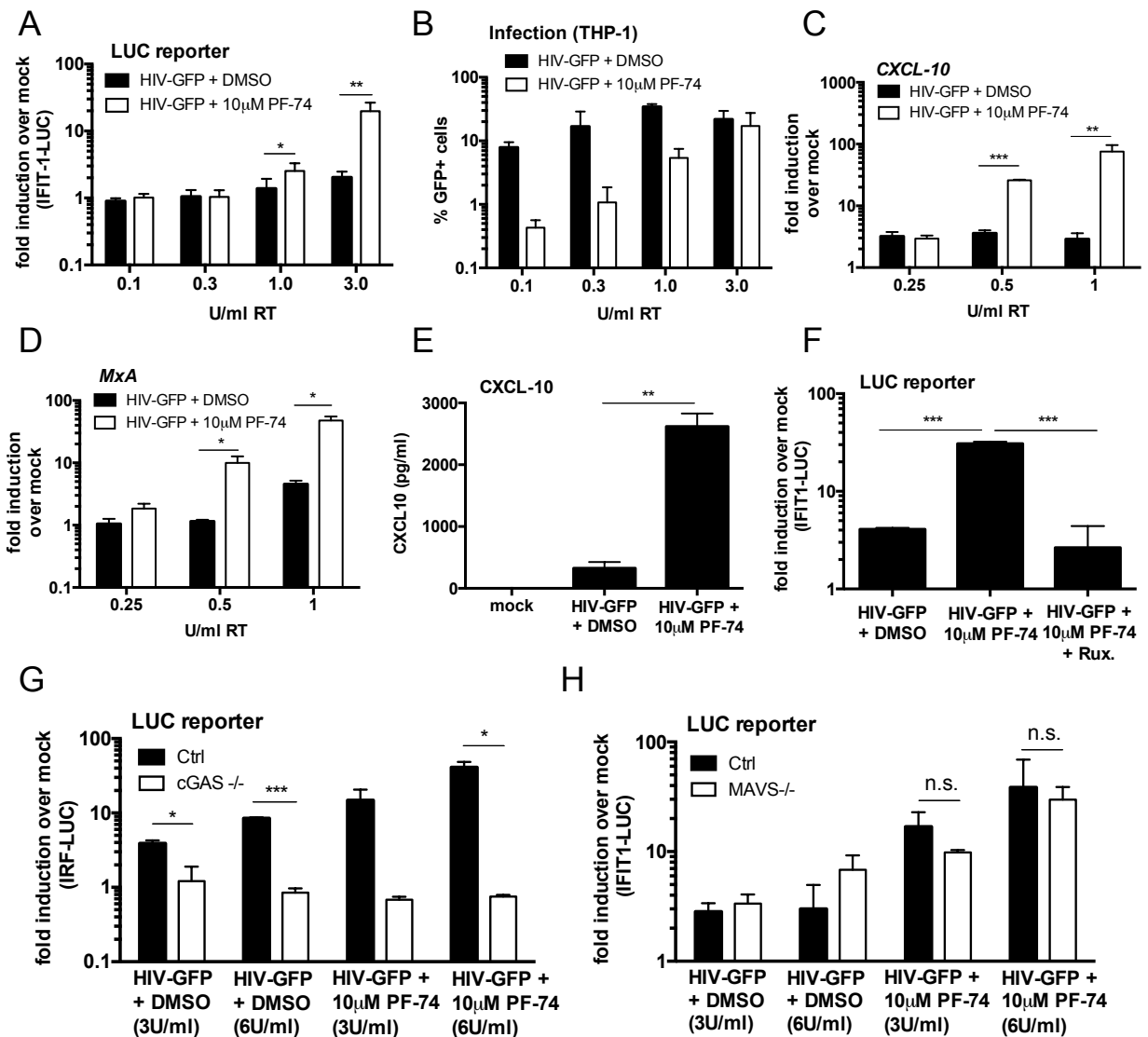
1018 Rescue of GFP infectivity was assessed by flow cytometry. Data are presented as singlet %

1019 GFP values. Statistical analyses were performed using 2-way ANOVA with multiple

1020 comparisons. \*  $P < 0.05$ .



**Fig 6: PF-74 treatment induces HIV-1 to trigger a DNA-sensing dependent ISG response**



1021

1022 **Fig. 6.** PF-74 treatment induces HIV-1 to trigger a DNA-sensing dependent ISG response. (A)

1023 IFIT-1 reporter activity from THP-1-IFIT-1 cells transduced for 24 h with increasing doses

1024 (0.1-3 U/ml RT) of HIV-1 GFP in the presence or absence (DMSO control) of PF-74 (10  $\mu$ M).

1025 Gaussia luciferase activity in the supernatant was measured and normalised to mock

1026 transduced cells to generate a fold induction. (B) Infection levels of cells from 6A. Cells were

1027 analysed for GFP positivity by flow cytometry. (C-D) ISG qPCR from THP-1-IFIT-1 cells

1028 transduced for 24 h with increasing doses of HIV-1 GFP (0.25-1 U/ml RT) in the presence or

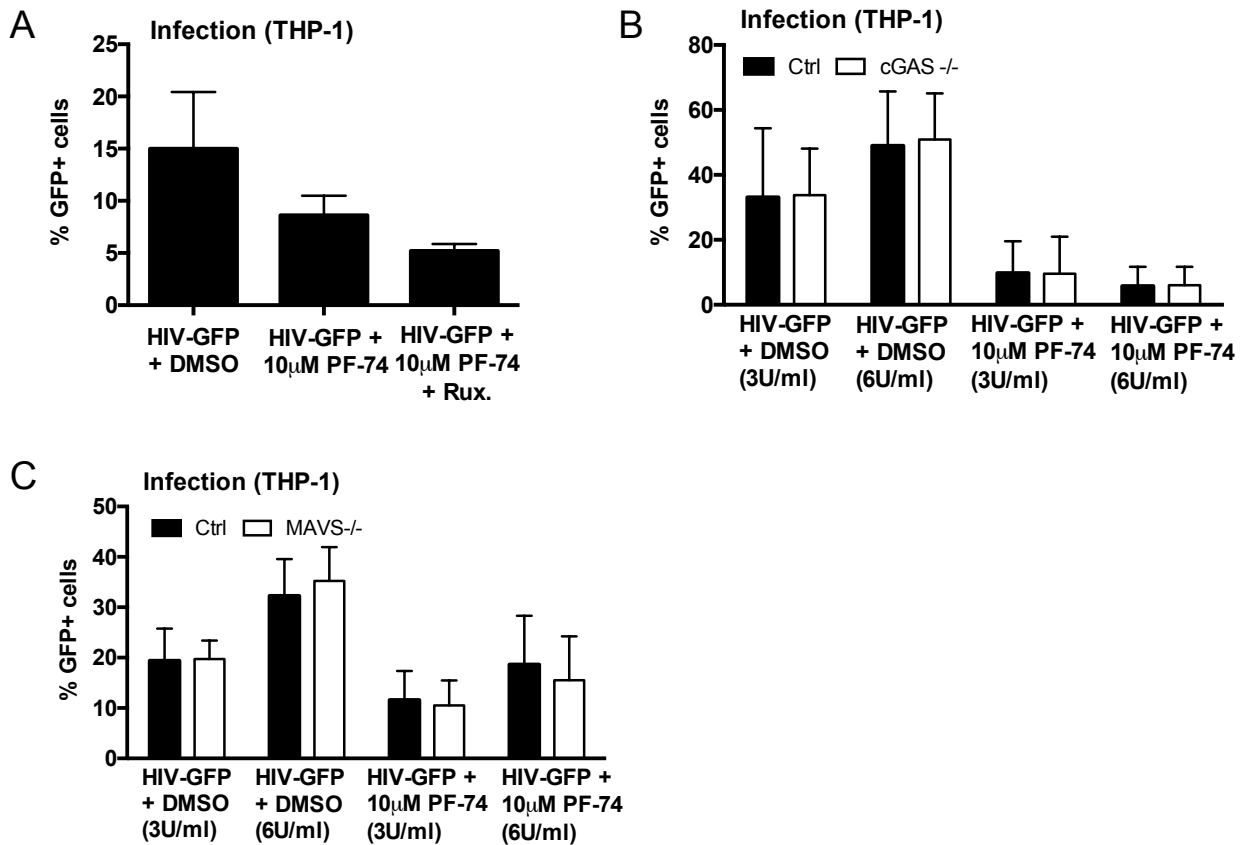
1029 absence (DMSO control) of PF-74 (10  $\mu$ M). Expression of *CXCL-10* (C) and *MxA* (D) was

1030 normalised to an internal control (*GAPDH*) and these values were then normalised to those

1031 for the non-transduced mock cells, yielding the fold induction over mock. (E) Level of CXCL-

1032 10 protein in the cell supernatants of THP-1-IFIT-1 cells transduced for 24 h with HIV-1 GFP  
 1033 (3 U/ml) in the presence or absence (DMSO control) of PF-74 (10  $\mu$ M) was measured by  
 1034 ELISA. (F) IFIT-1 reporter activity from THP-1-IFIT-1 cells transduced for 24 h with with HIV-1  
 1035 GFP (3 U/ml RT) in the presence or absence (DMSO control) of PF-74 (10  $\mu$ M) and ruxolitinib  
 1036 (Rux, 2  $\mu$ M) as indicated. (G) IRF reporter activity from THP-1 Dual shSAMHD1 cells lacking  
 1037 cGAS or a matching control (Ctrl) cell line that were transduced for 24 h with increasing doses  
 1038 of HIV-1 GFP (3 U/ml and 6 U/ml) in the presence or absence (DMSO control) of PF-74 (10  
 1039  $\mu$ M). (H) IFIT-1 reporter activity from THP-1-IFIT-1 cells lacking MAVS or a matching gRNA  
 1040 control (Ctrl) cell line that were transduced for 24 h with increasing doses of HIV-1 GFP (3  
 1041 U/ml and 6 U/ml) in the presence or absence (DMSO control) of PF-74 (10  $\mu$ M). Data are  
 1042 presented as mean  $\pm$  SD of replicate data (2-6 replicates per condition) repeated at least  
 1043 three times. Statistical analyses were performed using the Student's t-test, with Welch's  
 1044 correction where appropriate. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . See also Suppl. Fig. 8.  
 1045

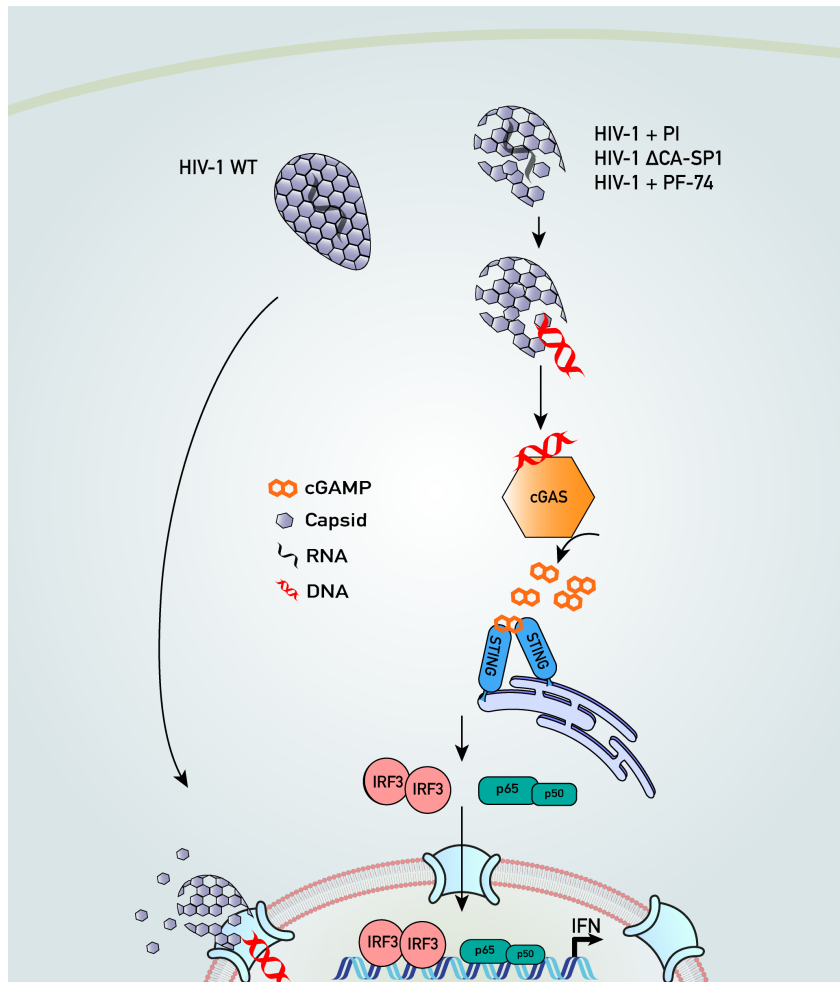
**Suppl. Fig 8:** PF-74 treatment induces HIV-1 to trigger a DNA-sensing dependent ISG response



1046

1047 **Suppl. Fig. 8.** PF-74 treatment induces HIV-1 to trigger a DNA-sensing dependent ISG  
1048 response. (A) Infection levels of cells from Fig. 6F. THP-1-IFIT-1 cells were transduced for 48  
1049 h with with HIV-1 GFP (3 U/ml RT) in the presence or absence (DMSO control) of PF-74 (10  
1050  $\mu$ M) and ruxolitinib (Rux, 2  $\mu$ M) as indicated. Cells were analysed for GFP positivity by flow  
1051 cytometry. (B) Infection levels of cells from Fig. 6G. THP-1 Dual shSAMHD1 cells lacking  
1052 cGAS or a matching control (Ctrl) cell line were transduced for 48 h with increasing doses of  
1053 HIV-1 GFP (3 U/ml and 6 U/ml) in the presence or absence (DMSO control) of PF-74 (10  
1054  $\mu$ M). Cells were analysed for GFP positivity by flow cytometry. (C) Infection levels of cells  
1055 from Fig. 6H. THP-1-IFIT-1 cells lacking MAVS or a matching gRNA control (Ctrl) cell line  
1056 were transduced for 48 h with increasing doses of HIV-1 GFP (3 U/ml and 6 U/ml) in the  
1057 presence or absence (DMSO control) of PF-74 (10  $\mu$ M). Cells were analysed for GFP  
1058 positivity by flow cytometry. Data are presented as mean  $\pm$  SD of replicate data (2-4  
1059 replicates per condition) repeated at least three times.  
1060

**Suppl. Fig 9: The HIV-1 capsid protects viral DNA from sensing by cGAS**



1061

1062

1063 **Suppl. Fig. 9.** Disrupting HIV-1 capsid formation causes cGAS sensing of viral DNA.

1064 After entry wild-type (WT) HIV-1 stays intact as it traverses the cytoplasm allowing it to

1065 synthesise its DNA without activating a type I IFN response. Conversely treatment of HIV-1

1066 with protease inhibitors (PI), capsid destabilising small molecule PF-74 or mutation of the

1067 protease cleavage site between capsid and spacer peptide 1 (HIV-1 ΔCA-SP1) leads to

1068 defective particles that fail to protect viral DNA from innate sensor cGAS. Binding of cGAS to

1069 viral DNA leads to the production of cGAMP that binds STING and stimulates IFN production

1070 through activation of the transcription factors IRF3 and p50/p65 (NF-κB).

1071