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ABSTRACT

11 Gene expression in HIV-1 is regulated by the promoters in 5' long-terminal repeat (LTR)
12 element, which contain multiple DNA regulatory elements that serve as binding sites for cellular
13 transcription factors. YY1 could repress HIV-1 gene expression and latent infection (1-4). Here,
14 however, we observed that virus production can be increased by YY1 over-expression and
15 decreased under YY1 depleted condition by siRNA treatment. To identify functional domain(s)
16 of YY1 activation, we constructed a number of YY1 truncated mutants. Our data show that full-
17 length YY1 enhances the viral transcription both through U3 and U3RU5 promoters. Moreover,
18 the C-terminal region (296-414 residues) of YY1 is responsible for the transcriptional
19 upregulation, which could be enhanced further in the presence of the viral Tat protein. The
20 central domain of YY1 (155-295 residues) does not affect LTR activity but has a negative effect
21 on HIV-1 gene expression. Taken together, our study shows that YY1 could act as a
22 transcriptional activator in HIV-1 replication, at least in the early stages of infection.

INTRODUCTION

23

24

25 Yin Yang 1 (YY1), a ubiquitous and multifunctional member of polycomb group (PcG) of
26 proteins, is known to function in chromatin remodeling (5). This 414 residue-long protein has an
27 N-terminal acidic transcriptional activation domain (1-154 residues), followed by a glycine-
28 lysine rich DNA transcriptional inhibition domain (170-200 residues), a small Recruit Polycomb
29 (REPO) domain (201–225 residues), and a C-terminal DNA binding domain with four GLI-
30 Krüppel-like zinc finger motifs (296-414 residues) that are responsible for DNA binding (6-9)

31 YY1 was first identified as a transcription factor and cDNA clone was isolated
32 simultaneously by two independent groups (9-11). Multiple diverse functions of YY1 have been
33 identified, such as, regulation of cell proliferation, embryogenesis, differentiation, tumor
34 suppression, oncogenesis, DNA damage and apoptosis (12, 13) and reviewed in (14, 15).
35 Further, YY1 could function as a transcription factor regulating several different eukaryotic and
36 viral genes including those of HIV-1, Adenovirus, adeno-associated virus (AAV),
37 cytomegalovirus, parvovirus and Human papillomavirus (HPV) (reviewed in (14, 16)). The
38 consensus core DNA sequence of YY1 recognition site is known as 5'-CAT-3's (17). Depending
39 on the promoter and the ability of YY1 to interact with other transcription factors or cofactors,
40 such as histone acetyltransferase (HAT), p300, CREB binding protein (CREBBP), histone
41 deacetylases (HDACs), enhancer of zeste 2 (EZH2) and DNA methyltransferases (DNMTs),
42 YY1 could function as either an activator or a repressor (3, 7, 18-21).

43 HIV-1 has a 5' long-terminal repeat (LTR) region, regulated by a promoter with multiple
44 DNA regulatory elements that could bind several transcription factors. HIV-1 LTR contains two
45 binding sites for YY1. The first region is at -17 to +27 position of LTR, through which YY1 was
46 shown to repress the LTR-driven reporter gene transcription (1). The repression, however,
47 requires a late SV40 factor (LSF), for which LSF and ZF motifs in the C-terminal domain (308-
48 368 residues) of YY1 are involved. Co-purification of HDAC1 with LTR-LSF-YY1 complex
49 demonstrated the involvement of the central domain (154-199 residues) of YY1 in recruiting
50 HDAC1 for the repression of HIV-1 transcription and thereby promoting establishment and
51 maintenance of its latent infection (3, 22). The second region is at -140 to -120 of LTR. Unlike -
52 17 to +27 region, YY1 binds this region directly in unstimulated conditions and leads to
53 transcriptional repression of LTR. This binding is decreased after T-cell stimulation, thereby
54 releasing the repression (4). Interestingly, YY1 mutant lacking HDAC1-interacting domain (155-
55 198 residues) could bind both -140 to -120 and -17 to +27 regions on LTR but not repress LTR
56 expression. These studies suggested that YY1 functions as a transcriptional repressor of HIV-1
57 LTR in conjunction with HDAC1 and a regulator leading to the latent infection of HIV-1.

58 Here, we investigated the effect and role of YY1 on the production of HIV-1 virus.
59 Unprecedentedly, we observed that YY1 could enhance HIV-1 U3 and U3RU5 mediated
60 promoter activity, leading to increased viral transcription and production. Our data strongly
61 indicate that YY1 could function as an activator, at least in the acute infection stage.

RESULTS

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64 **YY1 is important for HIV-1 replication**

65 To investigate the effect of YY1 on HIV-1 replication and production, 293T cells were co-
66 transfected with HIV-1 proviral DNA, pNL4-3_{GFP}, and a YY1 expression plasmid. HIV-1
67 production was determined at 24 h post-transfection by measuring the amount of Gag protein
68 production in cell lysate and viral production in cell supernatant using Western blotting and
69 p24^{Gag} ELISA analysis, respectively. Overexpression of YY1 enhanced the Gag protein
70 production in cells and virus in cell supernatant (Fig. 1A and 1B). An RFP expression vector was
71 included as a normalization control in all our transfection assays. We constructed an mCherry-
72 tagged YY1 expression plasmid and used in co-transfection with the pNL4-3_{GFP} to rule out
73 transfection efficiency based experimental bias. Intensity of GFP was measured in mCherry⁺
74 GFP⁺ cells by FACS analysis. YY1-mCherry expressing cells showed higher GFP intensity than
75 mCherry control (Supplementary figure 1). These data indicated clearly that YY1 could increase
76 HIV-1 gene expression, resulting in an enhancement of HIV-1 production. Since YY1 is a well-
77 known transcription factor, we verified the level of HIV-1 mRNA by northern blotting analysis.
78 Production of all HIV-1 mRNA derivatives, such as un-spliced (9 kb), single spliced (4 kb) and
79 multiple spliced (2 kb) RNAs, were increased in YY1 overexpressed cells (Fig. 1C).

80 Further, using RNA interference, we investigated the effect of YY1 on HIV-1 production.
81 After 24 h post-transfection with either siYY1 or siTat (as a positive control for knock-down
82 experiment), resulting cells and viral supernatants were harvested, and the virus level was

83 measured. Western blotting showed that transfection with siYY1, which repressed YY1 by over
84 80%, resulted in a concomitant decrease in levels of Gag and GFP proteins compared to that of
85 control siRNA treated cells (Fig. 1D). Further, p24^{Gag} ELISA showed that knock-down of YY1
86 caused about 50% reduction of the virus production compared to the control (Fig. 1E). Treatment
87 with siTat, which targets HIV-1 specific transcriptional activator, further supported the effect of
88 siYY1 on virus production. Quantitation by qRT-PCR showed about 40% decrease in Gag
89 mRNA (9 kb) in the presence of siYY1 (Fig. 1F). Altogether, these results suggest that YY1 has
90 a positive role in HIV-1 transcription and virus production.

91 **YY1 has a positive effect on HIV-1 replication in CD4⁺ T cells**

92 Further, to confirm the positive effect of YY1 on virus production, as observed in proviral
93 DNA transfection system, we investigated the effect of YY1 on HIV-1 infection cycle, using a
94 CD4⁺ T cell line MT-4 cells. At 24 h post-transfection of MT-4 cells with either YY1-mCherry
95 expression vector or siYY1, the cells were infected with HIV-1 NL4-3_{GFP} viruses. Three days
96 post-infection, we observed that MT-4 cells expressing the YY1-mCherry yielded more viruses
97 than the cells expressing mCherry alone (Fig. 2A and 2B). Conversely, when YY1 was depleted
98 by siYY1 RNA, virus production became lower than in the case of control siRNA (Fig. 2C and
99 2D). Notably, however, that effect of overexpression or knock-down of YY1 in MT-4 cells is
100 lesser than that observed in 293T cells, due to lower transfection efficiency of MT-4 cells.

101 To verify the requirement and positive effect of YY1 on HIV-1 virus production in MT-4
102 cells, we constructed and established a YY1 stably knocked-down MT-4 cell line, named MT-
103 4_shYY1. Equal numbers of MT-4 and MT-4_shYY1 cells were infected by different MOI of

104 HIV-1 NL4-3_{GFP} viruses, as indicated in figure 2E. Three days post-infection, virus infected
105 cells, expressing GFP, were captured by fluorescent microscope and analyzed by FACS analysis
106 (Fig. 2E and 2F) and Western blotting (Fig. 2G). Similar to siRNA treatment, MT-4_shYY1 cells
107 showed a lower level of virus production than control MT-4 cells. Quantification of virus level 3
108 h after infection (Supplementary figure 2) confirmed that same level of virus entered the cells,
109 indicating that the lower level of virus production in MT-4_shYY1 cells, compared to MT-4
110 cells, is not due to different numbers of virus entered. These data altogether demonstrated that
111 YY1 also functions positively in the virus infection cycle in CD4 + T cell.

112 **YY1 upregulates the HIV-1 promoter activity and trans-acting activity of HIV-1 Tat**

113 We observed that overexpression of YY1 increased mRNA level of HIV-1 (Fig. 1C) and
114 knockdown of YY1 resulted in the opposite (Fig. 1F). Thus, to investigate the direct influence of
115 YY1 on HIV-1 promoter further, we constructed a firefly luciferase (Fluc) expression plasmid
116 driven by U3, U3R, and U3RU5 (also referred to as LTR) region of HIV-1. U3 contains the
117 modulatory region (nt -454 to -104), a core enhancer (nt -105 to -79), and basal promoter (nt
118 -78 to -1) (23, 24). R (+1 to +82) contains transactivation response RNA structure (TAR) (+19
119 to +44), which is a Tat protein binding region, and Tat-TAR interaction leads to enhance the
120 efficiency of viral transcription and elongation (25, 26). Moreover, two binding sites for YY1
121 were reported in HIV-1 LTR, one resides in U3 region (-120 to -140) and the other in R region (-
122 17 to +27).

123 YY1 increased U3, U3R and U3RU5 driven Fluc reporter gene expression over three-fold
124 (Fig. 3A). Since U3R and U3RU5 contain the TAR, Fluc expression is considerably increased in

125 the presence of Tat as shown earlier (Fig. 3A). Unexpectedly, YY1 elevates the Fluc expression
126 under the condition already activated by Tat protein. To confirm if increased Fluc level is due to
127 increased RNA level, qRT-PCR analyses were performed. Similar to Fluc protein expression,
128 increase in YY1 increases Fluc RNA level (Fig. 3B). These data indicate role of YY1 as a
129 transcription activator in both conditions, in the absence and presence of HIV-1 Tat protein.

130 **YY1 has two regions with different activities.**

131 In order to determine the functional domains of YY1 responsible for the positive effect on
132 HIV-1 virus production, we constructed various truncated mutants of YY1 (Fig. 4A). The
133 expression levels of all the mutants were confirmed by Western blotting (Fig. 4B). Expression of
134 most of the mutants was detected by Western blotting except of 1-154 and 155-200 domains.
135 Therefore, expression of 1-154 and 155-200 domains was confirmed by immunostaining (Fig.
136 4C).

137 Further, each of the truncated mutants was then co-transfected individually with pNL4-
138 3_{GFP} and pDs-red, and the virus production levels were measured. Among the mutants, while the
139 296-414 domain alone showed a significant enhancement of virus production, the 155-295
140 domain resulted in a decrease (Fig. 4D and 4E). Northern blotting indicated that the increase of
141 virus production with the 296-414 domain is due to up-regulation of viral transcription as
142 observed with wild type YY1 expression (Fig. 4F). However, an apparent decrease in the viral
143 production with the 155-295 domain of YY1 did not correlate with the viral mRNA level.
144 Measurement of Fluc activity of U3RU5-Fluc with YY1 truncated mutants showed that in the
145 absence of Tat protein, only full-length YY1 significantly enhanced the Fluc activity, but in the

146 presence of Tat protein, 296-414 domain activated the Fluc activity as much as full-length YY1
147 (Fig. 4G). Transcription activation via 296-414 domain was confirmed by qRT-PCR (Fig. 4H).
148 The 296-414 domain did not affect U3 and slightly increased the U3RU5. Similar to protein
149 levels, Fluc mRNA levels were increased in the presence of Tat (Fig. 4G), indicating that the
150 increased virus production via 296-414 domain occurred in a Tat-dependent manner.

151 A previous study showed that phosphorylation of threonine residues at 348 and 378 abolish
152 DNA binding activity of YY1 (27). To analyze the importance of DNA binding activity on HIV-
153 1 production, we constructed the alanine substitution mutants on these sites, YY1_{T348A}, YY1_{T378A}
154 and a double mutation mutant YY1_{T348,378A}. We then determined the effect of these mutants on
155 virus production via co-transfection with pNL4-3_{GFP} into the 293T cells. Western blotting and
156 p24 ELISA show that all three mutants have abolished the activation ability of virus production
157 (Fig. 4I and 4J) and transcription activation (Fig. 4K), suggesting that the Threonine residues at
158 348 and 378 in 296-414 domain of YY1 are critical for a positive transcriptional activation of
159 YY1 on HIV-1 production.

160 These data suggest that YY1 might have bifunctional properties in HIV-1 production.
161 While 296-414 domain of YY1 has a transcriptional activator activity especially in the presence
162 of Tat, 155-295 domain may have some other role(s), like post-transcriptional or translational
163 repressor activity which needs to be elucidated further. Taken together, the data presented here
164 suggest strongly that full-length YY1 could function as HIV-1 transcriptional activator.

Discussion

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167 Here, using 293T cells in transfection assays and using MT-4 cell-line infection assays, we
168 report that overexpressing YY1 increased HIV-1 production and found that such an increase
169 came from transcriptional activation of HIV-1 (Fig. 1 and 2). Effect of YY1 on HIV-1 LTR
170 promoter was verified using constructs with U3, U3R and U3RU5 driven Fluc reporter gene
171 assay. We observed that YY1 enhances the U3 promoter activity, but higher enhancement was
172 observed with U3R and U3RU5 forms. The latter activity was also increased in the presence of
173 Tat transcriptional activator (Fig. 3). However, direct or indirect binding of YY1 to LTR has
174 been shown previously to repress LTR activity through the recruitment of HDAC1. The
175 contradicting observations might be due to the following: A) Unlike the previous studies, we
176 employed RFP expression plasmid as an internal control in our assays, which enabled relatively
177 accurate measurements in the transfection assays. B) We observed a decrease in the virus
178 production when YY1 is depleted as knock-down of YY1 decreased the virus production (Fig. 1
179 and 2). In the only knock-down experiment on latently HIV infected cell lines, 2D10 and J89, the
180 authors hypothesized that YY1 depletion leads to active transcription of HIV in both the cell
181 lines. However, because that was occurred only in 2D10 cells, the study concluded that
182 maintenance of HIV-1 latency is complex, involving multiple restrictive mechanisms. C) We
183 determined transient virus production within 24-48 h post-transfection of proviral plasmid or 72
184 h post-virus infection. However, previous studies have determined the virus production 5 days
185 (or even longer) after either transfection or infection.

186 Having established this phenomenon of YY1, we constructed various truncated or point
187 mutants of YY1 and analyzed their effect on HIV-1 replication. YY1 was shown to possess two
188 domains acting functionally different (Fig. 4). That is, regions spanning 155-295 and 296-414
189 residues of YY1 have negative and positive effect on HIV-1 replication, respectively. The 155-
190 295 segment contains a REPO domain (201-225) which is known to recruit the PcG proteins
191 onto DNA for transcriptional repression (8). However, we did not observe the transcription
192 repression upon over-expression of YY1 155-295 segment, other than a little increase of 2 kb
193 mRNA, a multiple spliced RNA of HIV-1 (Fig. 4F). However, although there is no detectable
194 decrease of 9 kb mRNA expressing Gag protein in 155-295 segment over-expressing cells, a
195 decrease in the Gag protein level in both cell and media was observed (Fig. 4D and 4E).
196 Therefore, 155-295 segment might affect the post-transcription step(s) of HIV-1 production.
197 However, 296-414 segment increased 9 kb mRNA level and thereby the virus production.
198 U3RU5 reporter gene analysis depicts that such an elevation is resulted from the overexpression
199 of 296-414 segment, as shown by enhanced Tat-dependent Fluc activity (Fig. 4G and 4H).
200 Therefore, apparently, YY1 is unable to increase the virus production as high as 296-414, due to
201 the inhibitory effect of 155-295 domain, which appears to be acting after the post-transcriptional
202 step.

203 A previous study showed that 170-200 residue region of YY1 is acetylated by p300 and
204 PCAF and 261-414 region of YY1 is acetylated by PCAF. They showed that acetylation sites are
205 for binding of HDAC proteins (HDAC1, HDAC2 and HDAC3). When YY1 is acetylated,
206 interaction with HDACs proteins is increased and HDACs deacetylate 170-200 region but not
207 261-414 region (19). To find out whether acetylation of 155-295 region would decrease the HIV-

208 1 production, we predicted the acetylation site(s) using Acetylation prediction software (GPS-
209 PAIL 2.0, <http://pail.biocuckoo.org/online.php>), which predicted highest probability of
210 acetylation on Lys178 followed by Lys179, and four acetylation sites in 160-181 region.
211 Therefore, we constructed 155-295_{K178A,K179A} and 155-295_{Δ160-181}. Further, 155-
212 295_{K283A,K286A,K288A} was constructed since 155-200 region does not have any effect on HIV
213 production (Fig. 4). Co-transfection with the said constructs and pNL4-3_{GFP} was performed and
214 the virus production was measured. We observed that these mutants resulted in further decrease
215 in the virus production than wild type (data not shown). These results suggested that acetylation
216 does not interfere with repression activity of 155-295 region, although some other sites might be
217 acetylated.

218 Tat protein is acetylated at Lys50 residue by p300 Histone acetyltransferase (28). A
219 previous study showed that acetylated Tat interacts with PCAF and this interaction
220 synergistically elevates the HIV promoter activity (29). Although we did not confirm the
221 physical interaction between and YY1-PCAF, it is possible that the region 296-414 reinforces the
222 Tat-PCAF interaction.

223 In summary, our data show clearly that a cellular factor, YY1, could have a positive effect
224 on HIV-1 replication, at least in HIV-1 actively replication cell or early infection. Therefore,
225 further studies are necessary to find out the precise requirement for switching the role of YY1 to
226 a repressor of HIV-1 transcription for the viral latency, as reported previously.

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MATERIALS AND METHODS

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230 See supplementary information for Material and Methods.

231

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232

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235

CONFLICTS OF INTEREST

236

237 The authors have no conflicting interests.

238

FIGURE LEGEND

239 **Figure1. YY1 enhances HIV-1 replication in 293T cell.** (A-C) 293T cells were transfected
240 pCMV/Con or pCMV/HA-YY1 with pNL4-3_{GFP} and pDs-Red. Cell lysate were subjected to
241 Western blotting (A) and for quantification of p24 levels, the viral supernatants were analyzed by
242 p24 ELISA, * $P < 0.05$, Student's t test, obtained from three independent experiments (B). HIV-1
243 mRNA was determined by Northern blotting (C). (D-F) 239T cells were transfected with either
244 control siRNA, siTat or siYY1 with pNL4-3_{GFP} and pDs-Red. Western blotting of cell lysate (D),
245 p24 ELISA of viral supernatant (E) and qRT-PCR of Gag RNA (F). The asterisks indicate a
246 significant difference from control, one-way ANOVA with Dunnett's multiple comparisons test,
247 *** $P < 0.0001$

248 **Figure2. YY1 enhances HIV-1 replication in MT-4 cell.** (A-B) MT-4 cells were transfected
249 pCDNA3/mCherry or pCDNA3/YY1-mCherry (Y-M). 24 h later, HIV-1_{NL4-3GFP} infected and
250 incubated for three days. (A) p24 ELISA result of viral supernatant. * $P < 0.05$, Student's t test,
251 obtained from three independent experiments. (B) Western blotting of cell lysate. (C-D) MT-4
252 cells were transfected either consiRNA, siTat or siYY1 then HIV-1_{NL4-3GFP} infected and
253 incubated as described above. (C) p24 ELISA result of viral supernatant. (D) Western blotting of
254 cell lysate. The asterisks indicate a significant difference from control, one-way ANOVA with
255 Dunnett's multiple comparisons test, ** $P < 0.01$, *** $P < 0.001$. (E-G) MT-4 or MT-4_shYY1
256 cells were infected HIV-1_{NL4-3GFP} with indicated concentration then incubated for three days. (E)
257 HIV-1 infected cell captured by fluorescent microscope, (F) GFP positive cell were analyzed by

258 FACS, (G) Western blotting. All experiments are performed more three independent
259 experiments.

260 **Figure3. YY1 up-regulates HIV-1 promoter activity and trans-acting activity of Tat**
261 **protein.** 293T cells were transfected pCMV/Con or pCMV/HA-YY1 with pGL/Fluc or pGL/U3-
262 Fluc or pGL/U3R-FLuc or pGL/U3RU5-Fluc, pDs-Red and pCMV-Tat (in the case mentioned).
263 24h later, cells were harvested and performed luciferase assay (A) and qRT-PCR (B). The
264 asterisks indicate a significant difference from control, one-way ANOVA with Dunnett's
265 multiple comparisons test, $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$ and n.s indicate non-
266 significant, obtained from more three independent experiments.

267 **Figure4. YY1 have two motifs with antagonistic roles on HIV-1 replication.** (A) Scheme of
268 YY1 and its truncated mutants used in this study. (B-C) A confirmation of protein expression by
269 Western blotting (B) and Immunofluorescent analysis (C). (D-F) 293T cells were transfected
270 pCMV/Con or indicated YY1 derivatives with pNL4-3_{GFP} and pDs-Red. Western blotting of cell
271 lysate (D), p24 ELISA of viral supernatant (E). Northern blotting of HIV-1 mRNA (F). (G) 293T
272 cells were transfected pCMV/Con or indicated YY1 derivatives with pGL/U3RU5-Fluc, pDs-
273 Red and pCMV-Tat. 24 h later, luciferase assay were performed. (H) 293T cells were transfected
274 pCMV/Con or HA-296-414 with pGL/U3-Fluc or pGL/U3RU5-Fluc, pDs-Red and pCMV-Tat.
275 24 h later, qRT-PCR.were performed. (I-K) 293T cells were transfected pCMV/Con or indicated
276 YY1 derivatives. Western blotting of cell lysate (I), p24 ELISA of viral supernatant (J) Northern
277 blotting of HIV-1 mRNA. The relative ratio (9kb/RFP) was calculated by measuring the band
278 intensity using Image J software (NIH) (K). The asterisks indicate a significant difference from

279 control, one-way ANOVA with Dunnett's multiple comparisons test, * $P < 0.05$, ** $P < 0.01$,
280 *** $P < 0.0001$ and n.s indicate non-significant, obtained from more three independent
281 experiments.

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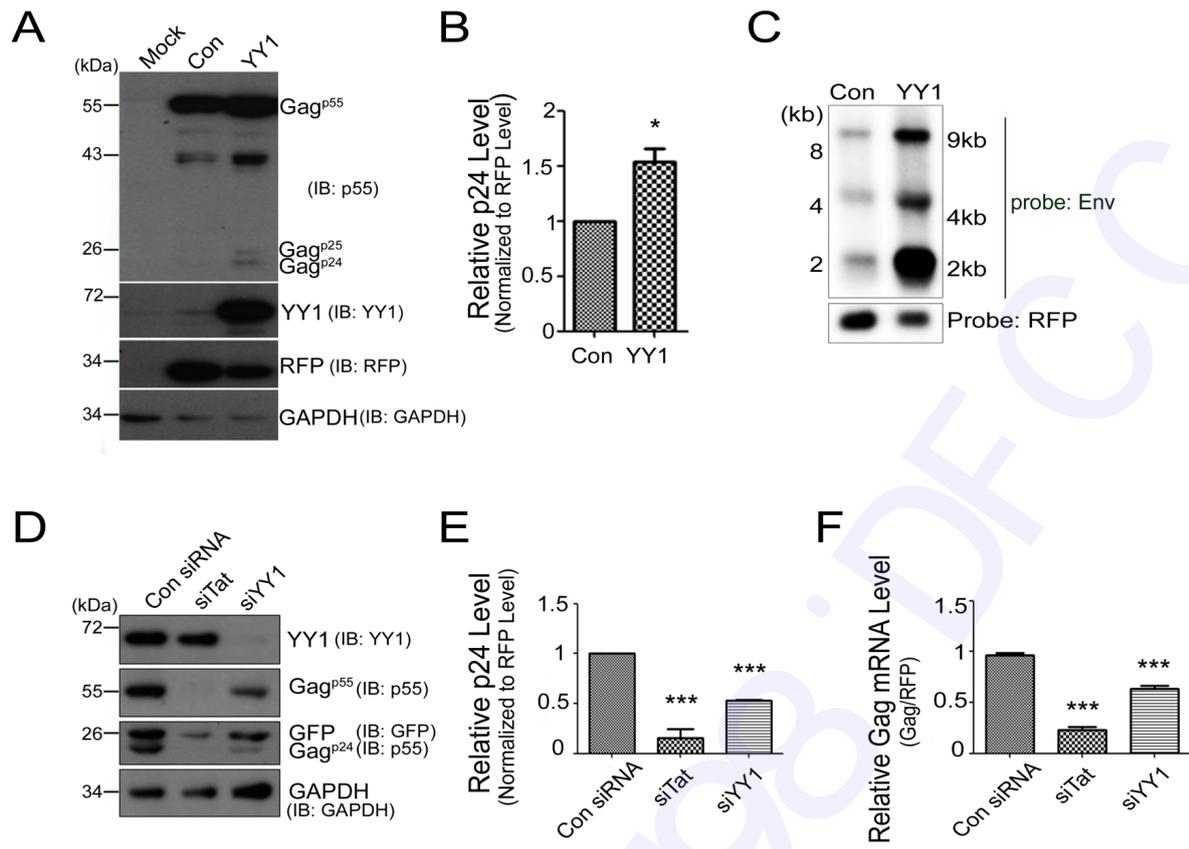


Fig. 1.

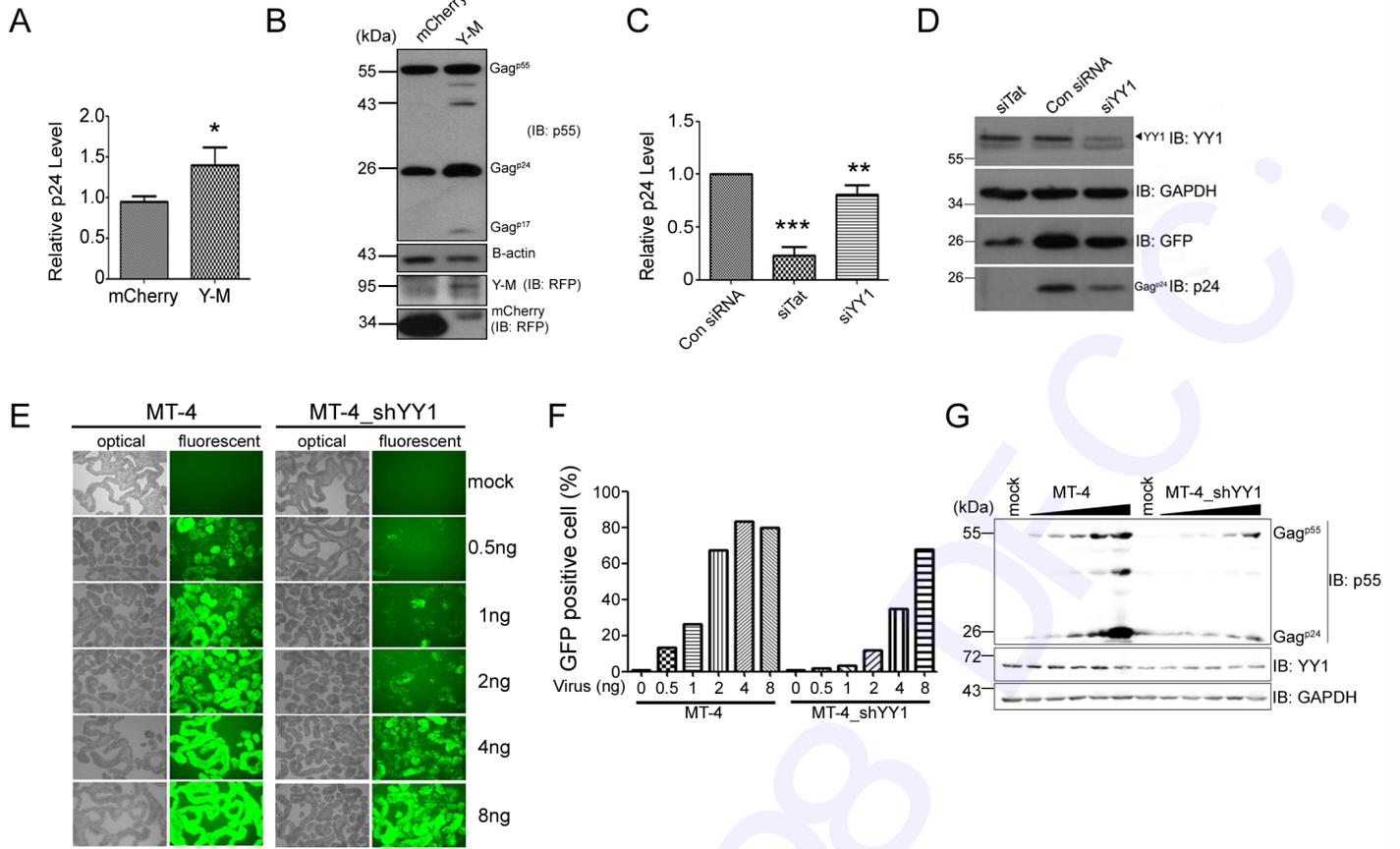


Fig. 2.

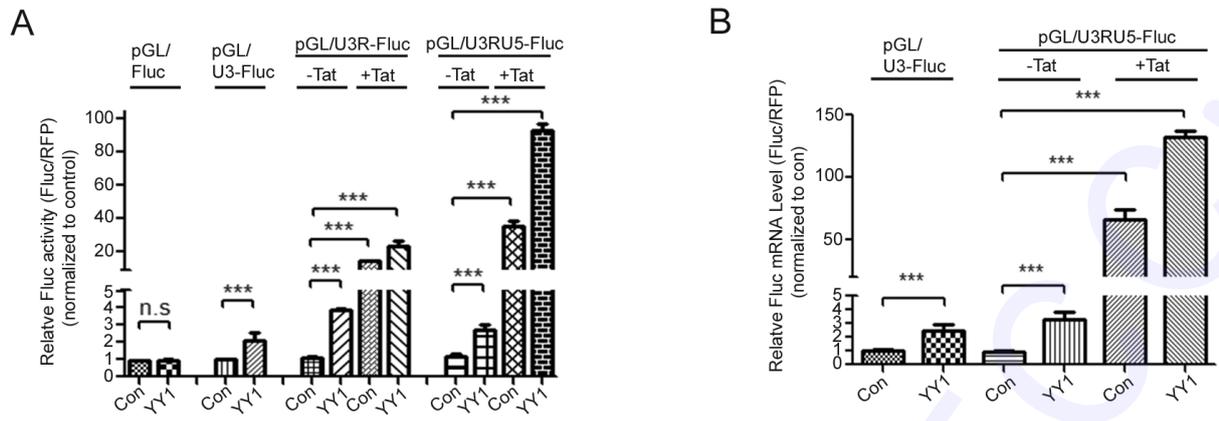


Fig. 3.

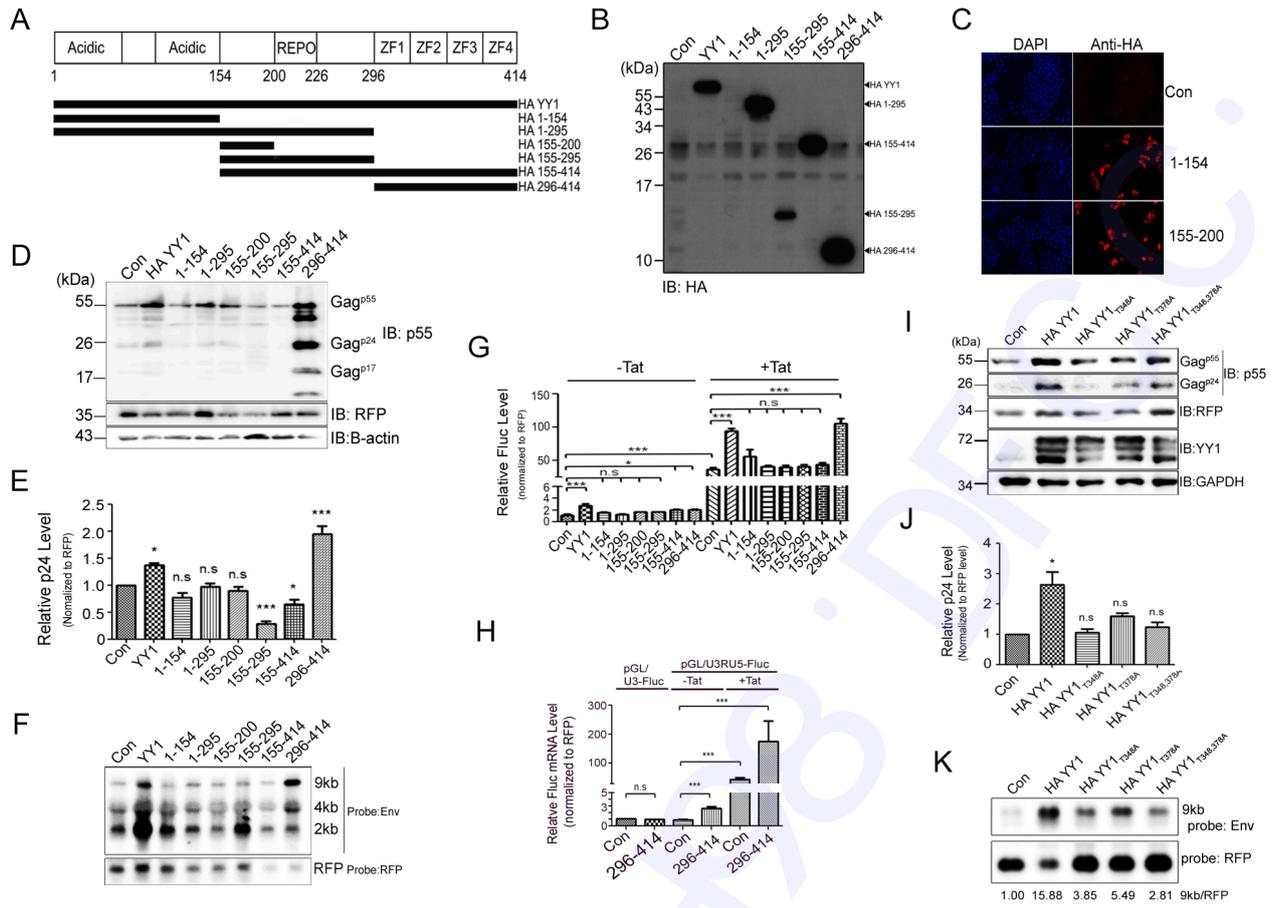


Fig. 4.

SUPPLEMENTARY INFORMATION

Manuscript Type: Article

Title: Human transcription factor YY1 could upregulate the HIV-1 gene expression

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Running title : YY1 could enhance the HIV-1 transcription

Keywords: Ying Yang 1 protein, Human deficiency virus type-1, Transcription activation, Long-terminal repeat region, Tat protein

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MATERIALS AND METHODS

Plasmid Construction

For the expression of YY1 and truncated mutants, YY1 and truncated form were amplified by PCR. All of PCR products insert to *BglIII/KpnI* site of pCMV-HA (Clontech). To construct YY1-mCherry, YY1 amplified by PCR, digested with *BglIII/KpnI* and made blunted end by klenow DNA polymerase (New England Biolabs) then insert to *HindIII*-klenow treated pCDNA3-mCherry (a kindly provided by Dr. de Rocquigny H). To create YY1_{T348A} and YY1_{T378A}, we used an overlap extension PCR method. For YY1_{T348,378A}, performed overlap PCR substituting T378 to A378 using YY1_{T348A} as a template. All of primer using overlap PCR also listed in Supplement Table 1. A pNL4-3_{GFP} was kindly provided by Dr. Y.C Sung [1]. A U3/Fluc, U3R/Fluc and U3RU5/Fluc were previously described [2]. For Tat expression, Tat amplified by PCR using pNL4-3_{GFP} as a template then insert to *EcoRI/EagI* site of pCMV-Myc (Clontech). All of primers used in this study are listed in Supplement Table1.

Cell Culture and stable cell line

HeLa and 293T cells were maintained in Dulbecco's modified Eagle medium (Hyclone) and MT-4 cells were maintained in RPMI-1640 medium (Hyclone), both supplemented with 10% fetal bovine serum (Tissue Culture Biologicals) and penicillin and streptomycin (Invitrogen), and incubated at 37°C in 5% CO₂. To generate MT-4_{shYY1} stable cell line, 293T cell were transfected with DNA mixture of pLP1, pLP2, pLP/VSVG (Invitrogen) and shYY1 plasmid (TRCN0000019895, Sigma-Aldrich) to produce the shYY1 lentivirus. At 30 h post-transfection, lentiviral supernatant were harvested and filtered by 0.45- μ m filter then transduced to MT-4 cells. Transduced cells were maintained and selected with media containing 5 μ g/ml of puromycin over two weeks.

Virus production and analysis

The 293T cells (2.5×10^5) in 12-well plate were transfected 200ng of pNL4-3_{GFP} and 50ng of pDs-Red with 1 μ g protein expression plasmid or 40nM siRNA using jetPEI (Polyplus) or Lipofector-EZ (Aptabio), respectively. 24 h later, cells and viral supernatant were harvested. Cell were washed with 1xPBS and lysed in RIPA buffer supplemented with protease inhibitor cocktail (P8340, Sigma-Aldrich), then measured fluorescence of GFP and RFP by fluorometer (Infinite F200, TECAN) and subjected to western blotting. The viral supernatant filtered with 0.45- μ m filter then analyzed by western blotting and p24 ELISA assay (XB-1000, Xpressbio). MT-4 cells (1×10^5) in 48-well plate were transfected 1 μ g of protein expression plasmid or 40nM of siRNA using Lipofector-EZ. 24 h later, 10ng of HIV-1_{NL4-3GFP} virus generated from pNL4-3_{GFP} transfected 293T cell were transduced and incubated for three days then analyzed cell and viral supernatant as described above. GFP positive cell indicating HIV-1 infection were captured by inverted fluorescence microscope (IX71, Olympus) and counted by FACS analysis (FACS Canto II, BD bioscience).

Immunofluorescence (IF) analysis

HeLa cells (4×10^5) in 24-well plate inserted 12mm Φ size of cover glass (0111520, Marienfeld) were transfected with the indicated plasmids using jetPEI. IF was performed as described previously (40).

Firefly luciferase (Fluc) assay

Fluc activity was measured by Luciferase assay system (E1500, Promega) and luminometer (Infinite F200, TECAN) following the manufacturers' instructions.

Northern blotting and Real-time quantitative PCR (qRT-PCR)

Total cell RNA was isolated using RNAiso Plus (TaKaRa). RNA were separated on a 0.7 % agarose gel containing 2.2 M formaldehyde then following of DIG Northern Starter Kit (Roche) manufacturer's instruction. The probe of Env (corresponded to 8368-8784 nt of pNL4-3) and RFP (corresponded to 1-400 nt of RFP) are generated by in vitro transcription using T7 polymerase. For qRT-PCR, cDNA generated using 5x M-MLV reverse transcriptase pre-mix (EBT-1511, Elpisbiotech) then subjected to qRT-PCR using LightCycler 480 SYBR Green 1 Master, white 96-multiwell plate and LightCycler480 instrument (Roche). All of primers used in qRT-PCR are also listed in Supplement Table1.

siRNA and Antibodies

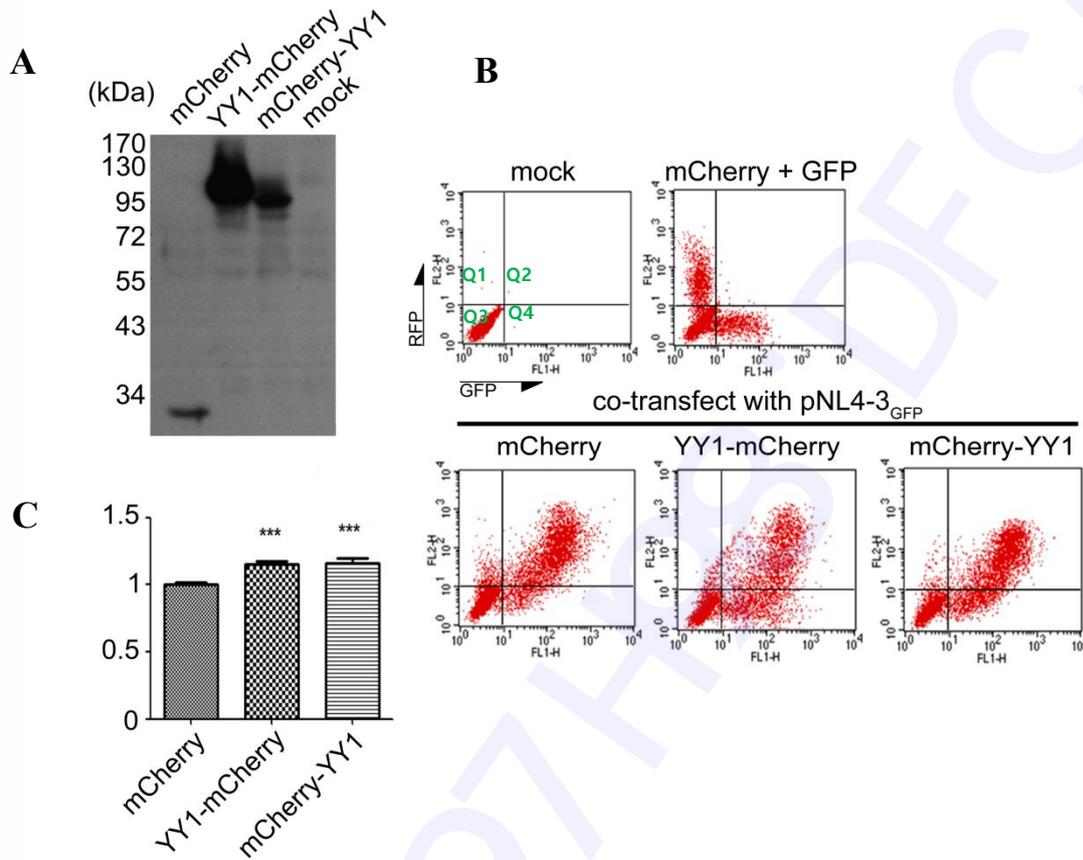
ON-TARGET_{plus} Non-targeting siRNA #1 (cat: D-001810-01-05) (control siRNA), ON-TARGET_{plus} SMARTpool YY1 (cat: L-011796-00) and custom siTat (sequence: 5'-CUGCUUGUACCAAUUGCUAUUUU-3') were purchased from Dharmacon. Primary antibodies used in this study include the following: rabbit anti-YY1 (sc-1703, SantaCruz); rabbit anti-p55 (ThermoFisher); mouse anti-HA (H-9658, Sigma-Aldrich); rabbit anti-RFP (632496, Clontech); mouse anti- β -actin (A00702, GenScript); mouse anti-GAPDH (A01622, GenScript). Secondary antibodies included: Immunopure Goat anti-Mouse IgG (H+L) HRP conjugate (31430, ThermoFishe) and Immunopure Goat anti-Rabbit IgG (H+L) HRP conjugate (31460, ThermoFisher).

Supplementary Table 1

Name of primer	Sequences of primer	Restriction enzyme
YY1 F	5'-AAAGATCTCTATGGCCTCGGGC-3'	<i>Bgl</i> II
YY1 R	5'-CAGGTACCTCACTGGTTGTTT-3'	<i>Kpn</i> I
155 F	5'-AAAAGATCTCTATGGCGGCCGGCAAGA-3'	<i>Bgl</i> II
296 F	5'-GGAGATCTCTATGATAGCTTGCCCTCATAA -3'	<i>Bgl</i> II
154 R	5'-CAAGGTACCTCACGCCACGGTGACCA-3'	<i>Kpn</i> I
200 R	5'-AAAGGTACCTCACGGGTCGGCGCCGCC-3'	<i>Kpn</i> I
295 R	5'-CAGGTACCTCATGTTCTTGAGCAT-3'	<i>Kpn</i> I
K 348A F	5'-CATGCCGAGAGAAGCCC-3'	-
K 348A R	5'-TTCTCTCCGGCATGAACCAGTT-3'	-
K 378A F	5'-GCCGGAGACAGGCC-3'	-
K 378A R	5'-GCCTGTCTCCGGCATGGATT-3'	-
Tat F	5'- AAAGAATTCGGATGGAGCCAGTAGAT -3'	<i>EcoR</i> I
Tat R	5'- AAACGGCCGCTATTCCTTCGGGCCTGT -3'	<i>Eag</i> I
Name of primer	Sequences of primer for qRT-PCR	
Gag qRT F	5'-GCAGCCATGCAAATGTAAAAGAG-3'	
Gag qRT R	5'-TCCCCTTGTTCTCTCATCTGG-3'	
RFP qRT F	5'-GAGATCCACAAGGCCCTGAA-3'	
RFP qRT R	5'-GGAGTCCACGTAGTAGTAGCC-3'	

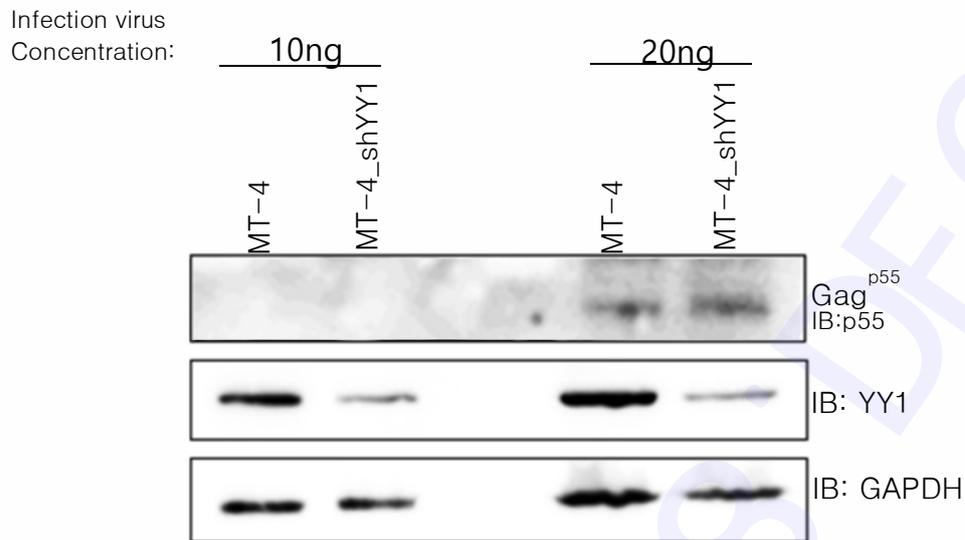
Primers used in this study. F: Forward. R:Reverse. Restriction enzyme sites are underlined.

Supplementary Figure 1



Supplementary 1. YY1 increases the GFP expression derived by HIV-1 LTR. (A) Confirmation of mCherry, YY1-mCherry, mCherry-YY1 expression by western blot (B) FACS analysis. mCherry + GFP; separately transfected mCherry and pNL4-3_{GFP} plasmid then mixed when cells were harvested. FACS result shown that Red and Green fluorescent detected in Q1 and Q4 region, respectively. Co-transfected cell; each mCherry or mCherry tagged YY1 plasmid were transfected with pNL4-3_{GFP}. Dual positive cells (mCherry⁺ GFP⁺) were detected in Q2 region. (C) GFP mean fluorescent value gained from Q2 region of (B).

Supplementary Figure 2



Supplementary 2. YY1 knock-down does not affect virus entry. 1×10^5 cell of MT-4 and MT-4_shYY1 cells were infected 10 and 20ng of HIV-1_{NL4-3GFP}, then cell were harvested and lysed at three hours post-infection. The same quantity of protein was loaded to SDS-PAGE, then immuno-blotted with indicated antibodies.

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