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Original Article

Adeno-associated virus Rep78 restricts adenovirus E1B55K-mediated p53 nuclear exportation

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Inactivation of p53 is needed during adenovirus type 5 DNA replication. E1B55K, an adenovirus early protein, has been reported to interact with p53 and inhibit p53 transactivation. Previous studies have shown that adeno-associated virus (AAV) type 2 could reduce the transforming potential of adenovirus by rescuing p53 from adenovirus-mediated degradation, but the details are not clear yet. We detected the Rep78–p53 interaction by co-immunoprecipitation assay. The co-localization assay revealed that Rep78 inhibits E1B55K-mediated p53 nuclear exportation. However, Rep78 did not detectably influence p53 stability and could not relieve the transcriptional inactivation of p53, as E1B55K could not be replaced from the p53–E1B55K complex by Rep78. Our results reveal a new possible mechanism that AAV-2 Rep78 inhibits adenovirus 5 by relocalizing p53 in the nucleus, which may shed some light on the regulatory mechanism of AAV-2 on its helper virus, adenovirus.

Keywords p53; Rep78; E1B55K; interaction

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Introduction

p53 is an important cellular transcription factor that regulates oncogenic transformation [1], cell cycle [2], and induction of apoptosis in response to stress and DNA damage [3]. An accumulation of p53 results in G1/S arrest or apoptosis [4]. Adenovirus type 5 replication requires cells to enter S phase [5]; therefore, it is important for adenovirus to inactivate p53-induced G1/S arrest. The protein E1B55K, an early adenoviral gene product, has been reported to form a complex with p53 and recruit p53 to the cytoplasm from the nucleus, thus inhibiting the transcriptional activity of p53 [6] and causing its ubiquitin-mediated degradation [7]. E1B55K can bind to the N-terminal transactivation domain of p53 [8]. Mutation of the p53 N-terminal hydrophobic residues Trp-23 and Pro-27 abolishes this interaction, but have no impact on the ability of the p53 protein to bind the p53-specific DNA element [9]. Moreover, E1B55K oligomerization and p53 tetramerization are required for the E1B55K–p53 interaction [10].

Adeno-associated virus (AAV) type 2 is a non-enveloped virus that carries a linear, single-stranded DNA genome. This virus becomes latent and cannot cause productive infection without the presence of a helper virus, such as adenovirus or herpes virus [11]. Rep78 is an important regulatory protein in all phases of the AAV life cycle, regulating DNA replication, virus integration, and gene expression. It is involved in reducing the transforming potential of other viruses such as adenovirus [12,13]. It has been reported that AAV could protect p53 from adenovirus-mediated degradation. When Rep78 was well expressed, p53 was rescued. In adenovirus and AAV co-infected cells, Rep78 and p53 could form a complex [14], but the precise mechanism of how AAV impacts E1B55K-mediated p53 inactivation is still unclear. In this study, we found that the AAV-2 protein Rep78 could restrict adenovirus 5 E1B55K-mediated p53 nuclear exportation, but it could not directly influence p53 stability and relieve the inhibition of p53 transcriptional activity.

Materials and Methods

Cell culture and transfection

p53-null H1299 and p53 wild-type U2OS cells were purchased from American Type Culture Collection (ATCC, Manassas, USA) and maintained in RPIM1640 medium (Gibco, Grand Island, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. DNA transfection was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) as described by the manufacturer.

Plasmid construction

The coding sequence of E1B55K and E4orf6 was cloned from the adenovirus 5 genome and was inserted into the

plasmid pCMV-Myc (Clontech, Mountain View, USA) between the *Xho*I and *Eco*RI sites in order to express Myc-E1B55K and Myc-E4orf6 fusion protein. RFP-E1B55K was generated by sub-cloning the E1B55K sequence into pDsRed-N1 (Clontech) between the *Xho*I and *Eco*RI sites. EGFP-p53 and pcDNA3.1-p53 plasmids were constructed by amplifying the open reading frame (ORF) of p53 from a human fetal brain cDNA library (Clontech), which was then inserted into pEGFP-C3 and pcDNA3.1 His-Myc(-), respectively, using *Xho*I and *Eco*RI. The ORF of Rep68 and Rep78 was amplified from the pRC plasmid (a generous gift from Dr H Buning of the Gene Center of the Ludwig-Maximilians University, Munich, Germany, which contains AAV-2 rep and cap genes controlled by their native promoters [15]) and then cloned into the pCMV-HA plasmid between the *Bgl*II and *Not*I sites, thereby generating HA-Rep68 and HA-Rep78. The plasmid RFP-Rep78 was generated by sub-cloning the Rep78 ORF into pDsRed-N1 between the *Xho*I and *Sma*I sites.

Immunoprecipitation and western blot analysis

For immunoprecipitation, cells were lysed with lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride, 0.5% protease inhibitor cocktail; Roche, Penzberg, Germany). The lysates were then immunoprecipitated with the indicated antibodies for 2 h at 4°C. Twenty microliters of pre-washed protein A/G Sepharose beads (Santa Cruz, Santa Cruz, USA) was added to each extract and shaken overnight. The beads were washed three times with lysis buffer and boiled in 2 × loading buffer. Protein samples were then separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, which was blocked in 5% skim milk in TBST and probed with the indicated antibodies. The following antibodies were used: anti-HA or anti-EGFP rabbit polyclonal antibodies and the mouse monoclonal antibodies anti-Myc or anti-p53 were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Sigma (St Louis, USA).

Confocal fluorescence assay

At 48 h post-transfection, H1299 cells were fixed with 4% paraformaldehyde and observed under a confocal fluorescent microscope (Leica, Heidelberg, Germany). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). The fluorescence of green fluorescent protein, red fluorescent protein and DAPI were observed at excitation wavelengths of 488 nm, 561 nm and 405 nm, respectively.

Cell fractionation

Nuclear and cytoplasmic protein extraction kit was purchased from Sangon Biotech (Shanghai, China). H1299 cells were transfected with the indicated plasmids. After 48 h post-transfection, 1×10^7 cells were collected and divided into nuclear and cytoplasmic fractions according to the standard protocol provided by the manufacturer. The anti-GAPDH (Millipore, Billerica, USA) and anti-LMNB1 (Proteintech, Chicago, USA) antibodies were used to confirm the purity of two fractions.

Dual-luciferase reporter assay

A luciferase construct under the control of the p53-dependent promoter (p53-TA-luc) (Clontech) was used to test the transcriptional activity of p53. U2OS cells were plated in 24-well plates and transfected with 500 ng of p53-TA-luc, 1 ng of SV40-Renilla luciferase, 10 ng of Myc-E1B55K, and 500 ng HA-Rep78 or empty vector. Then, cells were incubated for 48 h at 37°C, and the luciferase activity was measured according to the manufacturer's guidelines (Promega, Madison, USA).

Statistical analysis

The results are presented as the mean of three independent experiments, and statistical significance was evaluated using *t*-test. $P < 0.05$ was considered statistically significant.

Results

Rep78 interacts with p53

To test whether Rep78 could interact with p53, a co-immunoprecipitation assay was employed. H1299 cells were co-transfected with pcDNA3.1-p53 and HA-Rep68/78 or pCMV-HA. The mouse anti-p53 antibody was able to precipitate HA-tagged Rep78, while no interaction was observed in the empty vector transfected cells. Rep68 could not be detected in the co-immunoprecipitation assay either [**Fig. 1(A)**], which indicated the binding between Rep68 and p53 is weaker than that of Rep78 and p53. Thus, we further examined the subcellular localization of p53 and Rep78. H1299 cells were transfected with EGFP-p53 and RFP-Rep78 and imaged using a confocal fluorescent microscope. Both the red fluorescent Rep78 fusion protein and the green fluorescent p53 fusion protein were distributed throughout the nucleus [**Fig. 1(B)**], suggesting that they may interact with each other.

Rep78 inhibits the E1B55K-mediated nuclear export of p53

To investigate the subcellular distribution of p53 in the presence of E1B55K and Rep78, we fused EGFP to the N-terminus of p53 and detected its localization at 48 h post-transfection by confocal fluorescent microscopy [**Fig. 2(A)**]. The EGFP-p53 fusion protein was distributed throughout the nucleus when it was expressed alone, and in the presence of E1B55K, the subcellular localization of p53 changed from

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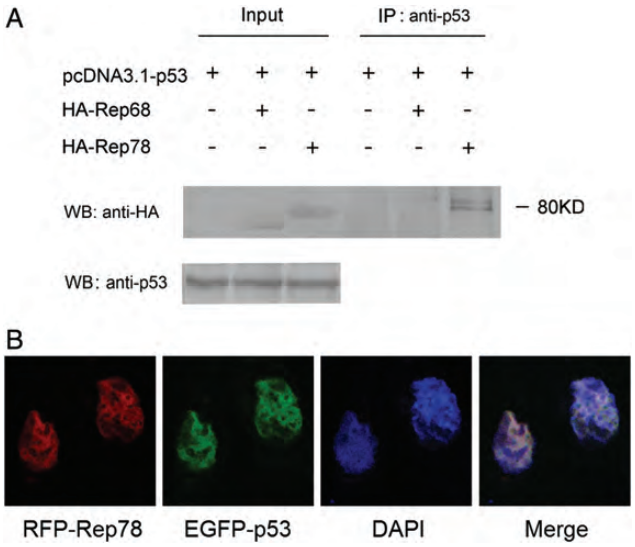

Figure 1 Interaction between Rep78 and p53 (A) Co-immunoprecipitation assay. H1299 cells were co-transfected with pcDNA3.1-p53 and HA-Rep68/78 or pCMV-HA. Mouse anti-p53 antibody was used for immunoprecipitation, and rabbit anti-HA antibody was used for western blot. The expression of p53 in input was also shown by an anti-p53 antibody. (B) Sub-cellular localization of Rep78 and p53. H1299 cells were co-transfected with EGFP-p53 and RFP-Rep78. The nuclei were stained with DAPI (×40).

the nucleus to the cytoplasm aggresomes. However, when p53, E1B55K, and Rep78 were co-expressed, most of the p53 protein would relocate to the nucleus in a distinct punctate manner. Rep78 itself had no effect on the localization of p53. One hundred cells from each experiment group were scored for complete/partial/no export of p53 to the cytoplasm when EGFP-p53 and E1B-55K is expressed with or without Rep78, and the statistical result was consistent with immunofluorescent images [**Fig. 2(B)**]. Western blot assay was also carried out to examine the relative abundance of p53 in both nuclear and cytoplasmic fractions. In the presence of E1B55K, p53 was accumulated significantly in the nuclear fractions upon expression of Rep78 [**Fig. 2(C, lanes 3, 4 and lanes 5, 6)**], suggesting that Rep78 could inhibit E1B55K-mediated p53 nuclear export. Interestingly, Rep78 could also cause p53 accumulation in the nucleus in the absence of E1B55K [**Fig. 2(C, lanes 1, 2 and lanes 7, 8)**].

Rep78 relocalizes the E1B55K protein from the cytoplasm to the nucleus

Further, we detected the localization of Rep78 or E1B55K along with EGFP-p53, respectively. The three proteins were co-expressed in H1299 cells in different combinations and detected at 48 h post-transfection by a fluorescent microscope. As shown in **Fig. 3(A)**, while E1B55K was found in large cytoplasmic inclusion bodies with p53 when expressed in H1299 cells, it was relocalized to nucleus with a particle distribution in the presence of Rep78, and the E1B55K nuclear punctuates still completely co-localized with p53. Similar results were observed in the absence of p53 expression, suggesting that p53 is not required for Rep78-mediated E1B55K relocalization (data not shown). However, E1B55K expression had no direct effect on Rep78's location. Rep78 was distributed throughout the nucleoplasm when it was expressed plus or minus an E1B55K expression vector [**Fig. 3(B)**].

Rep78 does not detectably influence p53 stability

Given the fact that p53 proteasome degradation was mainly carried out in the cytoplasm and Rep78 could sequester p53 in the nucleus, we further evaluated the effect of Rep78 on p53's degradation mediated by E1B55K-E4orf6 E3 ubiquitin ligase complex. H1299 cells were transfected with EGFP-p53, Myc-E1B55K, Myc-E4orf6, HA-Rep78, or empty vectors as indicated in **Fig. 4**. The amount of p53 protein was reduced significantly in the presence of E1B55K and E4orf6 together (**Fig. 4, lane 6**), which was consistent with a previous report [6]. This reduction was maintained in the presence of Rep78 (**Fig. 4, lane 7**). None of the three proteins affected the p53 levels when expressed alone (**Fig. 4, lanes 2, 4, and 5**), indicating that Rep78 could not protect p53 from E1B55K-E4orf6-mediated degradation.

Rep78 has no detectable effect on p53 inhibition by E1B55K

Because p53 stability has not been improved by Rep78, we then tested whether p53 transcriptional activity would be restored. A p53-specific transcriptional reporter system was used to measure p53 activity. Expression plasmids of E1B55K, Rep78, or empty vector were transfected into p53 wild-type U2OS cells along with the reporter plasmid. As shown in **Fig. 5(A)**, E1B55K could impair p53 transcriptional activity significantly, reducing p53 activity to only $31.66\% \pm 0.80\%$ ($P < 0.01$) compared with the control cells that were not transfected with E1B55K or Rep78. When Rep78 was co-expressed with E1B55K, p53 transcriptional inactivation was not obviously relieved, as the relative p53 transcriptional activity was $31.97\% \pm 1.02\%$ ($P > 0.05$) of that of untransfected controls. However, Rep78 itself induced a slight decrease in p53 transcriptional activity, which was $80.72\% \pm 5.59\%$ of the control group level ($P < 0.01$).

To explore the reason as to why Rep78 could not reverse the inhibitory effect of E1B55K on p53, a competing co-immunoprecipitation assay was used to detect the interaction of E1B55K and p53 when Rep78 was additively expressed. As shown in **Fig. 5(B)**, E1B55K still bound to p53 without a visible reduction when Rep78 was expressed simultaneously in H1299 cells, indicating that Rep78 could not replace E1B55K from the p53–E1B55K complex. We

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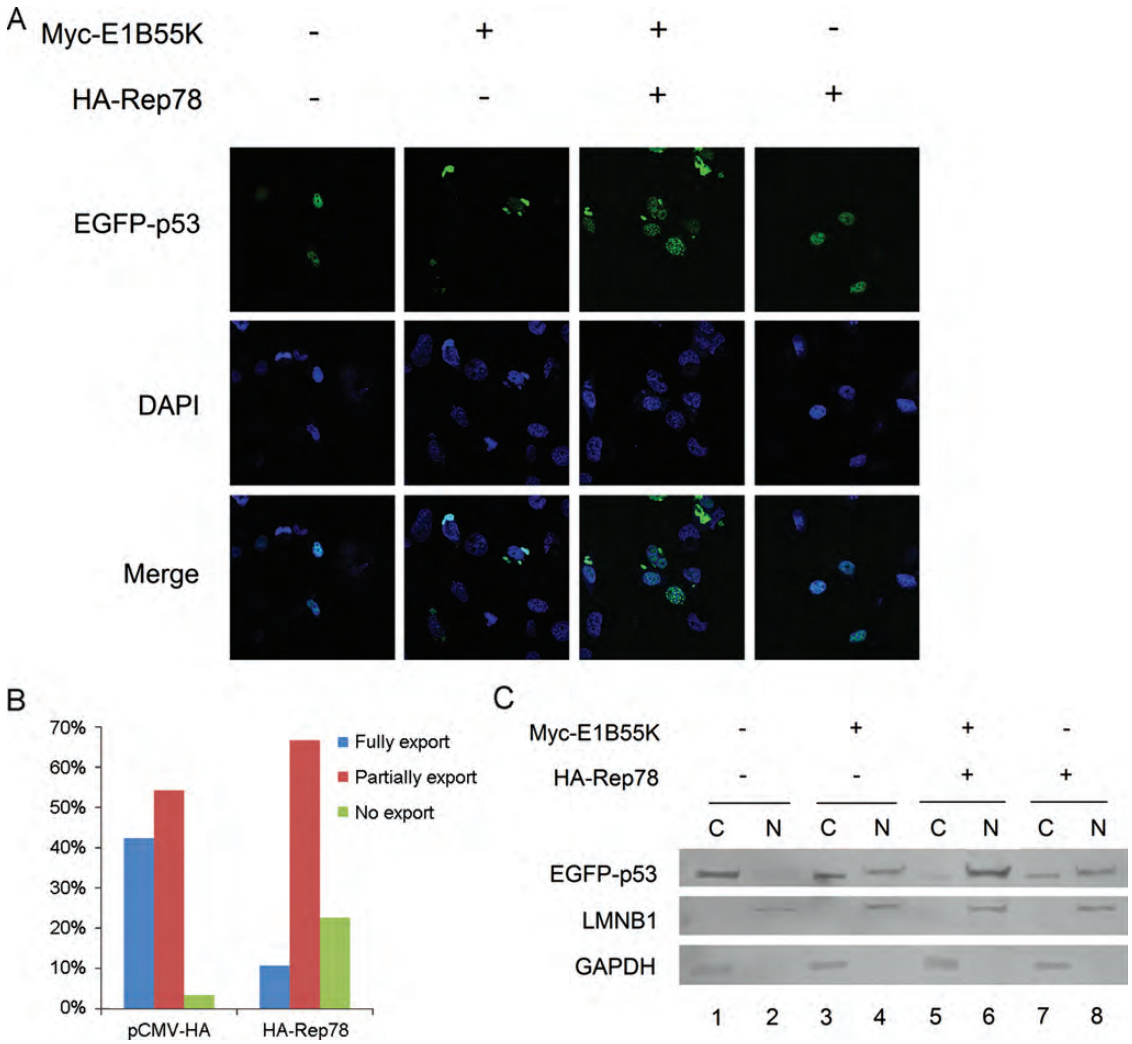

Figure 2 Rep78 inhibited E1B55K-mediated nuclear export of p53 (A) H1299 cells were transfected with EGFP-p53 and other indicated plasmids. At 48 h post-transfection, EGFP-p53 was detected using confocal fluorescent microscopy. The location of the nuclei was visualized by DAPI staining (×40). (B) Statistical result of one hundred cells when EGFP-p53 and E1B-55K was expressed with or without Rep78. (C) Western blot assay of relative abundance of p53 in the nuclear and cytoplasmic fractions. H1299 cells were transfected with EGFP-p53 and other indicated plasmids. Forty-eight hours later, the transfected cells were harvested and divided into nuclear (N) and cytoplasmic (C) fractions. p53 was detected by an anti-p53 antibody, and the anti-GAPDH and anti-LMNB1 antibodies were used to confirm the purity of two fractions.

also detected that Rep78 and E1B55K could co-precipitate with p53 simultaneously, suggesting that the region at which p53 physically interacted with Rep78 may be different from that of E1B55K [**Fig. 5(C)**].

Discussion

In the present study, we demonstrated that Rep78 but not Rep68 could interact with p53. Using confocal fluorescence assay, we demonstrated that when Rep78, E1B55K, and p53 were expressed simultaneously in H1299 cells, Rep78 could inhibit the cytoplasm recruitment of p53 by E1B55K, and it could also relocalize the E1B55K protein from the cytoplasm to the nucleus. The p53 sequestered in the nucleus showed a different punctate pattern from the diffuse distribution when p53 was expressed alone. This distribution of p53 was reminiscent of the typical pattern of promyelocytic leukemia nuclear bodies (PML-NBs), nuclear domains playing crucial roles in certain cellular events, including host antiviral defense [16,17]. E1B55K was reported to function as an E3 SUMO1-p53 ligase and to induce p53 to co-localize with E1B55K in PML-NBs, thereby greatly facilitating its nuclear export [18]. It seems that Rep78 might inhibit the nuclear exportation of p53 after p53 co-localizes with PML-NBs, although the precise mechanism is presently unknown.

For the degradation and inactivation of p53 were closely related to its cytoplasm localization, we further detected the protein level and the transcriptional activity of p53 when Rep78 and E1B55K were expressed together. It was found that Rep78 had no detectable effect on p53 degradation and functional inhibition mediated by E1B55K. The results of

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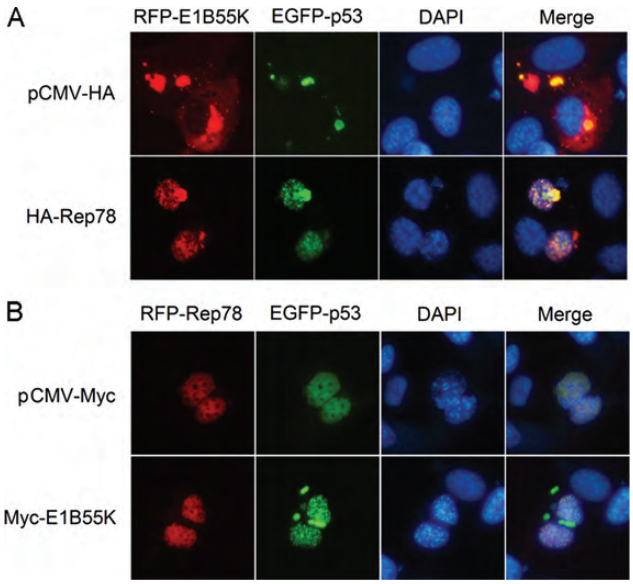

Figure 3 The localization of Rep78 or E1B55K along with EGFP-p53, respectively The three proteins were co-expressed in H1299 cells in different combinations and detected at 48 h post-transfection by a fluorescent microscope. The nuclei were stained by DAPI. (A) The localization of RFP-E1B55K and EGFP-p53 in the presence or absence of HA-Rep78. (B) RFP-Rep78 and EGFP-p53 were used to show the localization of Rep78 and p53 with or without E1B55K expression ($\times 40$).

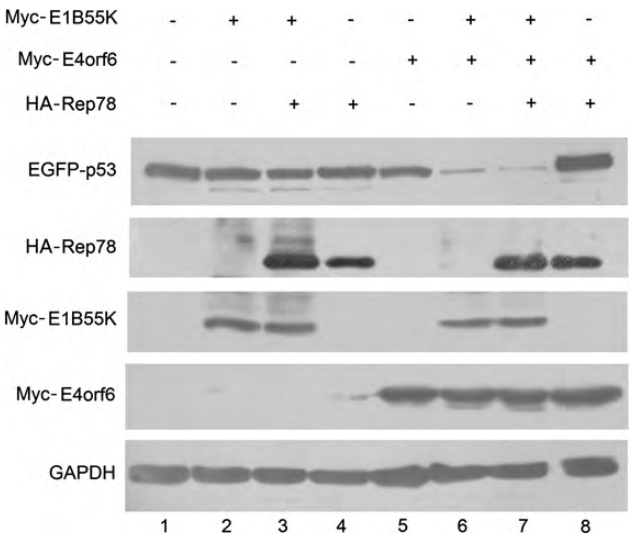

Figure 4 Rep78 could not enhance the stability of the p53 protein H1299 cells were transfected with 1 μg of EGFP-p53, 500 ng of Myc-E1B55K, 500 ng of Myc-E4orf6, and 1 μg HA-Rep78 or empty vector as indicated. At 48 h post-transfection, cells were lysed and subjected to western blot. Mouse anti-p53 antibody was used to detect p53 protein in the cell lysates.

the competitive co-immunoprecipitation assay indicated that although Rep78 could also bind to p53, it could not replace E1B55K from the p53–E1B55K complex. It has been also shown in **Fig. 3(A)**, while p53 was relocalized to nucleus by Rep78, E1B55K still co-localized with p53. Therefore, E1B55K still binds to the N-terminal transactivation domain

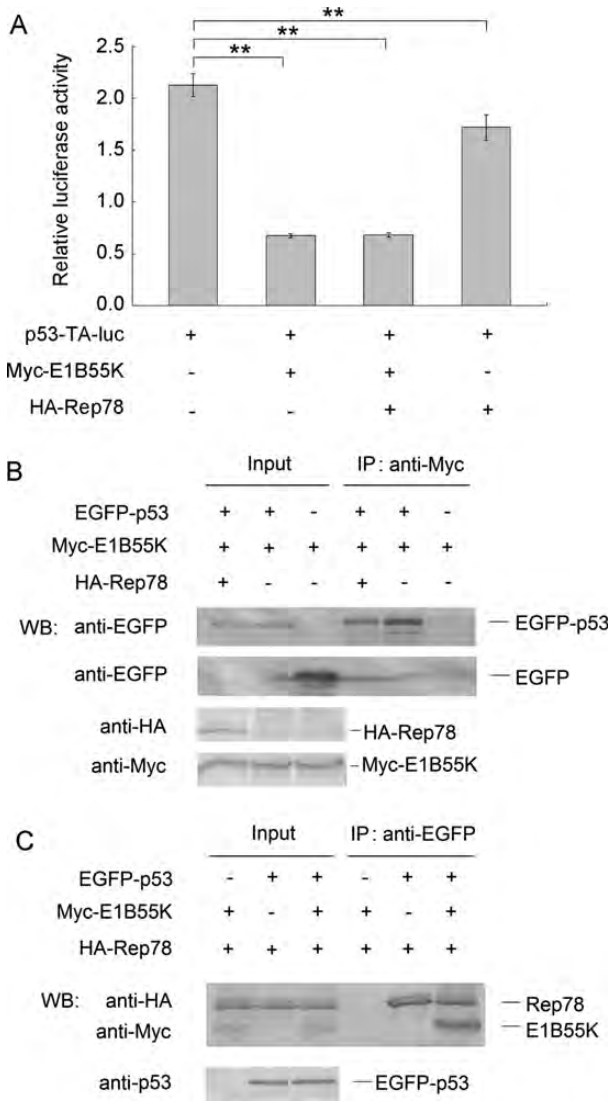

Figure 5 Rep78 had no detectable effect on p53 inhibition by E1B55K (A) Dual-luciferase activity assay. U2OS cells were transfected with 500 ng of p53-TA-luc, 1 ng of SV40-Renilla luciferase, 10 ng of Myc-E1B55K, and 500 ng HA-Rep78 or empty vector. ** $P \leq 0.01$. (B) Rep78 could not abolish the interaction of p53 with E1B55K. H1299 cells were co-transfected with EGFP-p53, Myc-E1B55K, and HA-Rep78 or empty vector. Mouse anti-Myc antibody was used for immunoprecipitation, and rabbit anti-EGFP antibody was used for western blot. (C) Rep78 and E1B55K could co-precipitate with p53 simultaneously. H1299 cells were co-transfected with indicated plasmids. Rabbit anti-EGFP antibody was used for immunoprecipitation, mouse anti-HA and mouse anti-Myc antibody were used for western blot.

of p53, which explains why p53 accumulation in the nucleus did not result in an increase in p53 stability and transcriptional activity. Alternatively, p53, E1B55K, and Rep78 may form a trimeric complex when they are expressed together, for E1B55K also binds to Rep78 [19].

While the interaction with Rep78 has little or no direct effect on p53's stabilization or transcriptional activation in the presence of E1B55K, Rep78 relocalizes both p53 and E1B55K in the nucleus. It is not known whether other

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interactions contribute to the following progress. Our results reveal a new possible mechanism that AAV-2 Rep78 inhibits adenovirus 5 by restricting p53 nuclear exportation, which may shed some light on the regulatory mechanisms of AAV by its helper virus, adenovirus.

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