A siRNA screening of UBE2 family demonstrated that UBE2R1 had a high repressive effect on HIV Tat protein

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ARTICLE INFO	ABSTRACT
Keywords: HIV Tat Transcription elongation UBE2R1 RNF20	HIV Tat is an essential protein required for the transcription elongation of HIV genome. It has been shown that Tat can be degraded by either proteasome or autophagy pathways. In this study, it was shown that proteasome inhibitor MG132 could significantly prevent HIV Tat protein degradation in Tat over-expressing HeLa cells but it had a moderate effect in preventing Tat protein degradation in Jurkat T cells. A screening of the available UBE2 siRNA family identified that UBE2R1 had a high repressive effect on Tat protein but not on Tat mRNA level. This study further showed that RNF20 might not be the E3 ligase of Tat but was required to maintain a high level of H2B-monoubiquitylation (H2Bub1) on HIV-1 genome for efficient elongation. Overall, our study indicated that UBE2R1 might be the potential ubiquitin E2 ligase for HIV Tat protein turnover and RNF20 regulated HIV expression in the transcription elongation level.

1. Introduction

HIV Tat recruits positive transcription elongation factor-b (P-TEFb) complex to stimulate RNA Polymerase II transcription elongation on HIV genome [1-3]. Thus, the proteins and compounds that can regulate Tat expression level are valuable target candidates in the pharmaceutical research of HIV/AIDS. Up-to-date, it has been shown that MG132, Didehydro-cortistatin A (dCA), Triptolide, Curcumin and JIB-04 can regulate Tat protein level [4-8]. MG132 was shown to prevent Tat protein from degradation, Triptolide and Curcumin were shown to promote Tat protein degradation [4,6,7], while dCA was reported to relocate Tat from nucleus to cytoplasm [5]. In our previous study, additionally, JIB-04 was shown to promote Tat degradation through the autophagy pathway [8]. To sum up, these publications suggest that Tat protein can be degraded through either proteasome or autophagy pathways in different cell lines.

Although MG132 has long been shown to prevent Tat from degradation in Tat over-expressing cancer cells [4], the cellular ubiquitin ligase E2 (UBE2) and E3 for Tat protein turnover have not been well characterized. The major degradation pathway for Tat in T cells seems to

be the autophagy pathway rather than the proteasome pathway [8]. RNF20 has also been shown to involve in HIV Tat transcriptional activation [9], with the molecular mechanisms to be uncovered. RNF20 is the E3 ligase for H2B monoubiquitylation [10] and H2Bub1 modification is required for efficient transcription elongation [11]. In this study, it was shown that proteasome inhibitor MG132 could significantly prevent HIV Tat protein degradation in Tat over-expressing HeLa cells but it had a moderate effect in preventing Tat protein degradation in Jurkat T cells. A screening of the available UBE2 siRNA family identified that UBE2R1 had a high repressive effect on Tat protein. Furthermore, it was shown that RNF20 knockdown reduced Tat protein level and RNF20 was required to maintain a high level of H2Bub1 on HIV-1 genome for efficient elongation. Overall, this study suggested that UBE2R1 might be the potential ubiquitin E2 ligase for HIV Tat protein turnover and RNF20 could regulate HIV transcription through H2B monoubiquitylation.

2. Materials and methods

2.1. Antibodies, inhibitors and western blotting

Specific antisera were obtained from the following sources: HIV-1 Tat (ab42359 & ab43014), Csn3 (sc-100693), Cyclin T1 (sc-10750), SHMT1 (ProteinTech, 14149-1-AP), UBE2R1 (ProteinTech, 10964-2-AP), UBE2Z (R&D, AF8154), RNF20 (ProteinTech, 21625-1-AP), H2B (Millipore, 07–371), H2Bub1 (Active Motif, 39624). Doxycycline was purchased from Sigma (D3447), while MG132 and JIB-04 were purchased from Selleckchem (S2619 & S7281). Western blotting was previously described [12].

2.2. 2D10 cell culture, RNAi and induction of HIV-1 in 2D10 cells

The detailed protocols had been described [8]. 2D10 Jurkat T cells (A gift of Dr. Jonathan Karn Lab to Dr. Katherine A. Jones lab) were cultured using RPMI-1640 medium plus 10% FBS and 1% antibiotic-antimycotic solution. Stemfect RNA transfection kit (Stemgent, 00–0069) was used for RNAi in 2D10 T cells. Recombinant TNFα was used to induce HIV-1 expression.

2.3. HeLa cell culture, RNAi, Dual-luciferase assay and rt-PCR

The detailed protocols had been described [8]. Tet-on-Tat-off HeLa cells and HeLa P4 cells (Dr. Katherine A. Jones lab) were cultured by DMEM medium plus 10% FBS and 1% antibiotic solution. RNAi with final 100 nM siRNA was tranfected by Lipofectamine 2000. Forty-eight hours after transfection, cells were lysed and analyzed by Dual-Luc assay and Western blotting. The sequences of siRNAs for UBE2 family were as follows:

Target	Sense	anti-Sense:
UBE2A	GAACAAACGGGAAUAUGAAtt	UUCAUAUUCCCGUUUGUUCtc
UBE2B	GGAACAAACGAGAAUAUtt	AUAUUCUCGUUUGUUUUCCtg
UBE2C	CCUACUCAAAGCAGGUCACtt	GUGACCUGCUUUGAGUAGGtt
UBE2D3	CCUAAUGACAGCCCAUAACtt	GAUAUGGGCUGUCAUUAGGtc
UBE2E1	CAAAGGCGAUAACAUCUAUtt	AUAGAUGUUAUCGCCUUUGgg
UBE2F	GGACUUCCGGAAUAAAGUGtt	CACUUUAUUCCGGAAGUCCtc
UBE2G1	GGUUUAAUAGAUGACAAUGtt	CAUUGUCAUCUAUUAAACCtg
UBE2R1	GGAUCGGGAGUACACAGACtt	GUCUGUGUACUCCCGAUCCtt
UBE2Z	CCUCGGGUCAAACUGAUGAtt	UCAUCAGUUUGACCCGAGGtg
RNF20	CUCUUAUCCCGGAAGCUAAtt	UUAGCUUCCGGGAUAAGAGct
Control N/A 4390844, SilencerR-select Negative Control#1		

Primers for qRT-PCR:

Tat	GAGCCAGTAGATCCTAGACTA	GAGATGCCTAAGGCTTTTGTC
UBE2A	CCTGCTTCTCCAGCCTCTTC	CCTCTTGAAGTCCCGCATGA
UBE2B	AGCAGAGAACTGATGCGACT	CAGCAGGCCCTGGCATTAAA
UBE2C	CGAGTTCCTGTCTCTCTGCC	CAGCTCCTGCTGTAGCCTTT
UBE2D3	TCCGGTTCTCGATGCTTCTC	CGTTTCAGCGCCATAGTGTG
UBE2E1	TCTCCCAGCGAGAGAGAACAC	GGGTCTAAAGTGATGTCCGCC
UBE2F	GACCGGGCATGGTGTTGG	ACCATCGTCACGCTTCAGTT
UBE2G1	TCCCCTCCGACCTCCTAAAA	CCATTAGGGTCTGCCAGCAT
UBE2R1	ACATCATCCGGAAGCAGGTC	CATCCTCATCGTCCCCGAAG
UBE2Z	GACAGACCTTCATGGGAGCC	GGAGCTTGCTTGGTTCTTGC

Chromatin Immunoprecipitation (ChIP).

The ChIP primers and protocols had been described [8,13]. Briefly, formaldehyde was added for cross-linking at 1% final concentration. About 0.5–2 μg of antibodies were used for ChIP. High salt at 5 M NaCl was added for reverse-crosslinking. At last step, DNA was purified with Qiagen quick-spin PCR Purification Kit and eluted in H2O.

3. Results

3.1. Proteasome inhibitor MG132 could significantly prevent HIV Tat protein degradation in Tat over-expressing HeLa cells but it had a moderate effect in preventing Tat protein degradation in Jurkat T cells

As previously reported, MG132 can prevent HIV Tat protein degradation [4]. Doxycycline was washed off from the Tet-on-Tat-off HeLa cells integrated with HA-Tat 86 for 16 h, and then the cells were treated with JIB-04 or MG132 for 8 h as indicated (Fig. 1A). The results showed that 1 μM of JIB-04 could greatly reduce Tat protein level [8], while MG132 had the opposite effect to prevent Tat protein from degradation in this Tat over-expressing physiological-unrelevant HeLa cells (Fig. 1A). For 2D10 Jurkat T cells, however, titrating MG132 up-to 6 h only slightly increased Tat protein level. Thus, it was indicated that Tat might be mainly degraded through autophagy pathway in physiological-relevant T cells, compared to both autophagy and proteasome pathways in Tat over-expressing HeLa cells (Fig. 1B). Our data suggested that Tat protein could be degraded through either autophagy or proteasome pathways but it might be mainly degraded by autophagy pathway in T cells.

3.2. A siRNA screening of UBE2 protein family showed that UBE2R1 had a high repressive effect on Tat protein level

To degrade Tat through the autophagy pathway in T cells, K63-type ubiquitin needs to be added by the E2 and E3 ubiquitin ligases to the Tat protein. Luckily, there are only about 20 ubiquitin E2 ligases in the cells. Therefore, we used all the available UBE2 family siRNAs in stock, including siRNAs to UBE2A, UBE2B, UBE2C, UBE2D3, UBE2E1, UBE2F, UBE2G1, UBE2O, UBE2R1 and UBE2Z (See Materials and Methods for the sequences), and performed a siRNA knockdown screening of UBE2 protein family to see which UBE2 had the most repressive effect on HIV Tat protein. The knockdown efficiency of each siRNA of the UBE2 protein family was all above 80% in mRNA level (Supplemental Fig. 1A). Moreover, the efficient knockdown in the protein level was shown by antibody targeting UBE2Z (Fig. 2A). Among all the UBE2 siRNA tested, UBE2R1/CDC34 knockdown had increased Tat protein level most significantly (Fig. 2A), suggesting that UBE2R1 might be a direct ubiquitin E2 ligase for Tat. Importantly, UBE2R1 siRNA was singled out and its knockdown effect was tested on Tat protein again in both HeLa and 2D10 T cells. The results confirmed that UBE2R1 knockdown could also greatly increase Tat protein level (Fig. 2B and C), indicating that UBE2R1 might also play an important role in the Tat-autophagy degradation pathway in T cells. Intriguingly, the mRNA of Tat only slightly increased when UBE2R1 was knocked down (Supplemental Fig. 1B), manifesting that UBE2R1 might regulate either the stability or translation level of Tat protein. In summary, these data showed that UBE2R1 might be a direct ubiquitin E2 ligase for Tat protein and further experiments are needed to explore this possibility.

3.3. Knockdown of RNF20 reduced Tat protein level and RNF20 was required to maintain a high level of H2Bub1 on HIV1 genome for efficient elongation

Considering the involvement of RNF20 in HIV Tat transcription [9], we tested the effect of RNF20 knockdown on Tat protein, and found that RNF20 knockdown reduced Tat protein level in 2D10 T cells (Fig. 3A), which was opposite to the effect of UBE2R1 on Tat. The result suggested that RNF20 might regulate HIV1 Tat through a distinct molecular mechanism. RNF20 has previously been shown to be the E3 ligase of H2B monoubiquitylation [10] and H2Bub1 is required for the efficient transcription elongation [11]. Consistently, our study showed that RNF20 knockdown reduced H2Bub1 protein level (Fig. 3A). Next, ChIP assay was performed to analyze the binding of RNF20 and H2Bub1 on HIV-1 genome. The efficient HIV-1 transcription elongation happened
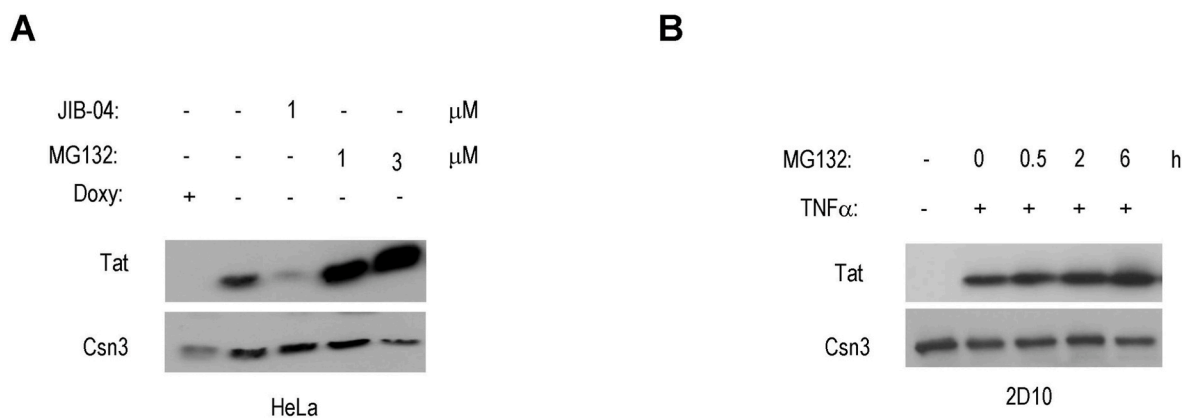
A   **B**

Fig. 1. Analysis of MG132 and JIB-04 on HIV Tat protein in HeLa and Jurkat T cells by Western blotting. **A.** The effect of 1 μM JIB-04 and 1-3 μM of MG132 on Tat protein level in Tet-on-Tat-off HeLa cells tested by Western blotting. Tat protein level was indicated by Tat antibody and Csn3 served as loading control. **B.** The effect of MG132 on 2D10 T cells activated by 10 ng/ml TNFα tested by Western blotting. MG132 was treated from 0 to 6 h. Tat protein level was indicated by Tat antibody and Csn3 served as loading control.
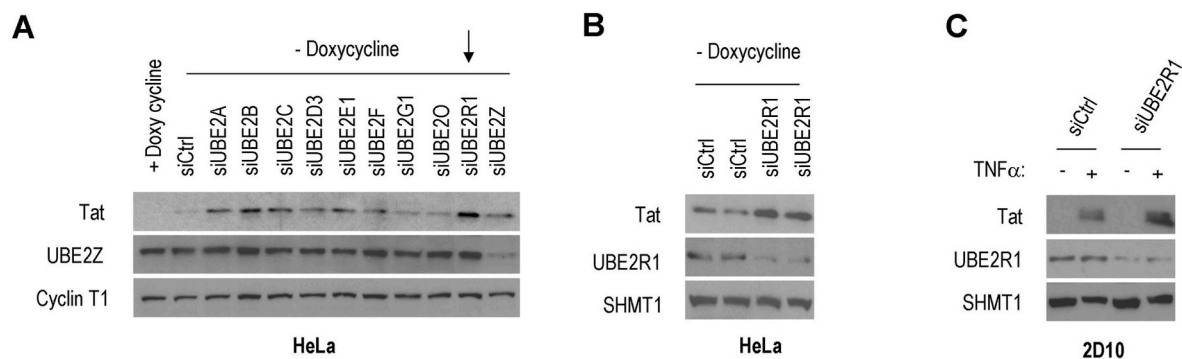

Fig. 2. The high repression effect of UBE2R1 on Tat protein was shown by a UBE2 family siRNA screening and Western blotting
A. The effect of indicated UBE2 family siRNA on Tat protein in Tet-on-Tat-off HeLa cells tested by Western blotting. UBE2Z band intensity was indicated by UBE2Z antibody. Cyclin T1 served as loading control. **B.** The effect of knockdown UBE2R1 by siRNA on Tat protein in Tet-on-Tat-off HeLa cells tested by Western blotting. The knockdown efficiency of UBE2R1 was indicated by the UBE2R1 antibody and SHMT1 served as loading control. **C.** The effect of knockdown UBE2R1 by siRNA on Tat protein in 2D10 T cells tested by Western blotting. The knockdown efficiency of UBE2R1 was indicated by the UBE2R1 antibody and SHMT1 served as loading control.

about 6 h after TNFα induction [8]. The result of ChIP showed that RNF20 and H2Bub1 were gradually enriched on HIV-1 genome during 0-6 h of TNFα induction, and the binding patterns of RNF20 and H2Bub1 were highly consistent with each other (Fig. 3B, top). The results indicated that RNF20 was needed to maintain a high level of H2Bub1 on HIV-1 genome for the efficient transcription elongation. Furthermore, RNF20 knockdown reduced the H2Bub1 level on HIV-1 genome (Fig. 3B, bottom), demonstrating that RNF20 was critical to maintain the efficient transcription elongation of HIV-1 genome. Overall, our results showed that although RNF20 might not be the E3 ligase of Tat, it was required to maintain a high H2Bub1 level on HIV-1 genome for efficient elongation.

4. Discussion

HIV-1 Tat is the essential protein that is required to support the efficient viral transcription elongation through recruiting the P-TEFb complex [14]. In our previous study, it was shown that Tat could be K63-ubiquitylated and degraded by JIB-04 through the autophagy pathway in Jurkat T cells [8]. Besides, it have been shown that Tat can also be degraded by the Ubiquitin Proteasome System (UPS) degradation pathway [4,6,7]. However, the ubiquitin E2 and E3 ligases for Tat have not been uncovered. UBE2O has been shown to interact with HIV Tat protein to reorganize the 7SK snRNP for transcription activation [15]. Our siRNA screening results showed that the knockdown of

UBE2R1 had a much greater effect in increasing HIV Tat protein level than those of other UBE2 ligases (Fig. 2A). It had been shown that UBE2R1/Cdc34 was a lysine48-specific E2 ligase and it could function to add ubiquitin without the downstream E3 ligase [16,17]. Thus, it would be very interesting and important to further investigate how over-expression of UBE2R1 affects Tat protein level and whether UBE2R1 is the direct ubiquitin E2 ligase that does not need a E3 ligase. Furthermore, UBE2R1 knockdown also increased Tat protein level in 2D10 Jurkat T cells (Fig. 2C), indicating that lysine48-specific proteasome pathway might also play an important function in T cells.

The cellular ubiquitin E3 ligase protein CHIP was recently shown to be likely the E3 ligase for HIV-1 Tat protein [18], suggesting that there might be multiple E2 ligases for Tat protein in cells. Besides CHIP, PJA2 has been shown to ubiquitinate the HIV-1 Tat protein with atypical chain linkages to activate viral transcription, Cullin 3 E3 ligase can also regulate HIV-1 Transcription [19,20], and RNF20 is involved in HIV transcription [9]. Subsequently, RNF20 was shown to be the ubiquitin E3 ligase for H2Bub1, and H2Bub1 was required to maintain the efficient transcription elongation [10,11]. Therefore, we wanted to explore the effects of RNF20 on the transcription elongation of HIV-1 genome. It was shown that RNF20 knockdown reduced HIV Tat level, and additionally, ChIP binding patterns of RNF20 and H2Bub1 to HIV genome were highly overlapping with each other. Our observations support the notion that RNF20 is required to maintain high H2Bub1 on HIV-1
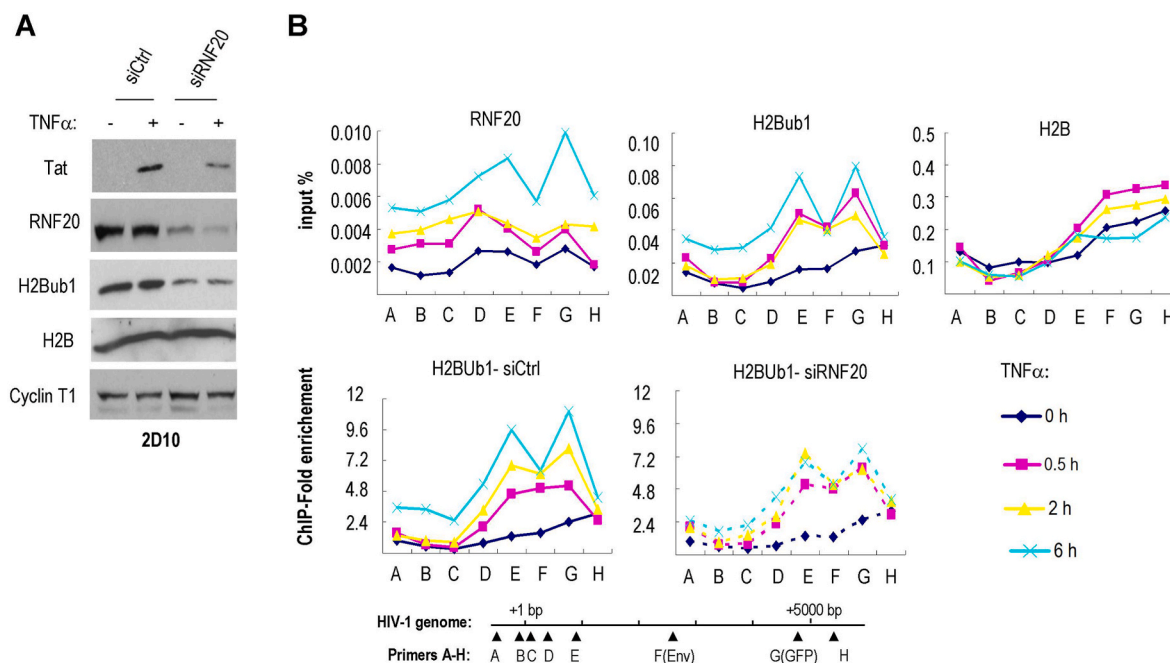
Fig. 3. Western blotting showed that RNF20 knockdown reduced Tat protein level and Chromatin-Immunoprecipitation (ChIP) with antibodies targeting RNF20, H2Bub1 and H2B showed that RNF20 was required to support the efficient transcription elongation of HIV genome
A. The effect of RNF20 knockdown on Tat and H2B-monoubiquitylation (H2Bub1) was demonstrated by Western blotting using 2D10 T cells. H2B and Cyclin T1 served as loading controls. **B.** Top, Chromatin-Immunoprecipitation (ChIP) of RNF20, H2Bub1 and H2B on the genome of HIV-1. ChIP was performed at the points of TNFα induction of HIV at 0 h (dark blue), 0.5 h (purple), 2 h (yellow) and 6 h (light blue). The locations of primers on HIV-1 genome were indicated by the diagram below. ChIP signals were normalized to input. Bottom, ChIP of H2Bub1 at the conditions of siCtrl or siRNF20, relative ChIP signals were normalized to fold-enrichments with location A signal manually set to 1-fold.

genome for efficient elongation. Since UBE2R1 and RNF20 are both ligases of ubiquitin with enzymatic activity, the development of inhibitors to such enzymes can potentially reactivate HIV from latency or inhibit HIV transcription. Either direction is of great interest to the field of HIV/AIDS drug discovery.

5. Conclusions

It was shown in this study that MG132 could prevent HIV-Tat protein from degradation more significantly in Tat over-expressing HeLa cells than in T cells. And it was likely that HIV Tat could be degraded through both proteasome and autophagy pathways. This study further showed that UBE2R1/CDC34 had a high repression effect on Tat protein, and RNF20 was required to support HIV transcription elongation by maintaining a high level of H2Bub1. The molecular mechanisms of how UBE2R1/CDC34 regulates HIV Tat protein level need to be further investigated.

Author contributions

Muyu Xu: Conceptualization, Methodology, Investigation, Data analysis, Writing, Co-Funding & Corresponding; **Jiying Zhang:** Writing, Editing, Reviewing & Proofing.

Declaration of competing interest

The authors have declared no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101366.

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