1   **Gene knockout shows that PML (TRIM19) does not restrict the early stages of HIV-1**

2   **infection in human cell lines**

3   Nasser Masroori[1], Pearl Cherry[1], Natacha Merindol[1], Jia-xin Li[1], Lina Poulain[1], Mélodie B.

4   Plourde[1], Lionel Berthoux[1,*]

5   Running title: PML does not restrict HIV-1 in human cells

6   [1] Laboratory of retrovirology, Department of Medical Biology and BioMed research group, Université du

7   Québec à Trois-Rivières, 3351 Boulevard des Forges, Trois-Rivières, QC G9A 5H7, Canada.

8   * Author to whom correspondence should be addressed; E-Mail: berthoux@uqtr.ca;

9   Tel.: +1-819-376-5011 (ext. 4466); Fax: +1-819-376-5084.

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11   Word count (abstract):

12   Word count (main text):

Abstract (250 words)

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14   The PML (promyelocytic leukemia) protein is a member of the TRIM family, a large group of

15   proteins that show high diversity in functions but possess a common tripartite motif giving the

16   family its name. We and others recently reported that both murine and human PML strongly

17   restrict the early stages of infection by HIV-1 and other lentiviruses when expressed in mouse

18   embryonic fibroblasts (MEFs). This restriction activity was found to contribute to the type I

19   interferon (IFN-I)-mediated inhibition of HIV-1 in MEFs. Additionally, PML caused

20   transcriptional repression of the HIV-1 promoter in MEFs. By contrast, the modulation of the

21   early stages of HIV-1 infection of human cells by PML has been investigated by RNAi with

22   unclear results. In order to conclusively determine whether PML restricts HIV-1 or not in human

23   cells, we used CRISPR-Cas9 to knock out its gene in epithelial, lymphoid and monocytic human

24   cell lines. Infection challenges showed that PML knockout had no effect on the permissiveness

25   of these cells to HIV-1 infection. IFN-I treatments inhibited HIV-1 equally whether PML was

26   expressed or not. Over-expression of individual hPML isoforms, or of mPML, in a human T cell

27   line did not restrict HIV-1. The presence of PML was not required for the restriction of

28   nonhuman retroviruses by TRIM5α, another human TRIM protein, and TRIM5α was inhibited

29   by arsenic trioxide through a PML-independent mechanism. We conclude that PML is not a

30   restriction factor for HIV-1 in human cell lines representing diverse lineages.

Importance (150 words)

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32   PML is involved in innate immune mechanisms against both DNA and RNA viruses. Although

33   the mechanism by which PML inhibits highly divergent viruses is unclear, it was recently found

34   that it can increase the transcription of interferon-stimulated genes (ISGs). However, whether

35   human PML inhibits HIV-1 has been debated. Here we provide unambiguous, knockout-based

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36   evidence that PML does not restrict the early post-entry stages of HIV-1 infection in a variety of

37   human cell types and does not participate in the inhibition of HIV-1 by IFN-I. Although this

38   study does not exclude the possibility of other mechanisms by which PML may interfere with

39   HIV-1, we nonetheless demonstrate that PML does not generally act as an HIV-1 restriction

40   factor in human cells and that its presence is not required for IFN-I to stimulate the expression of

41   anti-HIV-1 genes. These results contribute to uncovering the landscape of HIV-1 inhibition by

42   ISGs in human cells.

Introduction

PML/TRIM19 belongs to the tripartite motif (TRIM) protein superfamily that shares a conserved tripartite architecture: a RING domain, one or two B-boxes, and a coiled-coil domain (1). Due to the alternative splicing of the C-terminal domain, seven PML isoforms are present in human cells. Isoforms I to VI are primarily located in the nucleus, while PML VII is mostly cytoplasmic (2). PML is the major component of a nuclear substructure named PML nuclear body (PML NB). PML NBs are dynamic and their size, number, and composition change in response to cellular stresses or during the cell cycle. In addition to PML, these NBs recruit many other proteins in a transient fashion (3-6). TRIM5α, a cytoplasmic factor that restricts retroviruses in a species-specific, virus-specific manner (7), is actively shuttling between the cytoplasm and the nucleus and localizes to the PML NBs when present in the nucleus (8). PML is involved in many cellular activities including transcriptional regulation and tumor suppression (5, 9, 10).

IFNs are a multigene family of inducible cytokines released by host cells in response to pathogens, including viruses (11-13). IFN-I binding to its receptor leads to the transcriptional stimulation of a set of genes encoding antiviral proteins which inhibit the replication of a wide range of viruses (12, 14). The transcription of PML and of many NB-associated proteins (e.g. Daxx and Sp100) is up-regulated by IFN-I (15, 16). Conversely, it was recently proposed that PML is involved in the IFN-I-induced expression of ISGs by directly binding their promoter (17).

The involvement of PML in antiviral defense mechanisms against several DNA and RNA viruses has been extensively studied. PML was shown to restrict a complex retrovirus, the human foamy virus, by inhibiting viral gene expression (18). PML deficient cells are also more prone to infection with rabies virus (19). Moreover, PML was shown to interfere with the replication of

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66   poliovirus (20), encephalomyocarditis virus (EMCV) (21), herpes simplex virus type-1 (HSV-1),

67   adeno-associated virus (AAV) (22), influenza virus, and vesicular stomatitis virus (VSV) (23).

68   As a direct consequence, some viruses such as HSV-1 and the human cytomegalovirus have

69   evolved mechanisms to counteract PML, either by disrupting PML NBs and/or by inducing PML

70   degradation (24-26).

71   The role of PML in HIV-1 infection of human cells is controversial. As_2O_3, a drug that induces

72   PML oligomerization and degradation (27), was shown to increase the susceptibility of human

73   cells to N-tropic murine leukemia virus (N-MLV) and HIV-1 (28). A recent study proposed that

74   PML was an indirect inhibitor of HIV-1 early post-entry infection stages through its association

75   with Daxx, a constitutive partner protein in PML NBs (29). However, another group found that

76   the depletion of PML (but not that of Daxx) enhanced HIV-1 infection in human primary

77   fibroblasts, while having no effect in T cell lines such as Jurkat (30). PML was also found to

78   regulate HIV-1 latency. Specifically, PML degradation or NBs disruption resulted in the

79   activation of HIV-1 provirus transcription in a lymphoid model of HIV-1 latency (31), although

80   these results have not been independently confirmed. There is consensus, however, on the

81   existence of a PML-dependent restriction of HIV-1 in MEFs. In these cells, PML inhibits the

82   early post-entry stages of infection (32-34) and also promotes the transcriptional silencing of the

83   integrated provirus (34). Human PML (hPML) was able to reconstitute both restriction activities

84   in MEFs knocked out for the endogenous murine PML (mPML), in an isoform-specific fashion

85   (34). In addition, the inhibition of lentiviruses by IFN-I in MEFs involves PML (34). In this

86   study, we investigate the role of PML in the restriction of HIV-1 and other retroviruses in several

87   human cell lines, including T cells and myeloid cells, by gene knockout. We also examine the

88   role of PML in the IFN-induced restriction of lentiviruses in human cells. We show that PML is

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89   dispensable for the restriction of lentiviruses in human cells, is not involved in the IFN-I-

90   mediated inhibition of infection, and is not relevant to the inhibition of TRIM5α by As_2O_3.

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92   **Results**

93   **CRISPR-Cas9-mediated knockout of PML in human cells.** In order to stably and irreversibly

94   deplete PML in human cells, we designed two guide RNAs (gRNAs), hPML1 and hPML2, to

95   target the Cas9 nuclease towards exon 2 of *PML* (Fig. 1). The plasmid used in this study,

96   pLentiCRISPRv2 (pLCv2), can mediate knockouts through transfection and also through

97   lentiviral transduction. The control plasmid, pLCv2-CAG, targets the CMV-IE/chicken

98   actin/rabbit beta globin hybrid promoter, a nonhuman sequence (35). We used the Surveyor

99   assay (36) to reveal the presence of insertions/deletions (indels) in the PML gene of HEK293T

100   cells transiently transfected with pLCv2-hPML1 or pLCv2-hPML2. We could observe the

101   presence of PML DNA digestion products of the expected size in cells transfected with each of

102   the PML gRNAs but not in cells transfected with the control gRNA (Fig. 1), indicating that both

103   PML gRNAs generated double-strand breaks that were repaired by non-homologous end joining

104   (NHEJ).

105   **Knocking out PML in human monocytic cells has little to no effect on the permissiveness to**

106   **HIV-1 in the presence or absence of IFN-I.** Human monocytic THP-1 cells were stably

107   transduced with lentiviral vectors produced using pLCv2-hPML1 and pLCv2-hPML2. Following

108   puromycin selection to eliminate non-transduced cells, we performed a Western blotting (WB)

109   analysis of PML levels in bulk populations (Fig. 2A). The levels of hPML were not sufficiently

110   high to be detected in unstimulated cells (not shown), and therefore, the analysis was done using

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111   cells treated with IFN-β. In control cells we found several bands corresponding to hPML

112   isoforms, as previously reported (2). In the cells transduced with the hPML gRNAs, PML was

113   undetectable, showing that knockout was efficient with both gRNAs. This result is consistent

114   with the NHEJ-mediated mutagenesis observed in transfected HEK293T cells using both gRNAs

115   shown in Fig. 1. As both gRNAs showed similar efficiency, all the subsequent experiments in

116   this study were only performed with one gRNA, hPML1. We next infected PML knockout

117   (hPML1 gRNA transduced) and control cells (CAG transduced) with a single dose of HIV-1$_{NL\text{-}}$

118    $_{GFP}$ (37), a VSV-G-pseudotyped, Δ-Envelope HIV-1 vector expressing GFP in place of Nef (Fig.

119   2B). The percentage of GFP-positive cells following HIV-1$_{NL\text{-}GFP}$ challenge is directly

120   proportional to the cells' permissiveness toward infection by this virus. This system is thus well-

121   suited to analyze restriction activities taking place during post-entry steps and until integration.

122   These infections were performed in the presence or absence of IFN-β, owing to the reported role

123   of PML in stimulating the transcription of ISGs (38). In the absence of IFN-β, we found that the

124   PML-KO cells were slightly more permissive to infection by HIV-1$_{NL\text{-}GFP}$ compared with the

125   control cells (less than 2-fold). The addition of IFN-β very strongly inhibited (>20-fold) the

126   infection of THP-1 cells (Fig. 2B), and the low numbers of infected cells prevented a fine

127   analysis of the role of PML in this inhibition. However, the absence of PML clearly did not

128   prevent IFN-β from inhibiting HIV-1$_{NL\text{-}GFP}$, showing that PML was dispensable for this activity.

129   In order to obtain a more complete picture of the importance of PML in the permissiveness to

130   retroviruses in this immune cell line, we performed additional infections with this HIV-1 vector

131   as well as with GFP-expressing vectors derived from the macaque strain of the simian

132   immunodeficiency virus (SIV$_{mac\text{-}GFP}$), the equine infectious anemia virus (EIAV$_{GFP}$) and the B-

133   tropic murine leukemia virus (B-MLV$_{GFP}$). EIAV is restricted by TRIM5α in human cells (39),

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134   making it possible to analyze whether PML modulates the restriction of retroviruses by this well-

135   characterized restriction factor. Infectivity of the three lentiviral vectors (HIV-1, SIVmac, EIAV)

136   was slightly higher in the absence of PML at most virus doses used, whereas infectivity of the B-

137   MLV vector was unaffected by PML knockout (Fig. 2C). These results suggest that PML has a

138   small, barely detectable inhibitory effect on the infection of THP-1 cells by lentiviruses and does

139   not modulate TRIM5α activity. Treatment with IFN-β strongly decreased THP-1 permissiveness

140   to all four vectors, preventing us from measuring the -fold decrease in infectivity with accuracy

141   (Fig. 2C). However, it was clear that IFN-β efficiently inhibited infection in the presence or

142   absence of PML, indicating that PML is not crucial for the IFN-I-mediated anti-retroviral

143   response.

144   **Knocking out PML in human epithelial cells has little to no effect on the permissiveness to**

145   **retroviral infections in the presence or absence of IFN-I.** We then transduced epithelial

146   carcinoma HeLa cells with the the CAG or PML gRNAs. PML was efficiently knocked out, as

147   seen by WB (Fig. 3A). We also performed immunofluorescence microscopy to analyze the effect

148   of PML knockout on PML and SUMO nuclear bodies. A large part (but not all) of SUMO-1

149   localizes to PML bodies in normal conditions (40). As expected, signal corresponding to PML

150   nuclear bodies almost completely disappeared from the cells transduced with the PML gRNA

151   (Fig. S1). In addition, SUMO-1 punctate nuclear staining was strongly diminished but not

152   abolished (Fig. S1). We then challenged the stably transduced cells with GFP-expressing viral

153   vectors like we had done in THP-1 cells. We found that susceptibility to HIV-1, SIVmac, EIAV

154   and B-MLV vectors was identical whether PML was present or not (Fig. 3B-C). IFN-β inhibited

155   all four viral vectors, although the magnitude of this effect (~2- to 3-fold) was much smaller than

156   in THP-1 cells. IFN-β treatments had identical effects in PML-expressing and PML-knockout

157   cells, again showing that PML does not modulate this inhibitory pathway in human cells.

158   Rhabdomyosarcoma-derived epithelial TE671 cells were similarly knocked out for PML by

159   lentiviral transduction, and knockout was efficient (Fig. 4A). Similar to what we found in HeLa

160   cells, infectivity of the four vectors tested was identical whether PML was present or not (Fig.

161   4B). IFN-β decreased the permissiveness of TE671 cells to all four vectors, although we noticed

162   that IFN-β had a relatively smaller effect on HIV-1$_{NL-GFP}$ compared with the three other vectors

163   in TE671 (Fig. 4B). The IFN-β-induced inhibition of the four retroviral vectors in TE671 cells

164   was identical whether PML was present or not (Fig. 4B).

165   **A knock-in approach to suppress PML in human cells.** In order to achieve efficient knockout

166   by transient transfection without the need to isolate cellular clones by limiting dilution, we

167   constructed a plasmid to serve as donor DNA in homology-directed repair (HDR). This plasmid

168   contains two ~800bp-long PML homology arms surrounding a neomycin resistance cassette (Fig.

169   5A). It is expected that its co-transfection in cells along with Cas9 and the hPML gRNA1 would

170   lead to the knock-in of Neo^R in *PML* through HDR in a fraction of the cells. Selection in

171   neomycin then eliminates cells in which the knock-in did not occur. Even if not all alleles of a

172   given gene are successfully modified by knock-in, recent reports indicate that the remaining ones

173   are usually knocked out through NHEJ-dependent mechanisms (41). We designed PCR primers

174   for the specific amplification of the knock-in product and another pair to amplify the wild-type

175   (WT) or the NHEJ-repair knockout alleles (Fig. 5A). To validate this system, we co-transfected

176   TE671 cells with pLCv2.PML1 and the HDR donor plasmid, and randomly isolated a number of

177   neomycin-resistant cell clones of which a representative analysis is shown in Fig. 5B. The

178   knock-in product was detected as expected in all 7 clones while being absent in the parental

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179   cells. On the other hand, the band corresponding to WT or NHEJ-repaired alleles was less

180   intense in these clones relative to the parental cells, but was always present, suggesting that

181   HDR-mediated knockout did not affect all the *PML* alleles.

182   **PML is important for the efficient inhibition of SIVmac but not HIV-1 by IFN-I in**

183   **lymphoid cells.** We knocked out PML in Jurkat cells using the transfection approach that results

184   in the insertion of Neo^R in *PML*, as described above. We performed WB analyses to assess

185   knockout efficiency. (Fig. 6A). Treatment with the IFN-I species IFN-α, IFN-β and IFN-ω

186   stimulated PML expression in Jurkat cells. PML was efficiently knocked out (Fig. 6A),

187   validating the HDR-based approach. In the absence of IFN-β, PML had little effect on the

188   infectivity of all four vectors (<2-fold) (Fig. 6B). The effect of IFN-β treatment differed

189   according to the retroviral vector used (Fig. 6B). IFN-β treatment decreased HIV-1$_{NL-GFP}$

190   infectivity by ~3.5-fold in both control and PML-KO cells. IFN-β similarly decreased the

191   infectivity of SIV$_{mac-GFP}$ by about 4-fold, but only in the control cells. In the PML-KO cells, the

192   inhibitory effect of IFN-β on SIV$_{mac-GFP}$ infectivity was smaller (<2-fold). Interestingly, we

193   found the opposite situation upon challenge with the EIAV$_{GFP}$ vector: IFN-β treatment had no

194   effect on EIAV$_{GFP}$ infectivity in the WT Jurkat cells, whereas it significantly inhibited this vector

195   in PML-KO cells, especially at low vector doses. Finally, IFN-β decreased the infectivity of B-

196   MLV$_{GFP}$ in both WT cells and PML-KO cells, with no apparent specificity. Thus, Jurkat cells

197   provided a more complex situation with respect to the importance of PML in the antiviral effects

198   of IFN-β. In order to further study the contrasting phenotypes of the HIV-1 and SIVmac vectors

199   in these cells, we also analyzed the effects of IFN-α and IFN-ω. (Fig. 6C). We found that in

200   control cells, all three IFN-I species decreased infectivity of both the HIV-1 and the SIVmac

201   vectors, by 2- to 4-fold; IFN-β appearing to be the most consistently inhibitory IFN-I in these

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202   cells, similar to what we had observed in other cell lines (not shown) and to what was reported in

203   the literature (42). In PML-KO cells, HIV-1$_{NL\text{-}GFP}$ was inhibited by all three IFN-I species,

204   similar to the control cells. In contrast, IFN-I inhibition of SIV$_{mac\text{-}GFP}$ was much less efficient in

205   PML-KO cells (Fig. 6C, bottom right panel). Thus, PML is important for IFN-I to inhibit the

206   early infection stages of SIVmac, but not HIV-1, in Jurkat cells.

207   **Over-expression of murine or human PML in Jurkat cells does not affect the infectivity of**

208   **an HIV-1 vector.** Unlike the PML-KO THP-1, HeLa and TE671 cells, the PML-KO Jurkat cells

209   generated do not continuously express Cas9 or a PML-targeting gRNA. Thus, these cells

210   provided an appropriate model to test whether the over-expression of specific hPML isoforms in

211   a PML-KO background could inhibit HIV-1 or other retroviruses. In other words, this

212   experiment was designed to reveal a possible cryptic restriction activity associated with specific

213   PML isoforms that would normally not be apparent due to the presence of other isoforms. We

214   retrovirally transduced the isoforms I to VI of hPML into PML-KO Jurkat cells, separately.

215   Because HIV-1 is inhibited by mPML in MEFs (32-34), we also transduced mPML. A WB

216   analysis showed that all six isoforms of hPML were expressed, as was mPML isoform 2 (Fig.

217   7A). We then challenged the various cell cultures with the HIV-1, SIVmac, EIAV and B-MLV

218   vectors (Fig. 7B). We found that none of the PML isoforms had an effect on GFP transduction

219   by HIV-1$_{NL\text{-}GFP}$. Interestingly, several hPML isoforms and mPML slightly increased

220   permissiveness to SIV$_{mac\text{-}GFP}$, by ~2-fold. Permissiveness to EIAV$_{GFP}$ was significantly decreased

221   by over-expression of hPML-IV, hPML-VI and mPML. Finally, the presence of hPML-VI also

222   slightly inhibited infection by B-MLV$_{GFP}$ at least at some virus doses used (Fig. 7B). Thus,

223   although individual PML isoforms modulated the permissiveness to infection by the SIVmac,

224   EIAV and B-MLV vectors in a virus-specific fashion, none of them affected permissiveness to

225   infection by the HIV-1 vector.

226   **Restriction of N-MLV by TRIM5α and inhibition of TRIM5α by arsenic trioxide are**

227   **independent of PML.** Intriguingly, TRIM5α localizes at PML bodies when shuttling to the

228   nucleus, as demonstrated by pharmacological treatment interfering with its nuclear export (43).

229   The possibility of PML involvement in the inhibition of retroviruses by TRIM5α has been

230   envisioned but not proven. The infectivity of the EIAV vector used here (which is restricted 5- to

231   10-fold by human TRIM5α (44)) was not significantly affected by knocking out PML (Fig. 2-4),

232   suggesting that TRIM5α does not require PML. In order to increase sensitivity, we used an "N-

233   tropic" strain of MLV, which is even more strongly restricted by human TRIM5α (39, 45) than

234   EIAV, and of which restriction is counteracted by As_2O_3 in a cell context-specific fashion (46,

235   47). Thus, As_2O_3 greatly increases the infectivity of N-MLV but not B-MLV vectors in many

236   human cell lines. The mechanism of action of As_2O_3 against TRIM5α has not been determined

237   but it was thought to involve PML, since As_2O_3 is well-known as a specific inhibitor of PML

238   (27, 48). Interestingly, As_2O_3 also enhances the infectivity of HIV-1 in human cells, although the

239   magnitude of this effect is milder than what is found with N-MLV (46, 49). We infected HeLa,

240   TE671 and Jurkat cells with HIV-1$_{NL-GFP}$, B-MLV$_{GFP}$ and N-MLV$_{GFP}$ in the presence of

241   increasing As_2O_3 concentrations (Fig. 8A). In the absence of As_2O_3, N-MLV$_{GFP}$ infectivity was

242   barely detectable or undetectable in all three cell lines, reflecting the strong inhibition conferred

243   by TRIM5α in human cells. At the same virus dose, B-MLV$_{GFP}$ infected 3% to 5% of the cells.

244   PML knockout had no effect on the infectivity of the two MLV vectors, implying that PML is

245   not required for TRIM5α-mediated restriction of N-MLV. In presence of As_2O_3, N-MLV$_{GFP}$

246   infectivity was greatly enhanced, although the stimulating effect was partly reversed at high

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247   As_2O_3 concentrations in HeLa and TE671 cells (Fig. 8A). As_2O_3 effectiveness at counteracting

248   TRIM5α-mediated restrictions was found to decrease at high concentrations in previous studies

249   as well (47, 49). In contrast to N-MLV$_{GFP}$, B-MLV$_{GFP}$ was only slightly enhanced by As_2O_3. As

250   reported before, As_2O_3 modestly increased HIV-1$_{NL-GFP}$ infection of HeLa and TE671 cells,

251   although it had no effect on this vector in Jurkat cells (Fig. 8B). Knocking out PML had no

252   detectable effect on the As_2O_3-mediated stimulation of N-MLV$_{GFP}$ and HIV-1$_{NL-GFP}$ in the three

253   cell lines tested. We performed an additional infection of the HeLa cells with the N-MLV and B-

254   MLV vectors, this time at a fixed As_2O_3 concentration and varying virus doses. Again, we

255   observed that (i) PML had no effect on the infectivity of N-MLV$_{GFP}$ and B-MLV$_{GFP}$, (ii) As_2O_3-

256   mediated stimulation of N-MLV$_{GFP}$ was significantly stronger than that of B-MLV$_{GFP}$, regardless

257   of the virus dose, and (iii) knocking out PML had no impact on the effect of As_2O_3 on the MLV

258   vectors. These data demonstrate that PML is not involved in the restriction of N-MLV by

259   TRIM5α, nor is it involved in the mechanism by which As_2O_3 stimulates retroviral infections and

260   counteracts TRIM5α.

261   **PML is not required for TRIM5α-mediated restriction of HIV-1 in MEFs.** MEFs provide a

262   cellular environment in which PML restricts HIV-1, as seen by several laboratories (32-34). In

263   addition, PML also inhibits HIV-1 transcription in MEFs, an effect that we did not observe in

264   human cells (34). Thus, it would be conceivable for PML to have an impact on TRIM5α-

265   mediated restriction of HIV-1 in this specific cellular environment. To test this hypothesis, we

266   used PML-KO MEFs (34, 50). WT and PML-KO MEFs were stably transduced with the HIV-1-

267   restrictive Rhesus macaque TRIM5α or the non-restrictive human TRIM5α as a control. The

268   cells were also transduced with the C35A RING domain mutant of each TRIM5α ortholog,

269   which abolishes the RING domain-associated ubiquitin ligase activity (51). Colocalization of a

270   fraction of TRIM5α with PML NBs was seen in the presence of the nuclear export inhibitor

271   leptomycin B, consistent with published data obtained in human and canine cells (43), and

272   exposure of the cells to HIV-1 did not modify this pattern (Fig. 9A). The cells were then

273   challenged with HIV-1$_{NL-GFP}$ or with the relatively restriction insensitive SIV$_{mac-GFP}$ as a control

274   (52). HIV-1 was very strongly restricted by rhTRIM5α in both WT and PML-KO MEF cells

275   (Fig. 9B). As expected, C35A rhTRIM5α and hTRIM5α (WT or C35A) had little to no effect on

276   HIV-1$_{NL-GFP}$. SIV$_{mac-GFP}$ was moderately restricted by rhTRIM5α, and PML knockout did not

277   affect this inhibitory effect (in fact, restriction was slightly greater in the absence of PML) (Fig.

278   9B). In conclusion, PML is not required for rhTRIM5α to restrict HIV-1.

279

280   **Discussion**

281   Whether PML has an impact or not on the infection of human cells by HIV-1 has been an open

282   question for over 15 years. Trono and colleagues reported that PML is transiently exported in the

283   cytoplasm following exposure to HIV-1 and co-localizes with the incoming virus in HeLa cells

284   (53); however, this study did not include functional evidence for the involvement of PML in

285   HIV-1 infection. Another team found no effect of HIV-1 infection on the distribution of PML

286   bodies (54). As$_2$O$_3$, a known PML inhibitor, was found to enhance the infection of human cells

287   with HIV-1 (46, 53) but it also stimulated the infection of MEFs with HIV-1 vectors whether

288   PML was present or not (46). Interest for PML as a modulator of HIV-1 infection surfaced again

289   in recent years, as it was proposed to act as an HIV-1 restriction factor in mouse and human cells

290   (33). However, the data gathered so far by three different teams, including this study, suggest

291   that the restriction activity in human cells, if it exists, is cell type-specific. Dutrieux and

292   colleagues, using shRNAs, observed a modest inhibition of HIV-1 vector transduction conferred

293 by PML in HeLa cells (<2-fold). They also observed a small delay in HIV-1 propagation in

294 peripheral blood mononuclear cells, but the decrease in infectivity was not quantified (33). We

295 previously observed that knocking down PML in T-lymphoid Sup-T1 cells increases HIV-1

296 infectivity by 2- to 4-fold (34). On the other hand, Kahle and colleagues saw no effect of

297 knocking down PML on the infectivity of an HIV-1 vector in T lymphoid cell lines including

298 CEM, HuT78, Jurkat and Molt4 (32). They showed, however, that PML reduces HIV-1

299 infectivity in human foreskin fibroblasts by 2- to 3-fold (32). Taken together, those previous

300 papers showed that knocking down PML has either no effect or modest effects on HIV-1

301 infectivity in human cells. We were not able to efficiently knock out PML in Sup-T1 cells,

302 preventing us from drawing comparisons with our previous knockdown results. However, our

303 knockout experiments in Jurkat, THP-1, HeLa and TE671 are not consistent with PML being an

304 HIV-1 restriction factor in human cells.

305 Interestingly, the EIAV and B-MLV vectors were slightly inhibited by PML in Jurkat cells, as

306 judged by infectivity in PML knockout cells. Furthermore, over-expression of hPML-IV in the

307 PML-KO Jurkat cells had an inhibitory effect on $EIAV_{GFP}$ and to a lesser extent on $B\text{-}MLV_{GFP}$.

308 Thus, it is possible that this hPML isoform contributes to the inhibition of specific retroviruses in

309 this cell line. However, the effects seen are probably too small for us to characterize a

310 mechanism or identify determinants of the restriction.

311 A recent study by Kim and Ahn (38) uncovered an additional function for PML in human skin

312 fibroblasts: the stimulation of ISG expression through a direct association with their promoter.

313 Accordingly, we previously showed that PML was important for the efficient inhibition of HIV-1

314 by IFN-I in MEF cells (34). Although HIV-1 is also readily inhibited by IFN-I in a variety of

315 human cell types, as illustrated in our study, we find that this effect is not affected by knocking

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316   out PML. However, we cannot exclude the possibility that PML is involved in regulating IFN-I-

317   dependent transcription in specific cellular contexts such as skin fibroblasts (38). It is also

318   possible that PML stimulates the transcription of some ISGs but not others. In support of this

319   idea is our observation that SIVmac inhibition by IFN-I in Jurkat cells was significantly greater

320   in the presence of PML. SIVmac, but not HIV-1, is inhibited by an unidentified restriction factor

321   in Jurkat cells and other T cells, provisionally called Lv4 (55). It is conceivable that the gene

322   encoding Lv4 is specifically stimulated by IFN-I in a PML-dependent fashion in Jurkat cells.

323   This characteristic could be exploited to identify this gene, similar to the strategy that led to the

324   identification of Tetherin as a retroviral restriction factor (56).

325   In our previous study (34), we showed that PML inhibited HIV-1 transcription in MEFs but not

326   in Sup-T1 cells and in an IFN-I-independent fashion. We analyzed GFP mean fluorescence

327   intensity in all our experiments for this study, as a surrogate for HIV-1 gene expression levels.

328   Consistent with our previous findings, we observed no effect of PML on the GFP fluorescence

329   intensity following infection of THP-1, Jurkat, HeLa or TE671 cells with our various vectors

330   (not shown). We conclude that PML does not repress HIV-1 transcription in human cells. This

331   apparently contradicts a report by Giacca and colleagues that PML inhibits HIV-1 transcription

332   by directly binding the viral promoter (31). However, the latter study was based on the use of "J-

333   Lat" clones, which are Jurkat cells in which the HIV-1 provirus has become constitutively

334   repressed through unknown mechanisms (57). We propose that PML may be involved in the rare

335   silencing events leading to HIV-1 latency in Jurkat cells, and that PML is important for the

336   maintenance of silencing; however, PML is not a ubiquitous silencer of HIV-1 transcription.

337   Finally, our study shows that the As_2O_3-mediated stimulation of early retroviral infection stages

338   is completely independent of PML, and so is the inhibition of TRIM5α by this drug. This is not

16

339   entirely surprising, considering that TRIM5α can target N-MLV in human cells, and HIV-1 in

340   MEF cells, in the absence of PML. However, these results challenge conclusions from another

341   paper that used radioactively or chemically labelled arsenate compounds to show that PML was

342   the main target for this group of pharmacological agents (27). How, then, does As_2O_3 counteract

343   TRIM5α and, to a lesser extent, stimulate HIV-1 and B-MLV vectors in human cells? Perhaps

344   addressing this long-unanswered question will be helped by an observation that pre-dated the

345   isolation of TRIM5α. Indeed, PK11195, a compound which, like As_2O_3, affects mitochondrial

346   functions, also counteracts TRIM5α (49). Strikingly, these two drugs enhance autophagy (58,

347   59), an outcome possibly related to their effect on mitochondria. It is possible that As_2O_3-

348   induced autophagy accelerates the lysosomal degradation of TRIM5α and other cytoplasmic

349   restriction factors.

350

351   **Materials and methods**

352   **Cell culture**

353   Jurkat and THP-1 cells were maintained in RPMI 1640 medium (HyClone, Thermo Scientific,

354   USA). Human embryonic kidney (HEK) 293T, HeLa, MEF and TE671 cells were maintained in

355   Dulbecco's modified Eagle's medium (DMEM; HyClone). All culture media were supplemented

356   with 10% fetal bovine serum (FBS) and penicillin/streptomycin (HyClone).

357   **Plasmids and preparation of retroviral vectors**

358   The pMIP retroviral vector plasmids containing individual isoforms of hPML (pMIP-hPML-I to

359   VI) and the isoform 2 of mPML (pMIP-mPML) have been described in details in a recent

360   publication (34) and make use of materials generously provided by Roger D. Everett (60).

17

361 Retroviral vectors were prepared by co-transfection of 293T cells with pMIP-m(h)PMLs together

362 with pMD-G and pCL-Eco using polyethylenimine (PEI; Polyscience, Niles, IL) as detailed

363 previously (34). Virus-containing supernatants were collected 2 d later, clarified by low-speed

364 centrifugation and kept at -80 $^{\circ}$C.

365 To produce GFP-expressing retroviral vectors, 293T cells were seeded in 10 cm culture dishes

366 and transiently co-transfected with the following plasmids: pMD-G, pCNCG and pCIG3-B or

367 pCIG3-N to produce B-MLV$_{GFP}$ and N-MLV$_{GFP}$, respectively; pMD-G and pHIV-1$_{NL-GFP}$ to

368 produce HIV-1$_{NL-GFP}$; pMD-G and pSIV$_{mac239-GFP}$ to produce SIV$_{mac-GFP}$; or pONY3.1, pONY8.0

369 and pMD-G to produce EIAV$_{GFP}$ (see (34, 61) and references therein).

370 Design of gRNAs and transduction of lentiviral CRISPR-Cas9 vectors

371 The lentiviral expression vector plentiCRISPRv2 (pLCv2) was a gift from Feng Zhang (Addgene

372 plasmid # 52961) and can be used to simultaneously express a gRNA, Cas9 nuclease, and

373 puromycin resistance, either by transfection or lentiviral transduction (62). Two gRNAs (hPML1

374 and hPML2) targeting *hPML* (NG_029036) were designed using the Zhang lab online software

375 available at crispr.mit.edu. The sequences targeted are 5'CAATCTGCCGGTACACCGAC

376 (hPML1) and 5'CACCGGGAACTCCTCCTCCGAAGCG (hPML2). A gRNA targeting the

377 CAG hybrid promoter (target: 5'GTTCCGCGTTACATAACTTA) was used as a negative

378 control (35). The oligodeoxynucleotides (ODNs) needed for the generation of pLCv2-based

379 constructs were designed according to the Zhang lab protocol (62, 63) and are shown in Table

380 S1.

381 The lentiviral vectors were prepared by co-transfection of 293T cells with 10 µg of the

382 plentiCRISPRv2 construct together with 5 µg of pMD-G and 10 µg of pΔR8.9 (64). The viral

18

383   supernatants were collected at 1.5 or 2 d post-transfection and used to transduce various cell

384   lines. Stably transduced cells were selected by addition of 0.5 µg/ml puromycin (Thermo Fisher

385   Scientific) to the medium at 2 d post-infection and for 5 d. Control untransduced cells were

386   killed under these conditions.

Surveyor nuclease assay

388   To evaluate on-target modifications (indels) in *hPML*, a surveyor nuclease assay was performed.

389   293T cells were transfected with either plentiCRISPRv2-hPML1, -hPML2 or -CAG using PEI. 3

390   d later, the genomic DNA was extracted from the transfected cells using the QIAamp DNA mini

391   kit (Qiagen, CA, USA). Two pairs of primers were designed to amplify 637 bp and 725 bp

392   fragments on either side of Cas9 targets guided by gPML1 and gPML2 respectively (Fig. 1A).

393   The sequences of these ODNs are included in Table S1. PCR amplicons were heat-denatured at

394   95 °C, and re-annealed by slow cooling to promote formation of dsDNA heteroduplexes. The

395   heteroduplexes were then cleaved by surveyor nuclease S (Integrated DNA Technologies,

396   Coralville, IA), according to the manufacturer's instructions. Digestion products were visualized

397   by agarose gel electrophoresis.

Construction of the homology directed repair (HDR) plasmid and generation of PML-KO

Jurkat cells

400   We used pcDNA3.1+ as the backbone plasmid to prepare a HDR "donor" plasmid containing a

401   neomycin selection gene (Neo^R). First, the backbone plasmid was cut with BamHI and BglII,

402   then self-ligated in order to remove the cytomegalovirus promoter from upstream of the

403   multicloning site MCS1. Next, two ODNs were designed to introduce the second MCS (MCS2)

404   (see Table S1); these ODNs were annealed, and the resulting duplex ligated into the PciI cut site

405   of the plasmid, downstream of Neo^R, yielding pNMs-Neo.HDR. To construct the PML HDR

406   plasmid, homology arms corresponding to 800 bp-long regions immediately upstream and

407   downstream of the hPML gRNA1-mediated Cas9 cut site in *hPML* were designed. The arms

408   were amplified by PCR from genomic DNA extracted from 293T cells using the QIAamp DNA

409   mini kit (Qiagen). The sequences of ODNs used in the PCR reactions are provided in Table S1.

410   The 5' arm was cloned into MCS1 of pNMs-Neo.HDR which had been cut with NotI and XbaI.

411   The plasmid was then cut with Mfe I and Sbf I in order to clone the 3' arm into MCS2, yielding

412   pNMs-Neo.HDR-hPML.

413   Jurkat cells (300,000) were electroporated with 1.5 µg of pNMs-Neo.HDR-hPML together with

414   1.5 µg of pLCv2-hPML1 using an MP-100 microporator (Digital Bio Technology) according to

415   the manufacturer's instructions. The parameters were 1300 V, 2 pulses, 20 ms. 48 h later, cells

416   were placed in medium containing 1 mg/ml G418, and selection was carried out for 7 d.

417   **Antibodies and WB analyses**. Cells (1×10^6) were lysed at 4^oC in RIPA lysis buffer (1% NP40,

418   0.5% deoxycholate, 0.1% SDS, 150mM NaCl, 50 mM Tris-HCl pH 8.0). The lysates were

419   subjected to SDS-polyacrylamide gel electrophoresis, followed by WB analysis using mouse

420   anti-mPML mAb (36-1-104, Enzo life sciences, NY), rabbit polyclonal anti-hPML (A301-167A,

421   Bethyl Laboratories, TX), rabbit polyclonal anti-FLAG (Cell Signaling, MA, USA), or mouse

422   anti-β-actin antibody (Sigma, MI).

423   **Viral challenges and flow cytometric analysis**. Cells were seeded into 24-well plates at 3×10^4

424   cells/well and infected the following day with GFP-expressing retroviral vectors. HeLa and

425   TE671 cells were trypsinized at 2 d post-infection and fixed in 3% formaldehyde (Fisher

426   Scientific, MA, USA). The percentage of GFP-positive cells was then determined by analyzing

427   $1x10^4$ to $5x10^4$ cells on a FC500 MPL cytometer (Beckman Coulter, CA, USA) using the CXP

428   Software (Beckman Coulter).

429   **Pharmacological treatments**. A 0.1 M stock solution of As_2O_3 (Sigma) was prepared in 1 N

430   NaOH, as previously described (28), and diluted in the culture medium immediately before use.

431   Cells were treated for 15 min prior to infection. 16 h post-infection, the supernatants were

432   replaced with fresh medium devoid of drug. Recombinant human IFN-α was obtained from

433   Shenandoah biotechnology (Warwick, PA). Recombinant human IFN-β and IFN-ω were

434   obtained from PeproTech (Rocky Hill, NJ). IFN-I was added to cell cultures 16 h prior to

435   infection and at a final concentration of 10 ng/ml.

436   **Immunofluorescence microscopy.** HeLa or MEF cells were seeded on glass coverslips placed

437   in 3.5-cm wells. MEFs were treated with LMB (20 ng/ml) 3 h prior to infection then infected for

438   6 h with HIV-1_{NL-GFP}. The cells were permeabilized and fixed for 10 min in Triton X-100/4%

439   formaldehyde at room temperature (RT), followed by 4 washes with PBS. Cells were then

440   treated with 10% goat serum (Sigma) for 30 min at RT followed by 4 h of incubation with

441   antibodies against FLAG (Sigma, 1:150), hPML (Bethyl Laboratories, 1:150) or mPML (Enzo

442   Life Sciences, 1:150) in 10% goat serum at RT. They were then washed 4 times with PBS and

443   fluorescently stained with Alexa Fluor 488-conjugated goat anti-mouse or 594-conjugated goat

444   anti-rabbit (Molecular Probes, Eugene, OR) diluted 1:100 in 10% goat serum for 1 h at RT. The

445   cells were then washed 4 times with PBS before mounting in Vectashield (Vector Laboratories,

446   Peterborough, UK). Hoechst 33342 (0.8 µg/ml; Molecular Probes) was added along with the

447   penultimate PBS wash to reveal DNA. Images were acquired on an AxioObserver Microscope

448   (Carl Zeiss Canada, Toronto, ON) equipped with the Apotome module.

449

450 **Acknowledgements**

453

454 **References**

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622

623   **FIG 1** CRISPR/Cas9-mediated genome editing of PML in human cell lines. The Cas9 nuclease

624   was targeted to exon 2 of the PML gene (green) by two selected gRNAs whose binding sites are

625   shown in blue (PAM motifs are in red). Arrows indicate the positions of the binding sites for the

626   ODNs used in the PCR-Surveyor assay (blue arrows for gRNA1-, red arrows for gRNA2-guided

627   cut sites). The Surveyor assay is shown in the lower panel. Briefly, PCR products amplified from

628   cells transfected with pLCv2-hPML1, -hPML2, or pLCv2-CAG (Ctrl), were subjected to

629   denaturation, re-annealing and digestion with the Surveyor enzyme. Arrowheads indicate

630   cleavage products of the expected size.

631   **FIG 2** PML knockout has negligible effects on intrinsic or IFN-I-induced restriction of

632   retroviruses in THP-1 cells. (A) WB analysis of THP-1 cells transduced with pLCv2-based

633   vectors expressing Cas9 and a gRNA targeting either hPML or CAG. Stably transduced,

634   puromycin-resistant cells were treated with IFN-β (10 ng/ml). Cellular lysates were prepared 16

635   h later and analyzed by WB using antibodies against hPML and actin as a loading control. (B)

636   FACS plots from PML-knockout (KO) and control (Ctrl, CAG gRNA-transduced) THP-1 cells

637   infected with HIV-1$_{NL-GFP}$. Control or PML-KO THP-1 cells were treated with IFN-β or left

638   untreated and then exposed to HIV-1$_{NL-GFP}$ (10 μl). 2 d later, cells were analyzed by FACS and

639   the percentage of infected (GFP-positive) cells observed is indicated on each plot. (C) Virus

640   dose-dependent analysis of the role of hPML in the intrinsinc and IFN-I-induced restriction of

641   retroviruses. Control and PML-KO THP-1 cells were treated with IFN-β (10 ng/ml) for 16 h,

642   followed by infection with increasing doses of retroviral vectors. The percentage of infected cells

643   was assessed 2 d later by FACS.

644   **FIG 3** PML knockout has no effect on intrinsic or IFN-I-induced restriction of retroviruses in

645   HeLa cells. (A) HeLa cells lentivirally transduced with pLCv2 vectors expressing either the

27

646   hPML gRNA1 or (as a control) the CAG-targeting gRNA were treated with IFN-β (10 ng/ml).

647   Cellular lysates were prepared 16 h later and analyzed by WB using an anti-hPML antibody.

648   Actin was analyzed as a loading control. (B) FACS plots from transduced HeLa cells infected

649   with HIV-1$_{NL-GFP}$. Control and PML-KO HeLa cells treated or not with IFN-β were infected with

650   HIV-1$_{NL-GFP}$ (6 μl). The percentage of infected cells determined at 2 d post-infection is indicated

651   for each plot. (C) Virus dose-dependent analysis of the role of hPML in IFN-I-induced restriction

652   of retroviral infection. Control and PML-KO HeLa cells were treated with IFN-β, followed 16 h

653   later by infection with increasing doses of the indicated retroviral vectors. The percentage of

654   infected cells was assessed 2 d later by FACS.

655   **FIG 4** PML knockout has no effect on intrinsic or IFN-I-induced restriction of HIV-1 in TE671

656   cells. (A) WB analysis. TE671 cells were stably transduced with pLCv2-based vectors

657   expressing Cas9 and either the hPML-targeting gRNA1 or the CAG-targeting gRNA as a

658   control. The cells were treated with IFN-β (10 ng/ml) or left untreated as a control. Cellular

659   lysates were prepared 16 h later and analyzed by WB using an anti-hPML antibody along with

660   actin as a loading control. (B) Infection assay. Control (CAG gRNA-transduced) and PML-KO

661   TE671 cells were treated with IFN-β or left untreated. 16 h later, the cells were infected with

662   increasing doses of the indicated retroviral vectors. The percentage of infected cells was assessed

663   2 d later by FACS.

664   **FIG 5** HDR-mediated knockout of PML. (A) Schematic of the HDR plasmid and targeting

665   strategy for the knock-in of the Neomycin resistance gene at the PML locus. Two 800 bp-long

666   PML homology arms encompass the Neo^R expression cassette on plasmid pNMs-Neo.HDR-

667   hPML. The arms are complementary to the PML regions on either side of the gRNA1-mediated

668   Cas9 cleavage site. Co-transfection of pLCv2-hPML1 and pNMs-Neo.HDR-hPML may yield a

28

669   knock-in allele as indicated if DNA is repaired by HDR. If DNA is repaired by NHEJ, WT or

670   indel-containing alleles may be generated. Yellow and orange arrows indicate the binding sites

671   for the primers used to detect knock-in and WT/indel alleles by PCR (1 Kbp and 0.3 Kbp

672   products, respectively). (B) PCR analysis of Neomycin-resistant TE671 clones. TE671 cells were

673   co-transfected with pLCv2-hPML1 and pNMs-Neo.HDR-hPML, then grown in presence of

674   neomycin. Individual Neo^R clones were analyzed using the two primer pairs described in A.

675   **FIG 6** PML knockout has virus-specific effects on the restriction of retroviruses in Jurkat cells.

676   (A) Jurkat cells were co-transfected with pLCv2.hPML1 and pNMs-Neo.HDR-hPML.

677   Neomycin-resistant cells (KO) and parental untransfected cells (WT) were treated with IFN-α,

678   IFN-β or IFN-ω (10 ng/ml). Cellular lysates were prepared 16 h later and analyzed by WB using

679   an anti-hPML antibody. Actin was analyzed as a loading control. (B) Virus dose-dependent

680   analysis of the role of hPML in the intrinsic and IFN-I-induced restriction of retroviruses. PML-

681   KO and control Jurkat cells were treated with IFN-β for 16 h, followed by infection with

682   increasing doses of the indicated retroviral vectors. The percentage of infected cells was assessed

683   2 d later by FACS. (C) PML-KO and control cells were challenged with increasing doses of

684   HIV-1$_{NL-GFP}$ following treatment with IFN-α, -β or -ω for 16 h. The percentage of infected cells

685   was assessed 2 d later by FACS.

686   **FIG 7** Transduction of mPML or hPML isoforms in PML-KO Jurkat cells has virus-specific

687   effects on the permissiveness to retroviral vectors. (A) WB analysis of mPML and hPML

688   expression. PML-KO Jurkat cells were stably transduced with mPML or with FLAG-tagged

689   hPML-I to -VI separately. The empty vector (EV) was transduced as a control. Lysates prepared

690   from the different cell populations were analyzed by WB with anti-FLAG (left panel) or anti-

691   mPML (right panel) antibodies. Actin was probed as loading control. The arrowheads indicate

29

692   the expected size for each hPML isoform. (B) Susceptibility to transduction by retroviral

693   vectors. The cells were infected with multiple doses of the indicated retroviral vectors and the

694   percentage of GFP-expressing cells was determined 2 d later by FACS.

695   **FIG 8** PML is irrelevant for the As_2O_3-induced stimulation of retroviral infectivity in human

696   cells. (A) Effect of As_2O_3 on the permissiveness to retroviral vectors in the presence or absence

697   of PML. Control and PML-KO human cell lines were treated with the indicated amounts of

698   As_2O_3 for 15 min prior to infection with HIV-1, B-MLV and N-MLV vectors expressing GFP

699   (B-MLV$_{GFP}$ and N-MLV$_{GFP}$ have identical titers in non-restrictive CRFK cat cells). The

700   percentage of infected cells was assessed 2 d later by FACS. The values represent the means of

701   three independent infections with standard deviations (**$P<0.01$, ***$P<0.001$, N/D not

702   detected). (B) Virus dose-dependent infections. Ctrl and PML-KO HeLa cells were infected with

703   increasing doses of B-MLV$_{GFP}$ or N-MLV$_{GFP}$ vectors in the presence or not of 4 μM As_2O_3. 2 d

704   later, the percentage of infected cells was determined with FACS.

705   **FIG 9** PML is not required for the TRIM5α-mediated restriction of HIV-1 in MEFs. (A)

706   Immunofluorescence staining of mPML and rhTRIM5α in WT MEFs stably transduced with

707   FLAG-tagged rhTRIM5α. The cells were treated with either LMB (20 ng/ml) or PBS as a

708   control, 3 h prior to infection with HIV-1$_{NL-GFP}$ at a viral dose leading to approximately 10%

709   infected cells. 6 h later, the cells were analyzed by immunofluorescence microscopy using anti-

710   FLAG (red) and anti-mPML (green) antibodies. Nuclear DNA was stained using Hoechst 33342

711   (blue). Images are representative of multiple observations. Scale bar, 5 μm. (B) rhTRIM5α

712   restricts HIV-1 in the presence or absence of PML. PML-KO or WT MEFs stably transduced

713   with rhTRIM5α or huTRIM5α (WT or C35A mutant) were infected with HIV-1$_{NL-GFP}$ or SIV$_{mac-}$

714    $_{GFP}$, using virus amounts leading to infection of about 10% of the parental cells. 2 d later, the

30

715   percentage of infected cells was measured by FACS. The values represent the means of three

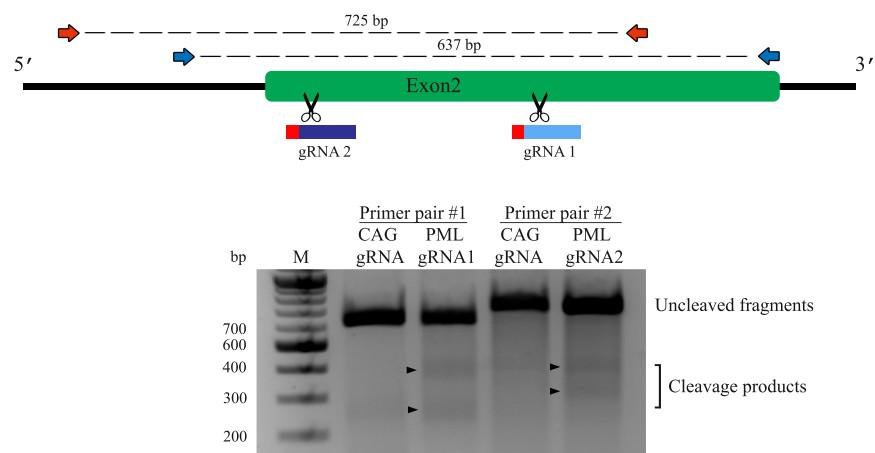716   independent infections with standard deviations (N/D, not detected).
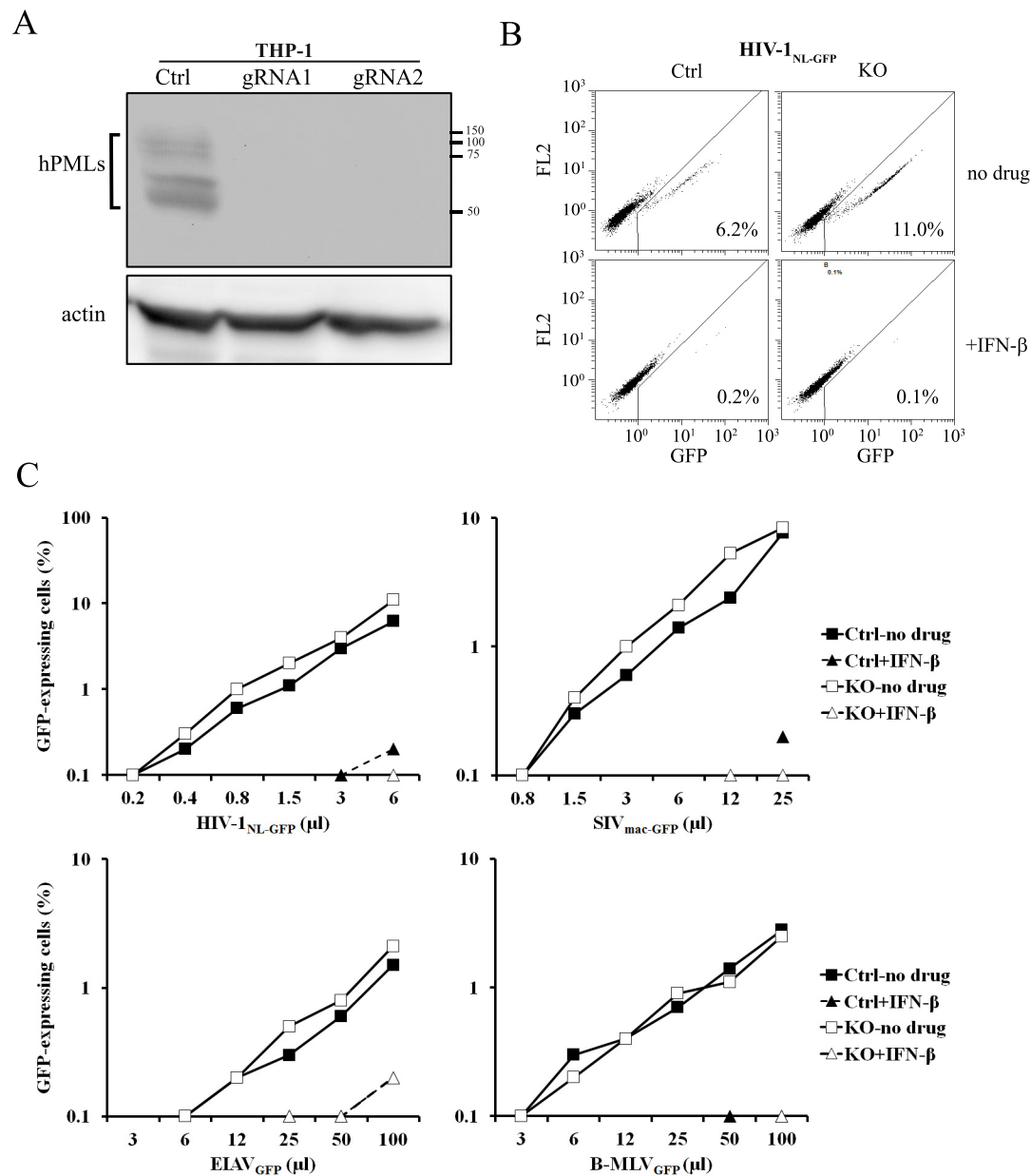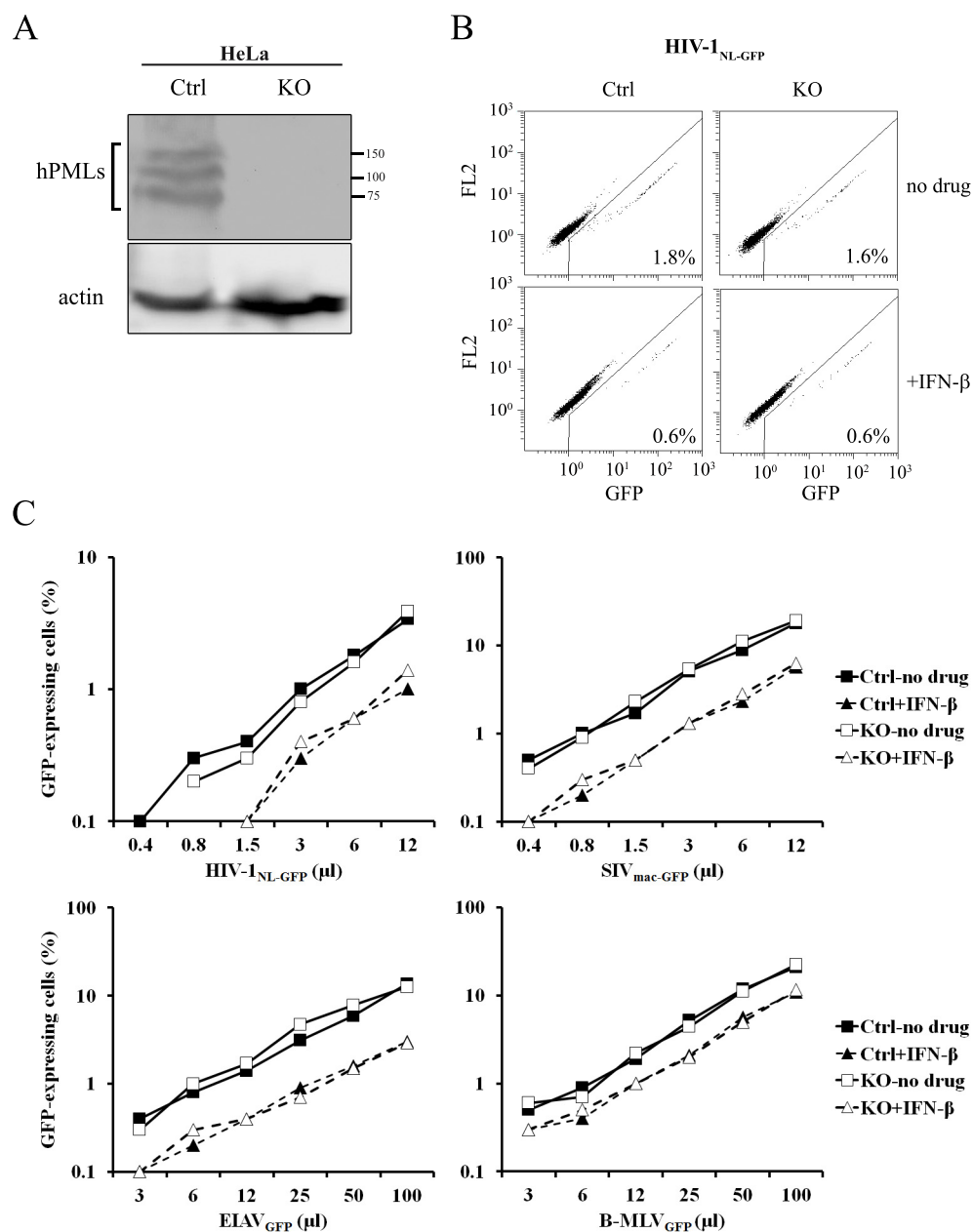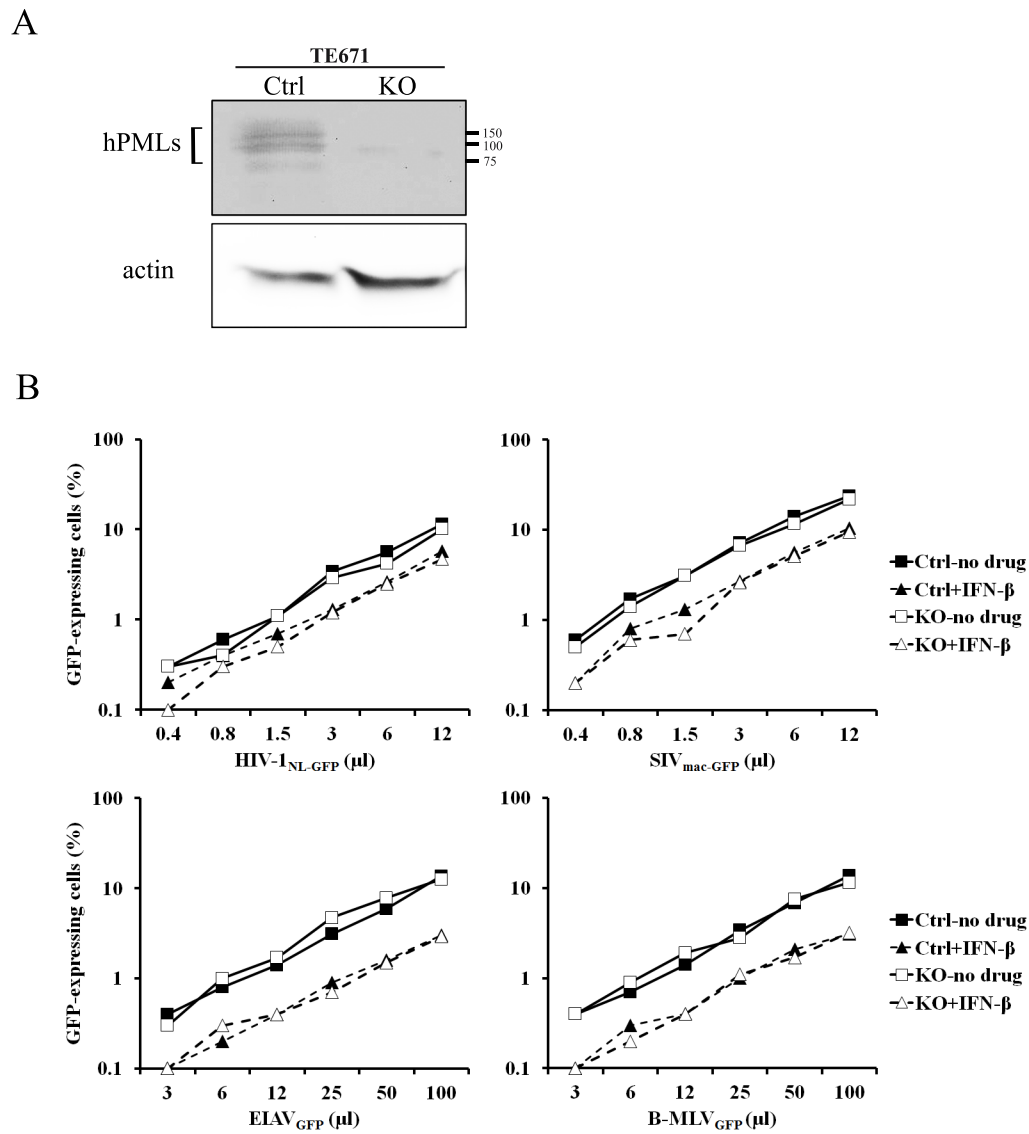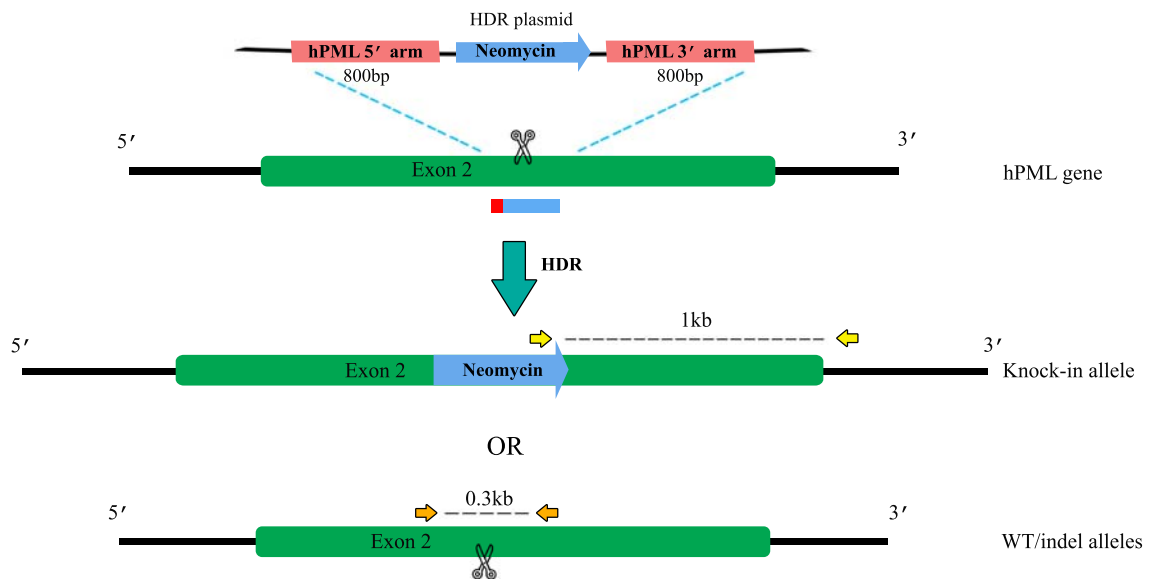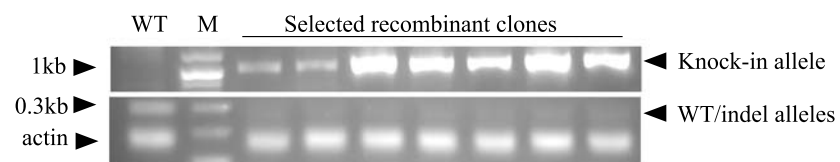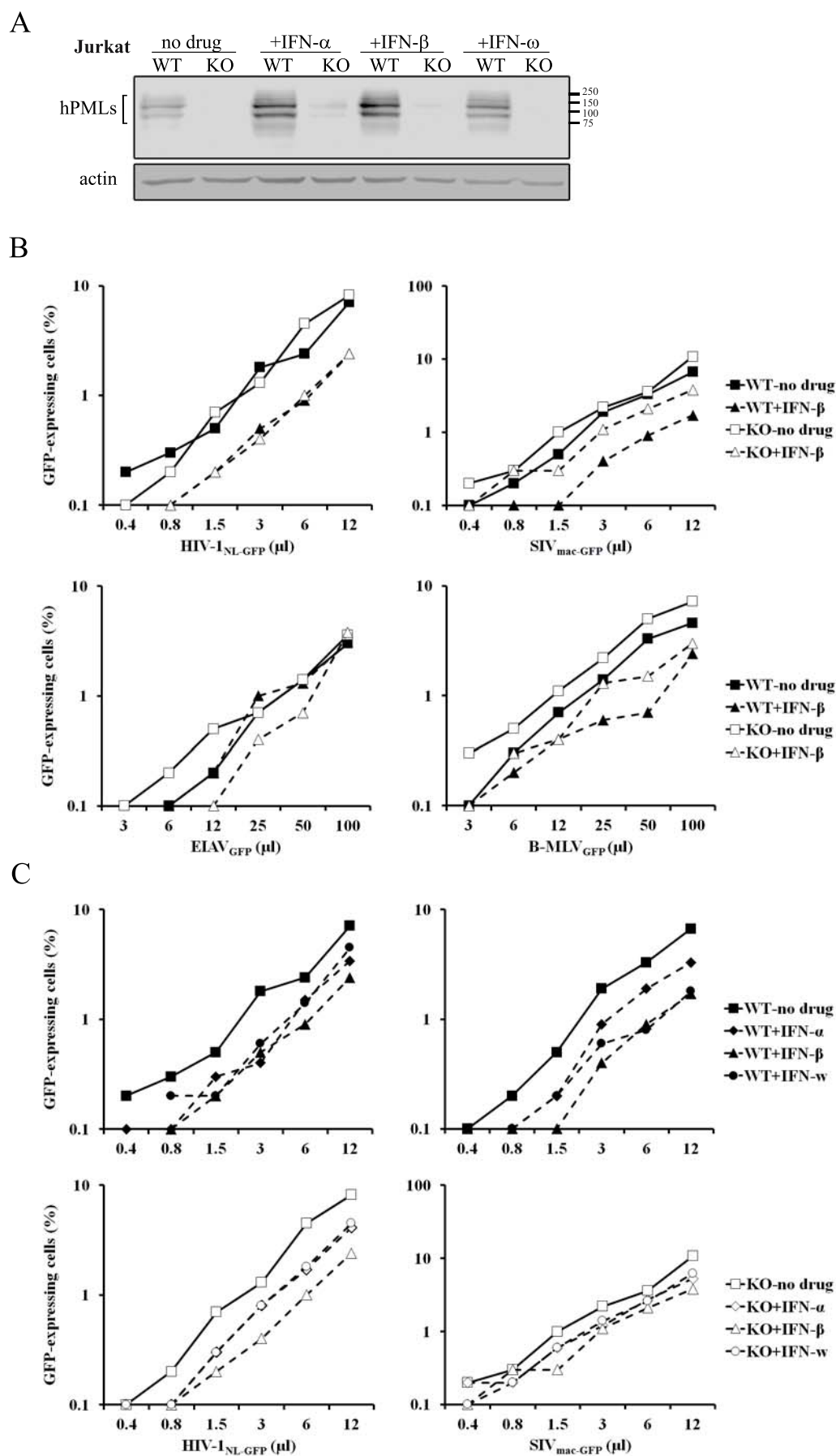
717

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

A

B

Figure 6

Figure 7

A

Jurkat-KO

B

Figure 8

A   HeLa

TE671

Jurkat

B

Figure 9

A

B