FoxO4 negatively controls Tat-mediated HIV-1 transcription through the post-transcriptional suppression of Tat encoding mRNA

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Abstract

The connection between the repression of human immunodeficiency virus type 1 (HIV-1) transcription and the resting CD4+ T cell state suggests that the host transcription factors involved in the active maintenance of lymphocyte quiescence are likely to repress the viral transactivator, Tat, thereby restricting HIV-1 transcription. In this study, we analysed the interplay between Tat and the forkhead box transcription factors, FoxO1 and FoxO4. We show that FoxO1 and FoxO4 antagonize Tat-mediated transactivation of HIV-1 promoter through the repression of Tat protein expression. No effect was observed on the expression of two HIV-1 accessory proteins, Vif and Vpr. Unexpectedly, we found that FoxO1 and FoxO4 expression causes a strong dose-dependent post-transcriptional suppression of Tat mRNA, indicating that FoxO should effectively inhibit HIV-1 replication by destabilizing Tat mRNA and suppressing Tat-mediated HIV-1 transcription. In accordance with this, we observed that the Tat mRNA half-life is reduced by FoxO4 expression. The physiological relevance of our findings was validated using the J-Lat 10.6 model of latently infected cells. We demonstrated that the overexpression of a constitutively active FoxO4-TM mutant antagonized HIV-1 transcription reactivation in response to T cell activators, such as TNF-α or PMA. Altogether, our findings demonstrate that FoxO factors can control HIV-1 transcription and provide new insights into their potential role during the establishment of HIV-1 latency.

INTRODUCTION

A major obstacle in human immunodeficiency virus type 1 (HIV-1) therapy is the ability of the virus to maintain a transcriptionally silent replication-competent provirus, which can escape viral immune clearance and antiviral drugs and persist for long periods of time [1–4]. These reservoir cells are generated during the acute phase of HIV-1 infection by the reversion to a memory resting state of a subset of CD4+ infected T cells harbouring the integrated HIV-1 genome, which leads to strong repression of HIV-1 transcription [4, 5]. During this transition, optimal transcription repression is achieved by multiple processes, including the inactivation or cytoplasmic sequestration of host transcription factors that are critical for T cell activation and viral transcription initiation, such as the nuclear factor-κB (NF-κB) [4, 6, 7]; proviral integration in transcriptionally silent heterochromatin [8–11]; a specific block to transcriptional elongation [12]; and post-translational modifications of histones affecting chromatin organization around the integrated provirus, including acetylation/deacetylation, methylation, phosphorylation and ubiquitination [3, 9, 10, 13]. In particular, deacetylation within the nucleosome positioned immediately downstream of the transcription start site leads to transcription elongation arrest by the RNA stem loop structure of the transactivation response element (TAR) [3]. In activated CD4+ T cells, the recruitment to the TAR element of remodelling factors and histone acetyltransferases by NF-κB and the virally encoded key regulatory protein Tat results in chromatin relaxation and transcription initiation through nucleosome hyperacetylation [3, 14]. Therefore, fluctuations in viral gene expression controlled by a Tat-positive or -negative feedback loop can contribute to the formation of either a productive or a latent infection. Although the connection between the repression of HIV-1 transcription and resting CD4+ T cells has been clearly established, little is known about the specific T cell factors that repress Tat-mediated transactivation of HIV-1 promoter.

The forkhead box O transcription factor family (FoxO1, FoxO3a, FoxO4 and FoxO6) are evolutionally conserved downstream effectors of the PI3K/c-Akt pathway, which play a critical role in response to environmental changes in a wide variety of processes, such as cell-cycle arrest, glucose metabolism, apoptosis and stress resistance [15–17].
Previous studies indicated that FoxO factors play an important role in the maintenance and integrity of stem-cell compartments and quiescent lymphocytes [18–24]. Indeed, the deletion of FoxO genes is associated with spontaneous T cell activation, maintenance of naive T cells in the peripheral lymphoid organs, lymphocyte trafficking alteration and an increased proportion of hematopoietic stem cells in the active phases of the cell cycle (S/G2/M) [19, 25–27]. In particular, deletion of the FoxO1 gene is associated with spontaneous T cell activation, maintenance of naive T cells in the peripheral lymphoid organs and lymphocyte trafficking alteration [19, 26, 27]. However, the loss of FoxO1 alone is not sufficient to allow uncontrolled lymphocyte proliferation, which suggests that other FoxO factors are involved in maintaining T cell quiescence [28]. Consistent with this, loss of FoxO1, FoxO3a or FoxO4 leads to similar phenotypes characterized by an increase in the proportion of hematopoietic stem cells in the active phases of the cell cycle (S/G2/M) [18]. In addition, FoxO3a has been shown to control the expansion and contraction of T cell populations through the regulation of interleukin-6 production after viral infection [29]. Complex and opposite functional cross-talks between FoxO factors and innate immune signalling pathways have been reported. FoxO1 was identified as a negative regulator of virus-triggered activation of the interferon (IFN)-β gene through the polyubiquitination and degradation of IRF-3 [30]. FoxO3 was reported to inhibit IRF-7 transcriptional activity and negatively regulate the type I IFN antiviral response limiting inflammatory cell injury [31]. Conversely, we provided evidence that FoxO4 triggers the activation of the innate immune signalling pathway in the coupling stimulation of TLR3 and RIG-like receptors by the synthetic double-stranded RNA analogue poly(IC) to IFN-β and IFN-induced gene induction [32]. Similarly, FoxO1 and FoxO3 were reported to activate innate immune response, in promoting antimicrobial peptides and pro-inflammatory cytokine production through distinct TLR-mediated signalling pathways [20, 23, 24, 35]. During lymphocyte activation, the direct phosphorylation on three conserved specific sites of FoxO factors by the serine/threonine kinase Akt triggers their nuclear exclusion and inactivation [34–36]. The fact that transcription of the HIV-1 provirus that is silent in quiescent T cells can be reactivated during lymphocyte activation allowed us to hypothesize that FoxO factors might be involved in the viral transcriptional repression in resting CD4+ T cells. We previously showed that the HTLV-1 Tax oncoprotein triggers the ubiquitination and proteasomal degradation of FoxO4 protein [37]. Because viral-encoded regulatory proteins can inactivate host factors involved in the antiviral response, our data suggested that FoxO4 might be a key factor in innate immune antiviral pathways and a good candidate for controlling HIV-1 transcription. To assess this hypothesis, we analysed the interplay between Tat and FoxO1 and FoxO4. We showed that FoxO1 and FoxO4 antagonize Tat-mediated transactivation of HIV-1 promoter through the repression of Tat protein expression. In agreement with this, FoxO1 and FoxO4 expression cause strong dose-dependent post-transcriptional suppression of Tat mRNA. In addition, using the J-Lat 10.6 model of latently infected cells, we demonstrated that the overexpression of a constitutively active FoxO4-TM mutant antagonized HIV-1 transcription reactivation in response to T cell activators, such as TNF-α or PMA. Altogether, our findings demonstrate that FoxO factors can control HIV-1 transcription and provide new insights into the potential role of FoxO factors during the establishment of HIV-1 latency.

RESULTS

FoxO1 and FoxO4 antagonize Tat-mediated transactivation of HIV-1 promoter

Different FoxO isoforms were reported to have highly specialized functions in the control of multiple cell types in the immune system, and to exert positive or negative effects on innate immune signalling pathways [20, 23, 24, 29, 30, 33, 38, 39]. We previously demonstrated that FoxO4 is a key factor in antiviral innate immunity pathways against RNA viruses such as Sendai virus and Newcastle disease virus [32, 37]. As in resting CD4+ T cells, active FoxO4 transcription factors are associated with the silencing of HIV-1 transcription, our data suggested that FoxO4 might be a key factor involved in the control of HIV-1 transcription by the viral transactivator Tat. To evaluate this hypothesis, the ability of FoxO4 to repress HIV-1 promoter was tested in a reporter assay. To this end, and to take into account the importance of provirus chromatin environment in the transcriptional activity of Tat, we first established a population of HeLa cell lines containing the stably integrated expression vector driving the luciferase gene under the control of HIV-1-LTR-5’ promoter (HeLa–LTR-5’–Luc). Then, a plasmid expressing the FLAG–Tat fusion protein was co-transfected in cells with various amounts of plasmids expressing FLAG–FoxO4. Luciferase activity was monitored and subsequently whole-cell extracts were prepared and analysed by immunoblot for Tat and FoxO4 expressions. As expected, we observed that Tat expression led to strong activation of the viral HIV-1 promoter (Fig. 1a). In addition, FoxO4 expression resulted in a dose-dependent repression of Tat-mediated HIV-1 promoter induction, with approximately 70% inhibition with the higher amount of FoxO4 (Fig. 1a). Similar data were obtained when the LTR-5’–Luc reporter was transiently co-transfected with Tat and FoxO4 expression vectors, demonstrating that the provirus chromatin environment was not required for the inhibition of HIV-1 promoter by FoxO4 (data not shown). Unexpectedly, immunoblot analysis indicated that the inhibition of Tat-mediated transactivation of HIV-1 promoter was associated with the strong repression of Tat protein expression, even with the smallest amounts of FoxO4 transfected (Fig. 1b). Importantly, it should be noted that because Tat protein is a potent activator of viral gene expression, Tat-mediated transactivation of LTR-5’–Luc reporter has always been measured, even without detectable Tat protein expression. These data suggested that FoxO4 could act at the level of Tat protein expression rather than through the direct
Fig. 1. FoxO1 and FoxO4 antagonize Tat-mediated transactivation of HIV-1 promoter through the downregulation of Tat protein expression. (a) HeLa cells containing a stably integrated HIV-1–LTR-5¢–luciferase reporter gene (HeLa–LTR-5¢–Luc) were transfected with 100 ng of a vector expressing the FLAG–Tat protein (FLAG–Tat) and various amounts (200 to 800 ng) of a vector expressing the transcription factor FoxO4 (FLAG–FoxO4). 48 h post-transfection, luciferase activities were quantified and the results from two independent experiments performed in duplicate are presented. The relative luciferase activity of each experimental condition is expressed in fold inductions relative to the luciferase activity observed in the absence of FLAG–Tat and arbitrarily set to 1. The HIV-1–LTR-5¢ promoter activity was significantly reduced in FoxO4-expressing cells versus cells transfected with an empty vector, as determined by a Student’s t-test for pairs (P<0.05). (b) Subsequently, whole-protein extracts were prepared and analysed by immunoblot using anti-FLAG and anti-tubulin-α antibodies. (c) HeLa–HIV–Luc was transfected with a vector expressing either 100 ng of Tat–HA or various amounts (100 and 400 ng) of HA–FoxO4. (d) HeLa cells were transfected with LTR-5¢–ΔTAR–Luc reporter with a vector expressing Tat–HA, in the absence or presence of HA–FoxO4. (e) HeLa cells were transfected with 200 ng of Tat–HA expression vector and either 50 or 200 ng of FLAG–FoxO4 or FLAG–FoxO4-TM expression vector. Whole-protein extracts were prepared and analysed by immunoblot
inhibition of viral promoter. To make sure, the basal (Tat-independent) luciferase level of HIV-1 promoter was analysed in presence and absence of FoxO4. As shown in Fig. 1(c), FoxO4 expression did not repress the basal HIV-1 transcription in the absence of Tat. To confirm this finding, the experiment was reconducted in HeLa cells transfected with the luciferase gene under the control of HIV-1–LTR–5’ promoter that lacked the sequence encoding the TAR element (HIV-1–LTR–5’–ATAR–Luc) and therefore insensitive to transactivation by Tat. As expected, in the absence of Tat transactivation, FoxO4 expression did not affect HIV-1 transcription (Fig. 1d). Altogether, these data demonstrated that FoxO4 mainly acted on HIV-1 transcription by suppressing Tat expression.

Phosphorylation of FoxO proteins by Akt on three conserved serine and threonine residues creates a binding motif for the 14-3-3 chaperone protein that interacts with FoxO and facilitates nuclear export and degradation in response to T cell activation [34]. The mutation of these three phosphophorylation sites on FoxO4 (Th32, Ser193 and Ser262) to alanine results in a permanent nuclear localization of the FoxO4 triple mutant protein (FoxO4-TM) that is constitutively active and refractory to negative inhibition [40]. To test whether Akt phosphorylation was required for FoxO4-mediated Tat downregulation, the previous experiment was reconducted using a FoxO4-TM expression vector. As shown Fig. 1(e), FoxO4-TM induced the repression of Tat expression more efficiently than wild-type FoxO4, suggesting that FoxO4 acted through a mechanism that requires its phosphorylation by Akt. Because the members of the FoxO family have redundant functions in the regulation of innate immune homeostasis, we next evaluated whether FoxO1 could affect the level of Tat expression. Similar to the data obtained with FoxO4, but to a lesser extent, we observed that FoxO1 downregulated Tat expression (Fig. 1f). Taken together, these data demonstrated the existence of a functional connection between HIV-1 transcription repression and active FoxO factors.

**FoxO4 controls Tat expression at a post-transcriptional level**

Our data led us to hypothesize that FoxO factor expression caused the proteolytic degradation of Tat protein. However, in our transient transfection experiments, the Tat fusion protein was expressed under the control of the cytomegalovirus (CMV) promoter. Therefore, to exclude the possibility that the repression of Tat expression could be due to the modulation of the viral promoter used to drive Tat–HA by FoxO factors, we analysed the effect of FoxO4 expression in a CMV–luciferase (CMV–Luc) reporter assay. We observed that FoxO4 did not negatively affect the transcriptional activity of CMV promoter (Fig. 2a). In addition, FoxO4 had no effect on the expression of two HIV-1 accessory proteins, Vif and Vpr, whose expression is also driven by the CMV promoter (Fig. 2b), strengthening the idea that FoxO acted specifically on Tat protein expression at a post-transcriptional level.

Although the actions of the FoxO factors can require binding to DNA, there is also evidence that they can affect the transcription of target genes via their association with activators or coactivators of transcription, or by inducing proteasomal degradation of transcription factors [15, 22, 41–43]. In particular, it has been shown that FoxO4 can suppress the cellular response to hypoxia, in part by downregulation of the steady-state levels of the HIF-1 protein, which suggests that FoxO4 could affect Tat protein stability [41]. Consistent with this, both proteasome-dependent and proteasome-independent mechanisms of Tat proteolysis were reported to be critical determinants of its activity [44–48]. Therefore, to evaluate whether FoxO4 induced proteasomal degradation of Tat, a part of the experiment described in Fig. 1(e) was reconducted in cells treated with 10 μM of the proteasome inhibitor MG132. In these experimental conditions, FoxO4-mediated Tat disappearance was weakly affected, revealing that the effects of FoxO4 were mainly independent of the ubiquitin/proteasome pathway (Fig. 2c). To test whether an alternative mechanism of proteolysis, such as lysosomal or caspase-mediated proteolysis, was involved in this mechanism, the experiments were reproduced using 3-MA or Z-VAD, two general lysosomal and caspase pathways inhibitors, respectively. Unexpectedly, neither 3-MA nor Z-VAD had an effect on Tat protein levels, indicating that FoxO4 did not seem to affect the stability of Tat protein through the three major degradation pathways (Fig. 2d). However, we cannot formally exclude the possibility that FoxO4 can affect Tat protein stability through another degradation mechanism.

Then, to address the potential contribution of endogenous FoxO1 and FoxO4 proteins to the transcriptional activity of HIV-1 promoter, we examined the functional consequences of small interfering RNA (siRNA)-mediated depletion of FoxO1 or FoxO4 for the induction of HIV-1 promoter by Tat. HeLa cells were transfected with siRNAs silencing either FoxO1 or FoxO4 for the induction of HIV-1 promoter by Tat. Consistent with the fact that the downregulation of Tat expression seemed to be less effective with FoxO1 as compared to FoxO4 (Fig. 1), the increase in transactivation...
appeared to be less important in FoxO1 knockdown cells (Fig. 3a). In accordance with our previous observations, the depletion of FoxO1 and FoxO4 was associated with a higher level of Tat protein expression, indicating that endogenous FoxO factors might be crucial for controlling the steady-state level of Tat protein in the cells (Fig. 3c). To confirm these data, a second approach using shRNA strategies to knockdown FoxO1 and FoxO4 was conducted. One shRNA targeting FoxO1 (sh-FoxO1) and two different shRNAs targeting FoxO4 (sh-FoxO4-1, sh-FoxO4-2), previously described for Fig. 2. FoxO4-mediated downregulation of Tat protein expression is independent of the main proteolytic pathways. (a) HeLa cells were transfected with 100 ng of a vector expressing the luciferase under the control of the CMV promoter (CMV-Luc) and various amounts (200 to 800 ng) of a vector expressing FLAG–FoxO4. 48 h post-transfection, the luciferase activity was quantified and the results from an experiment performed in triplicate are presented. Subsequently, whole-protein extracts were prepared and analysed by immunoblot using anti-FLAG and anti-tubulin-α antibodies. (b) HeLa cells were transfected with 200 ng of either the Tat–HA (Tat–HA), FLAG–Vif (FLAG–Vif) or FLAG–Vpr (FLAG–Vpr) expression vectors and either 400 or 800 ng of FLAG–FoxO4 (FLAG–FoxO4) expression vector. Whole-protein extracts were prepared and analysed by immunoblot using anti-HA, anti-FLAG and anti-tubulin-α antibodies. (c, d) HeLa cells transfected as described in Fig. 1(c), and then treated with either 10 µM of proteasome inhibitor MG132 for 3 h, 1 mM of lysosomal inhibitor 3-MA for 16 h, or 20 µM of caspase inhibitor Z-VAD for 3 h. Subsequently, whole-protein extracts were prepared and analysed by immunoblot using anti-HA, anti-FLAG and anti-tubulin-α antibodies.
their ability to specifically silence FoxO1 and FoxO4 proteins, were used [49]. As expected, the knockdown of FoxO factors was associated with increases to both HIV-1-LTR-5° transcriptional activity and Tat protein expression (data not shown).

Interestingly, the strong increases in Tat protein expression that were observed Fig. 3(c) did not result in equally large increases to its transactivation activity (Fig. 3b), suggesting that the quantity of Tat expressed in the cells was already at an over-saturated level. In order to test this possibility, we performed an experiment in which a suboptimal amount of Tat was transfected. First, a dose-dependent transactivation of LTR-5°-Luc reporter was performed to determine the optimal amount of Tat to be transfected. As shown in Fig. 3(d), the maximum level of transactivation is reached when around 10 ng of Tat expression vector is transfected. Interestingly, the level of LTR-5°-Luc transactivation was not directly proportional to the amount of Tat transfected in the cells. A 10-fold increase in the amount of Tat transfected (1 to 10 ng) only resulted in a 2.6-fold increase in the LTR-5°-Luc activity. Therefore, the results of this experiment makes it possible to account for the fact that the variations in luciferase activities presented in Fig. 3(b) do not exactly reflect the changes observed in the level of Tat protein expression. Because during virus replication Tat interacts with various cellular proteins to allow transcriptional elongation, these data suggested that Tat activity was limited by the quantity of various cellular factors essential for LTR transactivation. In accordance with
these explanations, when the siRNA experiment was reconducted with a suboptimal amount (1 ng) of transfected Tat vector, the increase to level of transactivation in the FoxO4 knockdown cells was around 2-fold, as compared to the cells transfected with the scramble siRNA control (Fig. 3e). Unfortunately, in these experimental conditions Tat remains undetectable in Western blot. However, this 2-fold increase in luciferase activity is compatible with a significant increase in Tat protein expression, as shown in Fig. 3 (c). Taken together, these data demonstrate that FoxO4 can negatively regulate HIV-1 transcription through the downregulation of Tat expression by a post-transcriptional mechanism, probably involving Tat messenger RNA (mRNA) degradation.

**FoxO1 and FoxO4 induce the specific post-transcriptional suppression of Tat mRNA**

Several reports have suggested that post-transcriptional mechanisms may contribute to HIV-1 latency [50, 51]. In particular, both the nuclear retention of spliced HIV-1 RNA and the miRNA-mediated degradation of viral RNA have been demonstrated to affect viral production [52, 53]. In addition, the zinc-finger antiviral protein ZAP has been shown to inhibit HIV-1 infection by selectively targeting spliced viral mRNAs for degradation [54]. Therefore, we set out to evaluate whether the disappearance of Tat protein in the presence of FoxO factors could result from the degradation of Tat mRNA. To this end, Tat was expressed in HeLa cells with various amounts of FLAG–FoxO4 or FLAG–FoxO1 and then total RNA were extracted and the levels of Tat mRNA were determined by Northern blot analysis. The levels of Tat mRNA were normalized to the levels of endogenous GAPDH mRNA. In parallel, the levels of Tat protein were analysed by immunoblot, as a control. We observed that FoxO1 or FoxO4 expression resulted in the disappearance of Tat mRNA, with a profile similar to that observed for the downregulation of Tat protein (Fig. 4). These effects were specific, as no variation in the levels of GAPDH mRNA was observed. The lower level of Tat mRNA observed in the presence of FoxO4 led us to assume that FoxO4 expression triggered a possible reduction in the Tat mRNA half-life. To test this hypothesis, Tat–HA was expressed alone or with FLAG–FoxO4 in HeLa cells. Then, the cells were exposed various times to high doses of actinomycin D (5 μg ml⁻¹) to shut off all transcriptional activity. Total RNA for each experimental condition was extracted and the survival of Tat mRNA was determined by RT-PCR. As shown in Fig. 5(a), FoxO4 expression led to a strong reduction in the half-life of Tat mRNA, as compared to Tat expressed alone. As expected, FoxO4 did not affect the half-life of GAPDH mRNA, which was used as negative control. These data demonstrated that FoxO4 expression induced post-transcriptional suppression of Tat mRNA. In addition, as the expression of Vif and Vpr proteins, whose parts of the RNA sequences, including the 3’ and 5’ ends, are identical to the complete Tat mRNA sequence (Fig. 5b), were not influenced by FoxO4, this suggested that FoxO4 specifically targeted the secondary structures of Tat mRNA, leading to its specific post-transcriptional suppression. However, we cannot formally exclude the possibility that FoxO4 could affect the Tat mRNA steady state through the modulation of cellular post-transcriptional processes that indirectly affect mRNA stability, such as 5’ capping or 3’ polyadenylation.

**The downregulation of Tat mRNA by FoxO is independent of the RNA interference (RNAi) pathway**

The control of mRNA degradation is an important part of the regulation of gene expression. During HIV-1 infection, micro RNA (miRNA)-mediated degradation of viral RNAs has been demonstrated to affect viral replication [53, 55–57]. In addition, FoxO1 was reported to regulate the expression of a microRNA cluster on the X chromosome [58], which suggests that there is a contribution from the RNA interference (RNAi) pathway in FoxO-mediated downregulation of Tat mRNA. To test this possibility, we examined the consequences of the knockdown of two endogenous proteins that are required for miRNA-mediated RNA silencing through the RNAi pathway, the dsRNA-specific ribonuclease Dicer and the human DEAD-box protein Rck/p54 [59–62]. HeLa cells were transfected with siRNAs previously described to have the ability to specifically silence Rck/p54 and Dicer protein expression [63]. Forty hours after the siRNA transfection, the cells were again transfected with Tat–HA and either the FLAG–FoxO4 or the FLAG–FoxO1 expression vector. As shown in Fig. 6(a, b), the knockdown of Rck/p54 or Dicer had no effect on the ability of FoxO1 and FoxO4 to downregulate Tat mRNA, indicating that an alternative mechanism of RNA catabolism could control Tat mRNA stability.

**The downregulation of Tat mRNA by FoxO4 is independent of the exosome pathway**

The mammalian RNA exosome complex is a highly organized system that plays a central role in the processing and degradation of various types of cellular RNA [64]. Recent findings have revealed its involvement in targeting viral RNAs for degradation, thereby restricting virus infections [65]. In particular, the zinc-finger antiviral protein ZAP has been shown to inhibit Moloney murine leukemia virus, Sindbis virus and HIV-1 replication by selectively targeting viral mRNAs for degradation by the exosome machinery [54, 66]. To test whether FoxO4 could affect Tat mRNA stability through the RNA exosome machinery, HeLa cells were transfected with siRNAs specific for Exosc4/hRrp41, a core structural component essential for exosome-mediated mRNA degradation [54, 67]. The knockdown efficiency of Exosc4/hRrp41 was validated by Western blot (Fig. 7a). The cells were then transfected again with Tat–HA and various amounts of FLAG–FoxO4. As shown Fig. 7(b, c), the depletion of Exosc4/hRrp41 had no effect on the ability of FoxO4 to repress both Tat protein and mRNA expression. Taken together, these data indicated that FoxO factors could control Tat expression through an unrelated mechanism of mRNA suppression.
FoxO4 expression antagonizes HIV-1 transcription reactivation in J-Lat 10.6 cells

On the basis of our results, we hypothesized that FoxO factors could negatively control the silencing of HIV-1 provirus transcription through the downregulation of Tat mRNA. Therefore we addressed whether overexpression of FoxO4 could antagonize provirus transcription reactivation in a well-established model of latently infected cells, the Jurkat J-Lat 10.6 cell line [10]. These cells harbour an HIV-1 provirus containing the green fluorescent protein (GFP) ORF instead of the nef gene. HIV-1 LTR reactivation in response to tumour necrosis factor alpha (TNF-α) or phorbol myristate acetate (PMA) can be monitored by flow cytometry analysis of GFP (Fig. 8a) [10]. Since TNF-α and PMA are potential activators of the serine/threonine kinase Akt pathway, which leads to the phosphorylation of FoxO proteins on three conserved residues, leading to their nuclear export and inactivation, J-Lat 10.6 cells were transfected with a vector expressing a FoxO4 mutant protein on the three Akt phosphorylation sites (FoxO4-TM), which were constitutively active and refractory to negative inhibition [34, 40]. Twenty-four hours after transfection, the cells were treated for 16 h with 10 ng ml⁻¹ of TNF-α or 10 nM of PMA, and the percentage of GFP positive living cells was quantified by flow cytometry. As a negative control, the cells were transfected with the empty pcDNA.3 vector. In these experimental conditions, the transfection efficiency in the Jurkat cells was about 45 %, as determined using the pmaxGFP vector from Amaxa (data not shown). As shown in Fig. 8(b), the expression of FoxO4-TM appeared to strongly reduce the percentage of GFP-expressing cells induced after TNF-α treatment (48.1 % with FoxO4-TM versus 68.4 % with pcDNA.3, see gate P2). Conversely, the number of GFP-negative cells increased by similar percentages (45.3 % with FoxO4-TM versus 27.2 % with pcDNA.3, see gate P1). In addition, although the displacement of the peak of GFP-positive cells seems modest, due to the logarithmic scale, the relative mean fluorescent intensity of the GFP-positive cells (Gate P2) was reduced by 25.03 % in FoxO4-TM cells (3699), as compared with control cells (4934). Similarly, FoxO4-TM significantly diminished the magnitude of HIV-1 reactivation after PMA stimulation (Fig. 8b). These data strengthen our hypothesis concerning the role of FoxO factors in the negative control of HIV-1 transcription.

DISCUSSION

Although latently HIV-1-infected CD4+ T cells are rare, they represent a major public health problem, because they persist even when viral replication is inhibited during antiretroviral therapies [1, 2]. The ability of resting memory CD4+ T cells to reactivate upon antigenic stimulation, such as from proinflammatory cytokines, provides favourable conditions for the reactivation of HIV-1 replication. The connection between HIV-1 transcription repression and the resting T cell state suggests that the host transcription factors involved in the active maintenance of the lymphocyte quiescence are likely to repress the viral transactivator Tat, thereby restricting HIV-1 transcription. Because FoxO factors play an important role in the maintenance of quiescent lymphocytes and are repressed during lymphocyte activation, they are good candidates for silencing HIV-1 transcription [18, 27].
In this study, we identified FoxO factors as cellular repressors of Tat-mediated HIV-1 promoter transactivation through a not yet reported post-transcriptional mechanism. We showed that overexpression of FoxO1 or FoxO4 led to strong inhibition of Tat protein expression through specific post-transcriptional suppression of Tat mRNA. Interestingly, siRNA-mediated depletion of FoxO1 or FoxO4 resulted in higher levels of Tat expression, indicating that endogenous FoxO is involved in the control of the steady-state level of Tat expression. During T-cell activation, the FoxO factors are regulated by protein kinase B (PKB/c-Akt)-mediated phosphorylation, resulting in their nuclear exclusion and inactivation [68]. Considering the fact that during the establishment of a latent infection by HIV-1 the reversion of infected CD4+ T cells to a resting memory state is associated with the nuclear translocation and activation of FoxO factors, our findings provide the first evidence for the role of FoxO factors in the repression of HIV-1 transcription, probably during the first step of acute infection [2, 69]. Indeed, it is obvious that numerous cellular factors are required to achieve an optimal block of the initiation and elongation of HIV-1 transcription. HIV-1 gene expression is largely extinguished, essentially because the transcription from the HIV-1 LTR promoter is heavily dependent on host transcription factors that are inactivated or activated in resting memory CD4+ T cells, such as the NF-κB or FoxO factors, respectively. In this context, the repression of Tat expression by FoxO factors early during the reversion of CD4+ T cells to resting memory cells might contribute to the transcriptional silencing of integrated HIV-1 proviruses, allowing a complete block of HIV-1 replication by mechanisms such as chromatin remodelling and epigenetic modifications. Consistent with this, using the J-Lat 10.6 model of latently infected cells, we demonstrated that the overexpression of a constitutively active FoxO4-TM mutant antagonized HIV-1 transcription reactivation in response to T cell activators, such as TNF-α or PMA. During the course of this study, HIV-1 suppression of FoxO1
activity was proposed to be a viral strategy to promote replication in memory-resting CD4+ T cells [70]. In addition, the authors showed that the FoxO1 inhibitor AS1842856 accelerates de novo viral gene expression, supporting the notion that FoxO1 can maintain silent transcription of HIV-1 through the downregulation of viral gene expression. In this context, our study provides the first evidence that FoxO factors can control HIV-1 replication through a post-transcriptional mechanism that brings to a strong decrease in the abundance of Tat-encoding mRNA.

We have previously shown that complex functional cross-talk between the HTLV-1 oncoprotein Tax and FoxO4 was crucial for Tax-mediated T-cell signalling reprogramming during HTLV-1 acute infection [37]. We demonstrated that Tax-mediated dose-dependent FoxO4 signalling deregulation may act as a trigger to initiate the long-term proliferation and transformation of infected cells [37]. So, it is tempting to speculate that functional cross-talk between Tat and FoxO factors can alternatively direct the cells towards a long-term proliferation or a resting state. In addition, we previously showed that FoxO4 triggers the activation of the innate immune signalling pathway in the coupling recognition of viral pathogen-associated molecular patterns for the downstream production of type I IFN, indicating that HIV-1 can take advantage of the component of the innate immune response to establish latent infection [32]. The loss of Tat expression by FoxO factors should likely enable latently infected cells to escape the host immune system, providing long-lasting living viral reservoirs that are protected from immune clearance and antiviral drugs. Finally, HIV-1 infection and Tat protein have been shown to activate phosphatidylinositol 3-kinase/AKT-dependent survival pathways associated with FoxO nuclear exclusion, suggesting that a subtle balance between Tat and FoxO factors can alternatively direct the cells towards virus-producing or latent-infected state [71–73].

Although both proteasome-dependent and proteasome-independent mechanisms for Tat proteolysis were reported to be critical determinants of its activity, the inhibition of the ubiquitin/proteasome, lysosomal or caspase-mediated proteolysis pathways did not affect the downregulation of Tat protein expression, suggesting that FoxO factors could repress Tat expression by promoting Tat mRNA post-transcriptional suppression. In agreement with this, we observed that FoxO expression induced a dose-dependent repression of Tat mRNA expression without affecting CMV-driven transcription. In addition, The Tat mRNA half-life is strongly reduced by FoxO4 expression, demonstrating that FoxO mainly triggered Tat mRNA post-transcriptional suppression. Mechanistically, various RNA catabolism

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**Fig. 6.** The post-transcriptional suppression of Tat mRNA by FoxO is independent of the RNA interference (RNAi) pathway. HeLa cells were transfected with either siRNA targeting Dicer (si-Dicer), RCK/p54 (si-RCK/p54), or a non-targeting negative control siRNA (scramble: si-Scr). 48 h after transfection, the cells were again transfected with 100 ng Tat–HA expression vector with and without 400 ng of FLAG–FoxO4 or FLAG–FoxO1 expression vector. (a) Whole-protein extracts were prepared from 10% of cells and analysed by immunoblot using anti-HA, anti-FLAG and anti-tubulin-α antibodies. (b) Subsequently, the total mRNA was purified and the level of Tat mRNA was quantified by Northern blot analysis. The levels of endogenous GAPDH mRNA were used as an invariant control.
pathways were described to affect the stability of the HIV-1 mRNA. In particular, cellular and viral micro-RNAs have been shown to restrict the translation of HIV-1mRNA by targeting mRNA to the miRNA-induced silencing complex [53, 74]. In the same way, the IFN-induced ZAP protein was reported to inhibit HIV-1 infection by recruiting both the 5' and 3' mRNA degradation exosome machinery to selectively target multiply spliced viral mRNAs for degradation [54]. Using siRNA-mediated depletion of endogenous proteins required for miRNA silencing, Dicer and RCK/p54 and the core exosome components, hRrp41, we demonstrated that neither of these two main mechanisms was involved in the downregulation of Tat mRNA by FoxO4 and FoxO1, suggesting that another cellular ribonuclease mediated Tat mRNA degradation. Interestingly, the expression of Vif and Vpr, whose part of the RNA sequences, including the 3’ and 5’ ends, is identical to that of Tat mRNA, were not influenced by FoxO4. These data strengthen the idea that FoxO4 recognized specific secondary structures of Tat mRNA, leading to their specific degradation. However, we cannot formally exclude the possibility that FoxO4 could affect the Tat mRNA steady state through the modulation of cellular post-transcriptional processes that indirectly affect mRNA stability, such as 5' capping or 3' polyadenylation.

Interestingly, evidence suggests that the ZAP protein works in concert with other IFN-induced genes to confer maximal protection against alphavirus infection [54, 75]. Tat protein has been shown to bind with a specific set of human mRNAs in T cells, which suggests that Tat may interfere with RNA coding for proteins whose activity affects the virus life cycle, providing a selective advantage to the virus [76]. Among these mRNA, one encodes for ISG20, a 3' to 5' exoribonuclease induced by interferon, which exhibits strong antiviral activities against various RNA viruses, including hepatitis A and C viruses, vesicular stomatitis virus, influenza virus, encephalomyocarditis virus and HIV-1 [77–81]. Furthermore, we previously reported that ISG20 is associated with macromolecular nuclear complexes required for the biogenesis of various small nuclear ribonucleoproteins, which suggests that it is involved in the maturation or catabolism of cellular RNAs [82]. Altogether, these data suggested that Tat mRNA could be a target for ISG20 exonuclease activity. Despite the fact that we observed that ISG20 expression induced the repression of Tat mRNA expression, we failed to determine its involvement in the mechanism of action of FoxO4 using the Exo II ISG20 dominant-negative mutant [77, 79]. Because human genomes encode a plethora of ribonucleases, often with overlapping activities, identifying the ribonucleases involved in the mechanism of action of FoxO4 will be difficult and will require the development of high-throughput screening [83].

As they are obligatory intracellular parasites and use cellular biosynthetic machinery to replicate, viruses need to maintain cell viability to a sufficient degree for them to be able
replicate or establish persistent infections. Evolution has selected both viruses for growth, despite the antiviral defence set-up, and specific recipient cells for a cellular environment that is favourable to both infections and host survival. In this context, our data provide the first demonstration that FoxO-mediated Tat mRNA post-transcriptional suppression may be required for the establishment of viral latency by HIV-1.

**METHODS**

**Cell culture, transfections and luciferase reporter assay**

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; GibcoBRL) supplemented with 10% foetal bovine serum (FBS) and antibiotics. Human Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% FBS. The following reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: J-Lat full-length clone 10.6 (041214) from Dr Eric Verdin [10]. Transient transfection experiments were performed in HeLa cells using the calcium phosphate method or jetPei reagent according to the manufacturer’s instructions (Polyplus-transfection). J-Lat cells were transfected by electroporation in Ingenio electroporation solution transfection reagent (Mirus) using the Nucleofactor 2b device from Amaxa (program X-005) according to the manufacturer’s instructions (Lonza). For each experimental condition, an equal amount of DNA was transfected into the cells with empty vectors to balance the total amount of DNA. The percentage of GFP-positive living cells was quantified by flow cytometry analysis in a BD FACS Canto (Becton Dickinson), and the data were processed with FlowJo software. The luciferase activity was measured with a luciferase reporter gene assay (Roche Diagnostics) in a Berthold Technologies luminometer. Each experiment was performed in duplicate and the standard deviations corresponded to two distinct experiments.

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**Fig. 8.** FoxO4-TM inhibits TNF-α- and PMA-induced HIV-1 transcription in the J-Lat 10.6 cell line. (a) J-Lat 10.6 cells were stimulated for 16 h with 10 ng ml⁻¹ of TNF-α or 10 nM of PMA and the percentage of GFP-positive living cells was quantified by flow cytometry. (b) J-Lat 10.6 cells were transfected with either a vector expressing FLAG–FoxO4-TM protein (FLAG–FoxO4-TM) or an empty pcDNA.3 vector (pcDNA.3). 24 h post-transfection the expression of FLAG–FoxO4-TM was verified by immunoblot using anti-FLAG antibody. Then, the cells were treated with 10 ng ml⁻¹ of TNF-α or 10 nM of PMA and the percentage of GFP-positive living cells was quantified by flow cytometry and processed with FlowJo software. Typical results from four independent experiments are presented. The percentage of GFP-positive (gate P2) and GFP negative (gate P1) living cells is indicated on the histograms and in the table.
Plasmid constructs

The pMT2–HA–FoxO4 and pGLO–FLAG–FoxO4, and shRNA–FoxO1 and shRNA–FoxO4 expression vectors were kindly provided by Dr B. M. Burgering (University Medical Center Utrecht, The Netherlands). The pMXs-puro–FLAG–FoxO4–TM was kindly provided by Dr H. Matsuzaki (The Salk Institute, La Jolla, USA). The FLAG–FoxO4–TM fragment was subcloned in the pcDNA-3 vector to generate the pCDNA-3–FLAG–FoxO4–TM expression vector. The plasmid expressing Tat (BH10 clone; 101 amino acids) fused to the FLAG tag (pcDNA3.1/Tat–FLAG) was a gift from M. Benkirane (Institut de Génétique Humaine, France). The pcDNA3–FLAG–FoxO1 plasmid (Addgene plasmid #13507) was a gift from Kunliang Guan [84], and the pcDNA3–Tat–HA plasmid (Addgene, plasmid #14654) was a gift from Matja Peterlin [85]. The pcDNA3–FLAG–Vpr and pcDNA3–FLAG–Vif plasmids were gifts from L. Expert (CPBS, Montpellier, France). The HIV–1–LTR–5′–Luc and HIV–1–LTR–5′–ATAR–Luc reporter plasmids were kindly provided by Dr S. Nisole (Institut Cochin, France).

siRNA sequences and siRNA transfection protocol for knockdown in HeLa cells

The si-scramble, si-FoxO4: CCAGGTATAGAGAAAGGTC TA, si-FoxO1: AACCAAGTAGCGCTTGATACAA and si-EXOSC4: ACGCTCCACAGTGTATCTA (Qiagen) were transfected at 10 nM with INTERFErin, according to the manufacturer’s reverse-transfection protocol instructions (Polyplus-transfection). The cells were incubated for 48 h at 37 °C and then transfected with the plasmid expressing Tat under the CMV promoter (Tat–HA) with FoxO4 or FoxO1 with jetPei reagent (Polyplus-transfection). Forty-eight hours later the cells were lysed and analysed by immunoblot.

Total RNA isolation, Northern blot and reverse transcriptase polymerase chain reaction (RT–PCR)

Total RNA was extracted using the TRI Reagent solution (Molecular Research Center, Inc) and analysed by Northern blot using DIG High Prime DNA Labeling and Detection Starter kit II (Roche) according to the manufacturer’s instructions. For RT–PCR, total RNA was purified using the High Pure RNA Isolation kit (Roche) and Tat mRNA was amplified with the forward ATGGAGCCAGTAGCTCC TAG and reverse CTCCGCCTCTCTGCTGAT primers. GAPDH mRNA was amplified with the forward CACCACTGCAGCAC and reverse CCCTGGTGGTGCAT primers. The PCR products were subjected to electrophoresis on 1.6 % agarose gels containing ethidium bromide. The levels of Tat mRNA were normalized to the GAPDH endogenous gene level. Stained gels were captured using a digital camera and the band intensity was quantified using ImageJ Analysis Software.

Western blotting analysis

Cells were resuspended in loading buffer (10 mM Tris–HCl pH 6.8, 1 % SDS, 5 mM EDTA and 50 % glycerol) and incubated for 5 min at 95 °C. The proteins were fractionated on SDS-PAGE and transferred onto a PVDF membrane. Protein extracts were analysed by immunoblot using monoclonal mouse anti-FLAG M2, anti-HA, anti-tubulin-α (Sigma), and the rabbit polyclonal antibodies anti-FoxO4 (Bethyl laboratories.Inc) and anti-FoxO1 (Cell Signaling). The anti-hRrp41 antibody was kindly provided by Dr G. Pujol (University of Nijmegen, The Netherlands). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, the blots were revealed using the Western Lightning TM Plus-ECL reagent (PerkinElmer) or the SuperSignal West Pico detection kit (Pierce).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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