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β-Actin facilitates etoposide-induced p53 nuclear import

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As a tumor suppressor, p53 preserves genomic integrity in eukaryotes. However, limited evidence is available for the p53 shuttling between the cytoplasm and nucleus. Previous studies have shown that β-actin polymerization negatively regulates p53 nuclear import through its interaction with p53. In this study, we found that DNA damage induces both β-actin and p53 accumulation in the nucleus. β-actin knockdown impaired the nuclear transport of p53. Additionally, β-actin could interact with p53 which was enhanced in response to genotoxic stress. Furthermore, N terminal deletion mutants of p53 shows reduced levels of association with β-actin. We further identified Ser15, Thr18 and Ser20 of p53 are critical to the β-actin: p53 interaction, which upon mutation into alanine abrogates the binding. Taken together, this study reveals that β-actin regulates the nuclear import of p53 through protein–protein interaction.

Keywords. β-actin; p53; interaction; DNA damage; nuclear localization

Abbreviations: γH2AX, phosphorylated histone H2AX; MDM2, the Murine Double Minute 2 oncogene; Mot2, mortalin 2; ETO, etoposide; FBS, fetal bovine serum; DAPI, 4′,6′-diamidino-2 phenylindole

1. Introduction

Genome integrity needs to remove any abnormal DNA lesions. The tumor suppressor p53 is critical to the maintenance of DNA integrity and can trigger cell cycle arrest, DNA repair and apoptosis (Gottlieb and Oren 1996; Beckerman and Prives 2010). The transport of p53 from the cytoplasm to nucleus is essential for its function. Previous studies have shown that several proteins regulate the nuclear translocation of p53. The Murine Double Minute 2 oncogene (MDM2) enhances p53 export from nucleus and promotes its degradation by ubiquitination (Alarcon-Vargas and Ronai 2002). Additionally, the hsp70 family member mortalin 2 (Mot2) inhibits p53 nuclear import through a direct interaction (Wadhwa *et al.* 1998). Recent studies have shown that microtubules and their associated motor protein dynein participate in the transportation of p53 into the nucleus following DNA damage (Giannakakou *et al.* 2000). However, p53 trafficking during different cell stress states is still sophisticated and largely uncharacterized.

β-actin participates in multitudinous cellular events ranging from cell motility, cell morphology changes, cell polarity, and transcriptional regulation. It was previously shown that the β-actin-binding RPEL motif regulates the nucleo-cytoplasmic shuttling of p53 in cancer cells (Guettler *et al.* 2008). Furthermore, wild-type p53 associates with cytoplasmic β-actin filaments during DNA synthesis (Olivier *et al.* 2002). Recent reports suggest that β-actin polymerization negatively regulates p53 nuclear import (Wang *et al.* 2013) and

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the monomeric forms of actin regulate the cytoplasmic-nucleo translocation of p53 in response to DNA damage (Saha *et al.* 2016). These preliminary findings suggest that cytoskeletal β-actin may be a key regulator in p53 transport.

In the present study, we demonstrate that β-actin facilitates p53 nuclear import and coordinates with p53 to regulate p21 expression in response to genotoxic stress. N-terminal deletion mutants of p53 and point mutations at Ser15, Thr18 and Ser20 of p53 which may situate at the β-actin: p53 interface abolished the interaction. Taken together, our study demonstrates that β-actin directs p53 nucleo-cytoplasmic shuttling through protein–protein interactions.

2. Materials and methods

2.1 *Cell culture and transfection*

U2OS and PC3 cells (American Type Culture Collection) were cultured in DMEM with 10% FBS at 37°C with 5% CO_2. U2OS cells and PC3 cells were transfected with plasmids or siRNAs using Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacturer's instructions. To introduce DNA damage, cells were incubated in 20 μM etoposide (ETO) for 1 h.

2.2 *Antibodies and reagents*

Rabbit antibodies against HA (Y-11), p53 (FL-393) and p21 (C-19) and mouse antibody against p53 (DO-1) were purchased from Santa Cruz Biotechnology. Mouse anti-β-actin antibody (A5441) and Rabbit anti-GAPDH antibody (G9545) were purchased from Sigma Aldrich. Mouse anti-γ-H2AX antibody (05-636) was obtained from Upstate. Etoposide was purchased from Sigma Aldrich. Mouse antibodies against Tubulin (66031-1-Ig) and LaminB (66095-1-Ig) were obtained from Proteintech.

2.3 *Plasmids and SiRNAs*

HA-wt p53, HA-Δ1-41 p53, HA-S15A p53, HA-T18A p53, HA-S20A p53, and HA-S15A/T18A/S20A p53 were subcloned from the coding sequences extracted from U2OS cells into the EcoRI and XhoI sites of pcDNA-HA3.1. PCR primers are shown in table 1.

siRNA duplexes were obtained from Dharmacon (China). The sequence of β-actin siRNAs were

Table 1. Sequences of primers used in p53 mutant construction

Name	Sequences
wt p53	F: atg gag gag ccg cag tca R: tca gtc tga gtc agg
Δ1-41 p53	F: gat ttg atg ctg tcc ccg
S15A p53	F1: atg gag gag ccg cag tca gat cct agc gtc gag ccc cct ctg gca cag gaa aca ttt tca gac cta F2: gaa aca ttt tca gac cta R: tca gtc tga gtc agg
T18A p53	F1: atg gag gag ccg cag tca gat cct agc gtc gag ccc cct ctg agt cag gaa gca ttt tca gac cta F2: gaa gca ttt tca gac cta R: tca gtc tga gtc agg
S20A p53	F1: atg gag gag ccg cag tca gat cct agc gtc gag ccc cct ctg agt cag gaa aca ttt gca gac cta F2: gaa aca ttt gca gac cta R: tca gtc tga gtc agg
S15A/T18A/ S20A p53	F1: atg gag gag ccg cag tca gat cct agc gtc gag ccc cct ctg gca cag gaa gca ttt gca gac cta F2: ctg gca cag gaa gca ttt gca gac cta R: tca gtc tga gtc agg

UUGGCGCUUUUGACUCAGGA and UGUAAG GUA AGGUGUGCACU. The sequence of the control siRNA was UUCUCCGAACGUGUC ACGUTT.

2.4 *Whole cell protein extraction and nuclear/cytoplasmic extraction*

U2OS cells transfected with control or β-actin siRNAs for 48 h were treated with 20 μM ETO for 1 h. Whole cell proteins were extracted with RIPA lysis buffer as previously described (Qi *et al.* 2016). Nuclear and cytoplasmic proteins were extracted according to CelLyticTM NuCLEARTM Extraction Kits (Sigma).

2.5 *Immunoprecipitation and Western blotting*

U2OS cells treated with the ETO or PC3 cells transfected with pcDNA3.1 vector, HA-wt p53, HA-Δ1-41 p53, HA-S15A p53, HA-T18A p53, HA-S20A p53 or HA-S15A/T18A/S20A p53 plasmid were lysed. Whole cell lysates were precleared with Protein A/G–Sepharose for 2 h and incubated with the indicated antibodies at 4° overnight. Samples were then incubated for a further 4 h with Protein A/G–Sepharose. For immunoprecipitations with lambda phosphatase, we added lambda phosphatase

to the whole cell lysates for 30 min. Immunoprecipitated complexes were detected by Western blotting.

2.6 *Immunofluorescence assays*

U2OS cells seeded onto coverslips were transfected with control siRNA or β-actin siRNAs. Forty-eight hours post-transfection, the cells were treated with 20 μM ETO for 1 h and fixed in 10% formaldehyde for 10 min. Cells were washed with PBS and permeabilized with 0.5% Triton X-100 for 5 min. Cells were incubated with 10% FBS for 1 h labeled with primary antibodies for 1 h, washed with PBS, and labeled with secondary antibodies for 1 h. The coverslips were stained with DAPI for 5 min and images were captured using a fluorescence microscope.

2.7 *Dual-luciferase assays*

PC3 cells were transiently transfected with 200 ng pGL4.2-p21-luc plasmid and 20 ng pREP7-Rluc control plasmid, together with 500 ng pcDNA3.1 vector, myc-β-actin, HA-p53 or myc-β-actin/HA-p53 expression plasmid. Twenty-four hours post-transfection, cells were lysed using dual-luciferase assays (Promega) and relative luciferase activity was analyzed using a Turner Designs TD 20/20 luminometer. The data were obtained from three independent experiments.

2.8 *Statistical analysis*

Statistical analysis was performed using an unpaired t-test or one-way ANOVA in GraphPad 6. $P < 0.01$ (**) and $P < 0.001$ (***, ****) indicate significant difference.

3. Results

3.1 *β-actin facilitates p53 nuclear transport in response to DNA damage*

The tumor suppressor p53 is a short-lived protein and genotoxic stress can increase its half-life (Bullock *et al.* 1997). Previous studies have found that β-actin interacts with p53 (Jiang *et al.* 2001) and its polymerization negatively regulates p53 nuclear import (Wang *et al.* 2013). We used ETO to evoke cellular DNA damage and enhance the nuclear accumulation of p53. As shown in figure 1A, ETO treatment led to obvious increase of p53 expression, and p53 was mostly transported into the nucleus. Notably, the localization of β-actin in the nucleus also increased. When the expression of β-actin was silenced by siRNA, the nuclear accumulation of p53 significantly decreased. Western blot analysis showed that β-actin knockdown had no obvious effects on p53 expression (figure 1B). Gamma H2AX levels increased after ETO treatment suggesting that DNA damage had occurred. We further investigated if β-actin silencing influences the nuclear trafficking of p53 by Nuclear/Cytoplasmic fractions. Figure 1C shows that β-actin and p53 were enriched in the nuclear fraction following ETO treatment and β-actin knockdown led to decreased accumulation of p53 in the nucleus. These data indicate that β-actin regulates the nuclear import of p53.

3.2 *Influence of β-actin on p53 leads to alteration in p21 expression*

Upon DNA damage, p53 translocates to the nucleus and binds to the promoter of p21, resulting in cell cycle arrest or apoptosis (Waldman *et al.* 1995). Thus, p21 is a downstream marker of p53 activity. We next examined the effects of β-actin depletion on p21 expression. As shown in figure 2A, β-actin knockdown resulted in significantly decreased p21 expression. Previous studies have shown that β-actin regulates IL6-induced p21 expression through its interaction with PolII (Tian *et al.* 2016). We therefore investigated whether the influence of β-actin depletion on p21 expression is mediated by p53. For this purpose, PC3 cells carrying p53-null were transfected with pcDNA3.1, myc-β-actin, HA-p53 or myc-β-actin/HA-p53 respectively and the p21-luc activity was measured. Western blotting was used to investigate the expression of myc-β-actin and HA-p53. It was observed that myc-β-actin expression alone had no obvious effects on p21-luc activity compared to vector controls. HA-p53 expression alone enhanced p21-luc activity and combined myc-β-actin/HA-p53 expression promote p21-luc activity compared with HA-p53 alone (figure 2B). These data reveal that β-actin coordinates with p53 to regulate p21 expression alteration.

3.3 *β-actin interacts with p53*

Our preliminary findings suggested that the nuclear accumulation of p53 and β-actin increase in response to DNA damage. We therefore investigated whether there is any protein–protein interaction involved. U2OS cells with or without ETO treatment were lased and subjected

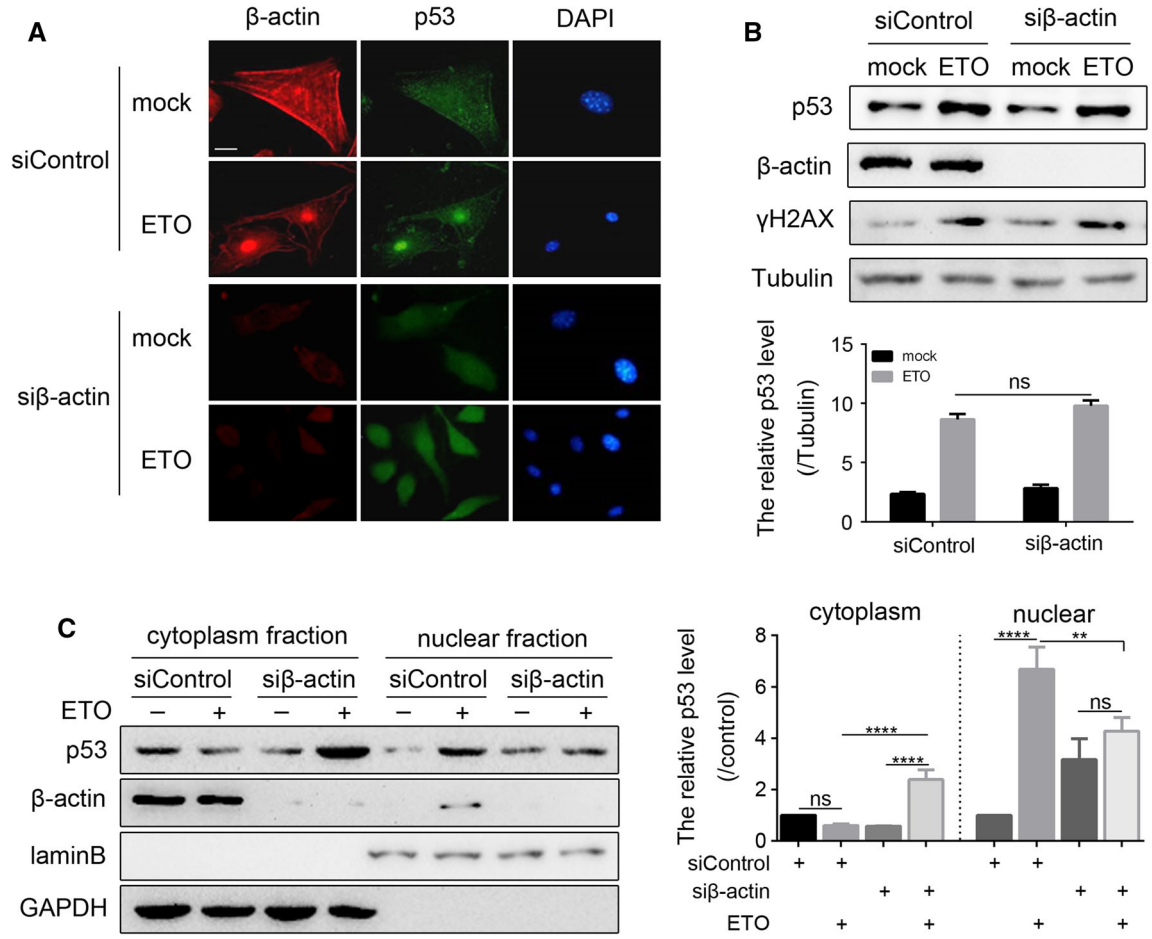

Figure 1. β-actin knockdown decreased the nuclear translocation of p53 in response to DNA damage. (**A**) U2OS cells were transfected with indicated siRNAs and treated with ETO. Cells were immunostained with anti-p53 and anti-β-actin antibodies. Images were captured using a fluorescence microscope. Scale bar = 10 μm. (**B**) U2OS cells transfected with indicated siRNAs were treated with ETO. The whole cell lysates were extracted and detected by Western blotting with the indicated antibodies. Relative p53 levels were compared with Tubulin (loading control) from three independent experiments and analyzed by ImageJ. (**C**) U2OS cells were treated as in (**B**). The cytoplasm and nuclear fractions were extracted and detected by Western blotting. GAPDH was used as loading control for the cytoplasmic fraction and Lamin B was used as loading control for the nuclear fraction. Relative p53 levels were compared with cytoplasm or nuclear loading control from three independent experiments and analyzed by ImageJ.

to co-immunoprecipitation assay using anti-p53 and anti-β-actin antibodies. As shown in figure 3, p53 was detected in β-actin immunoprecipitated complexes, whilst β-actin was identified in p53 immunoprecipitated complexes. Moreover, the interaction between p53 and β-actin increased obviously after ETO treatment. These results suggest that β-actin interacts with p53 which increases in response to ETO treatment.

3.4 *β-actin: p53 interaction involves the N terminal S15, T18 and S20 sites of p53*

p53 contains four essential domains: a disordered amino-terminal domain, a proline-rich domain, a DNA-binding domain and an unstructured carboxy-terminal domain (Saha *et al.* 2015). Impaired p53 nuclear import leads to the cytoplasmic retention of p53 (Xu *et al.* 2011).

The phosphorylation of Ser15, Thr18 and Ser20 amino acids at the N terminal following DNA damage is vital for p53 stability and the interation between p53 and other proteins (Liu *et al.* 2001; Kar *et al.* 2002; Polley *et al.* 2008). Hence, we assessed whether the N terminal deletions or mutations impaired the binding of p53 to β-actin. We generated short deletions and point mutations within this region (Δ1-41, S15A, T18A, S20A and S15A/T18A/S20A mutant p53) (figure 4A) and analyzed their influence on the β-actin: p53 interaction in transiently over-expressed PC3 cells. As

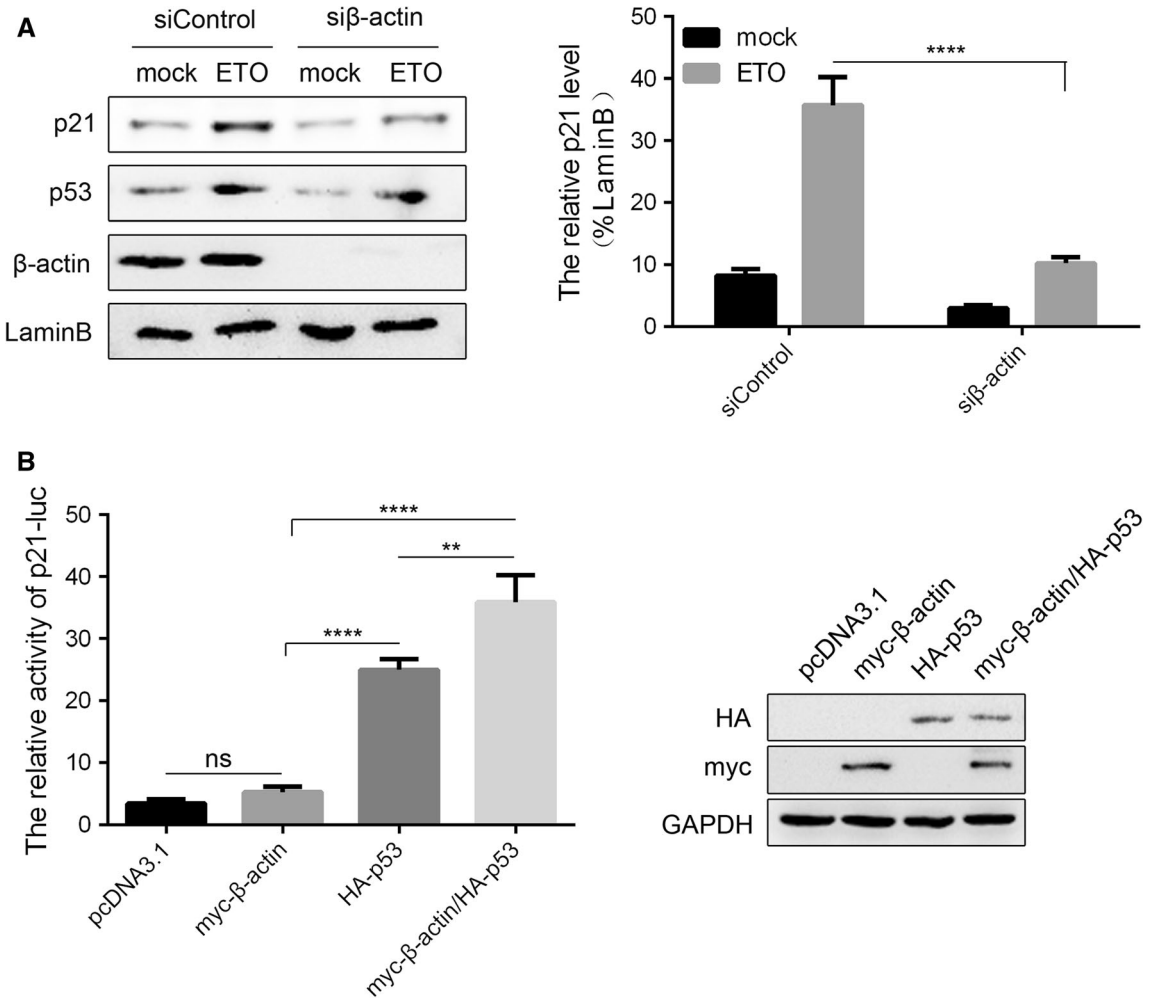

Figure 2. β-actin coordinates with p53 to regulate p21 expression. (**A**) U2OS cells transfected with the indicated siRNAs were treated with ETO. The extracted whole cell lysates were detected by Western blotting with the indicated antibodies. The relative p21 levels were compared with LaminB from three independent experiments and analyzed by ImageJ. (**B**) PC3 cells were transfected with the indicated plasmids and luciferase activity was assessed. Data are the means ± S.D. from three independent experiments. The expression of the plasmids was detected by Western blotting.

shown in figure 4B, the p53 mutants exhibited a varying degree of interaction with β-actin. Wt p53 could interact with β-actin, while the interaction was significantly diminished for Δ1-41 p53 mutant. However, S15A, T18A and S20A site mutants showed no obvious reduced affinity towards β-actin. Interestingly, when all the three sites were mutated to Ala, the β-actin: p53 interaction was impaired.

To further investigate the importance of the N-terminal domain in actin regulated p53 nuclear translocation, PC3 cells were transfected with HA-wt p53, HA-Δ1-41 p53 and HA-S15A/T18A/S20A p53 and pretreated with Cytochalasin D (CD) to block actin polymerization. We found that only HA-wt p53 could be imported into the nucleus in response to DNA damage, while Δ1-41 mutant p53 and S15A/T18A/S20A p53 showed little nuclear translocation. Moreover, CD treatment led to increased nuclear translocation of p53 (figure 4C). These findings suggest that β-actin interacts with the N terminal amino acids of p53, which are required for its nuclear import.

4. Discussion

The tumor suppressor p53 controls an array of cellular events to maintain the genome integrity (Joerger and Fersht 2010). Previous studies have reported that wild-type p53 is stabilized and translocated into the nucleus under genotoxic stress (Sakaguchi *et al.* 1998). However, the specific mechanisms of p53 shuttling are not fully understood.

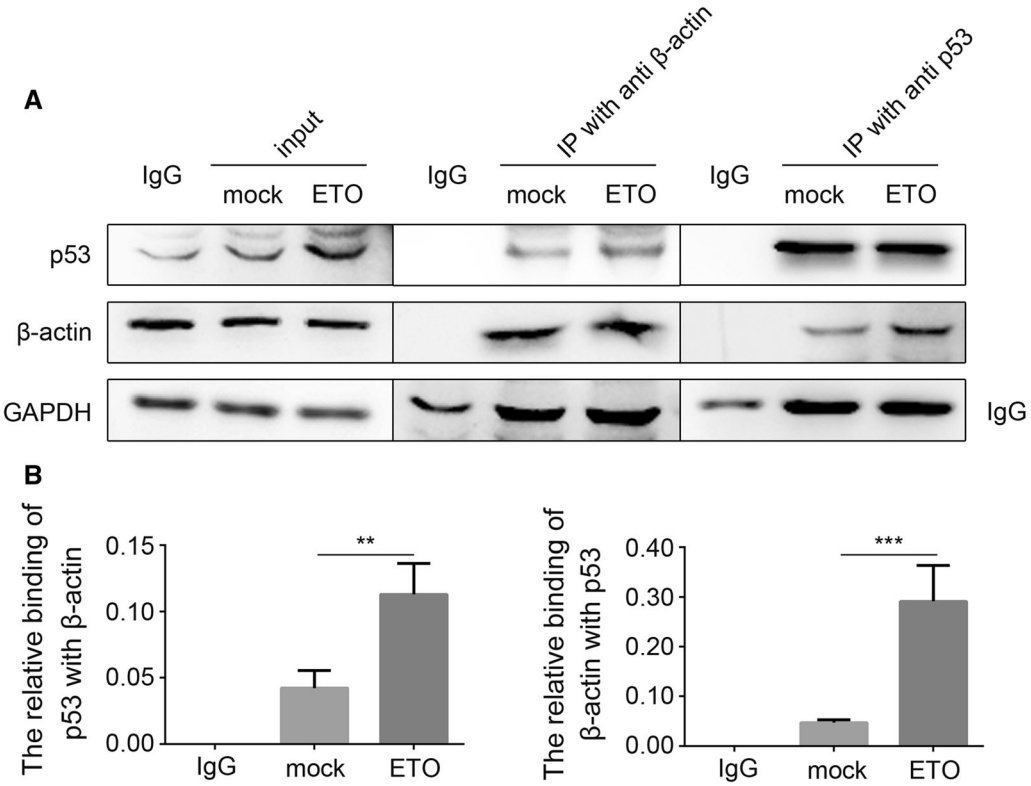

Figure 3. β-actin interacts with p53. (**A**) U2OS cells were treated with ETO. And co-immunoprecipitations were performed using the indicated antibodies. Immunoprecipitation complexes were analyzed by Western blotting with the indicated antibodies. (**B**) Relative binding of p53 to β-actin and the relative binding of β-actin to p53 in (**A**) from three independent experiments were analyzed using ImageJ.

Previous studies reported that MDM2 promotes p53 nuclear export (Alarcon-Vargas and Ronai 2002) and Mot2 inhibits p53 nuclear import through its interaction with p53 (Wadhwa *et al.* 1998). In this study, β-actin could interact with p53 and facilitate its import into the nucleus. Meanwhile, we found that β-actin silencing led to decreased p53 import but nuclear import was not completely prevented. This data suggested that β-actin partly contribute to p53 nuclear shuttling. Further studies investigating the crosstalk between β-actin, MDM2, Mot2 and other partners are required to fully understand the regulation of p53 trafficking. Several studies have shown that the polymeric forms of actin negatively regulate the nuclear import of p53 (Metcalfe *et al.* 1999; Wang *et al.* 2013) and that the monomeric forms of actin promote the nucleo-cytoplasmic shuttling of p53 in response to doxorubicin stress (Saha *et al.* 2016). Interestingly, we found that actin polymers were disrupted after ETO treatment. Additionally, CD treatment which blocks the polymerization of actin led to the increased nuclear translocation of p53. This inconsistent function of actin indicates that the status of β-actin is dynamic and is likely to play different roles under genotoxic stress or at different stages.

p53 consists of a N terminal domain (Met1-Asp42), a proline-rich domain (Asp61-Ser94), a DNA-binding domain (Th102-Lys292) and a carboxy-terminal domain (Saha *et al.* 2015). Mutations in the p53 DNA-binding domain result in altered p53 translocalization (O'Brate and Giannakakou 2003; Saha *et al.* 2016). In this study, we found that the N terminal (1-41 aa) of p53 is critical for the β-actin:p53 interaction, particularly at Ser15, Thr18, and Ser20 that mediate the translocation of p53. These findings suggest that the interaction between β-actin and p53 is not limited a single domain. Since Ser15, Thr18 and Ser20 are phosphorylated in response to DNA damage, we also detected the interaction in the absence and presence of lambda phosphatase. Lambda phosphatase treatment led to an impaired interaction between actin and p53 (unpublished data). The detailed mechanism and key interacting amino acids require further assessments.

In conclusion, we have confimed a role of β-actin in p53 nuclear translocation and found that Ser15, Thr18, and Ser20 amino acids of p53 are critical to β-actin:

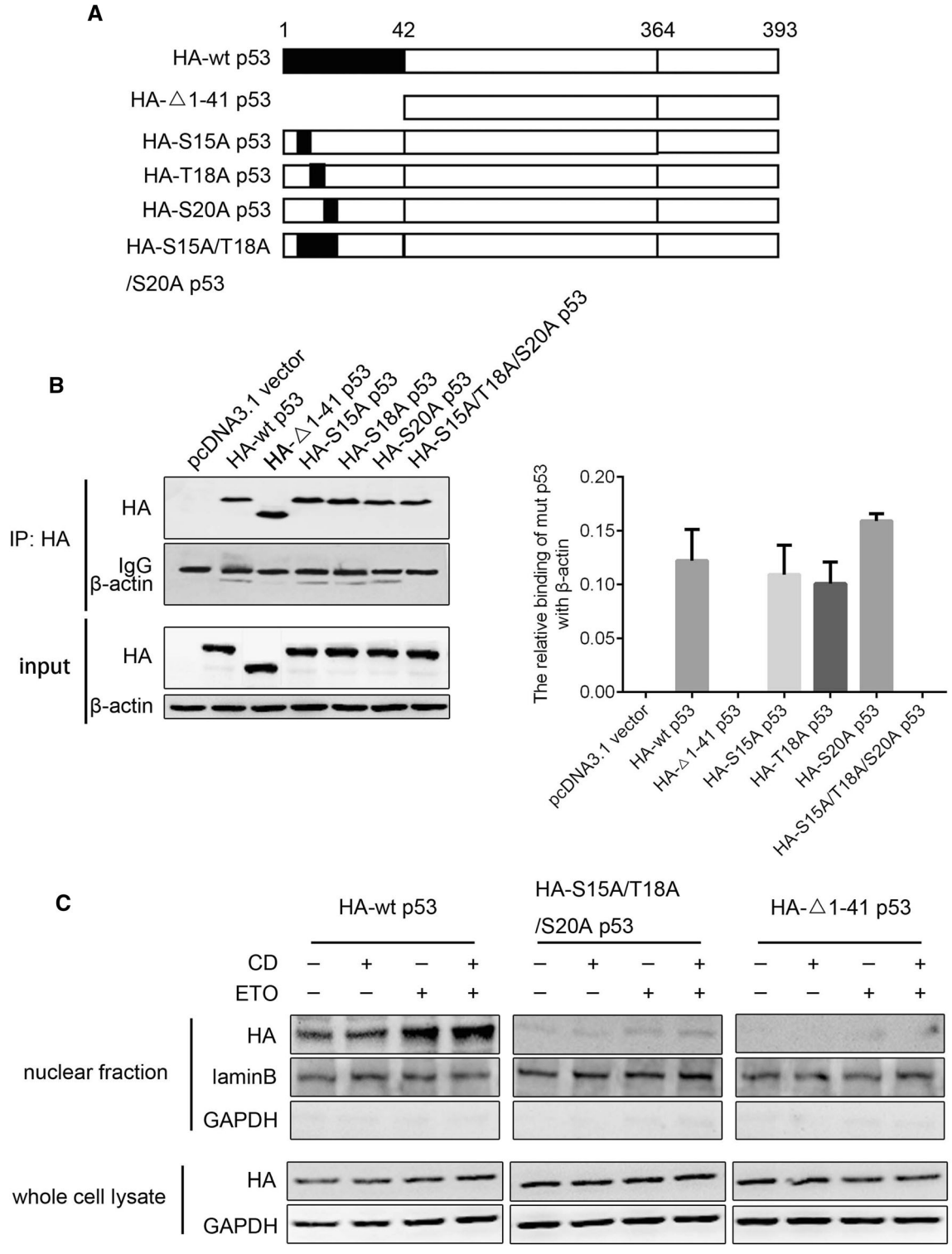

Figure 4. The N-terminal S15, T18 and S20 sites of p53 are critical to the β-actin:p53 interaction. (**A**) Diagram of HA-p53 mutants. (**B**) PC3 cells were transfected with the indicated plasmids. Forty-eight hours later, immunoprecipitations were performed using anti-HA antibody in whole cell lysates. The immunoprecipitation complexes were analyzed by Western blotting with the indicated antibodies. (**C**) PC3 cells were transfected with indicated plasmids and treated with ETO or CD. Cells were lysed with RIPA for whole cell lysates and extracted for nuclear fraction. Western blotting was performed with the indicated antibodies. Tubulin and Lamin B were used as loading controls for whole cell lysates and nuclear fractions.

p53 interaction. Further studies are now required to illustrate the crosstalk of other proteins associated with these events to reveal whether β-actin regulates p53 both in the perinucleus and inside nucleus import.

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