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De-dimerization of PTB is catalyzed by PDI and is involved in the regulation of p53 translation

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ABSTRACT

Polypyrimidine tract-binding protein (PTB) is an RNA binding protein existing both as dimer and monomer and shuttling between nucleus and cytoplasm. However, the regulation of PTB dimerization and the relationship between their functions and subcellular localization are unknown. Here we find that PTB presents as dimer and monomer in nucleus and cytoplasm respectively, and a disulfide bond involving Cysteine 23 is critical for the dimerization of PTB. Additionally, protein disulfide isomerase (PDI) is identified to be the enzyme that catalyzes the de-dimerization of PTB, which is dependent on the CGHC active site of the a' domain of PDI. Furthermore, upon DNA damage induced by topoisomerase inhibitors, PTB is demonstrated to be de-dimerized with cytoplasmic accumulation. Finally, cytoplasmic PTB is found to associate with the ribosome and enhances the translation of p53. Collectively, these findings uncover a previously unrecognized mechanism of PTB dimerization, and shed light on the de-dimerization of PTB functionally linking to cytoplasmic localization and translational regulation.

INTRODUCTION

Polypyrimidine tract-binding protein (PTB) is an RNA binding protein that preferentially binds to pyrimidine-rich sequences to regulate pre-mRNA splicing, mRNA polyadenylation, translation, localization and stability (1). PTB has been found to be a master suppressor of neuronal reprogramming (2), and a critical regulator of T cell activation (3–5) and B cell receptor (BCR)-mediated antibody production (6). PTB is also involved in oncogenesis by regulating glycolysis (7), cell apoptosis (8) and cell proliferation (9). Structurally, PTB contains an N-terminal nuclear localization signal, a nuclear export signal and four RNA recognition motifs (RRMs) (10). PTB shuttles between nuclear and cytoplasm (11), which export requires phosphorylation of PTB at Serine-16 by PKA (11). Moreover, it has been reported that PTB exists as homo-dimer and monomer (12). Several studies have revealed that RRM2 (10,12), Cysteine 23 (13) and the interaction between RRM3 and RRM4 (14) contribute to the formation of PTB dimer. However, the molecular mechanism of PTB dimerization remains unclear.

The thioredoxin superfamily is a large family of dithiol/disulfide oxidoreductases, which catalyzes the formation and breakage of the disulfides in mammalian protein polymerization (15). Formation of the disulfide bond is an oxidation reaction requiring terminal electron acceptors like glutathione disulfide (GSSG) and NAD(P), while breakage of the disulfide bond is a reduction reaction that needs terminal electron donors like glutathione (GSH) and NAD(P)H (16). Protein disulfide isomerase (PDI) is the first identified and the most highly expressed member of the mammalian thioredoxin superfamily (11). PDI is responsible for the formation, cleavage and isomerization of disulfides in the endoplasmic reticulum (ER), which is critical for the correct folding of proteins (15). PDI is highly expressed in many cancer types and is considered as a potential target for the treatment of cancer (17). Some reports show that the inhibition of PDI activates the unfolded protein response and suppresses the expression of DNA repair and DNA damage response genes in glioblastoma (18). Therefore, whether the thioredoxin superfamily members are involved in PTB dimerization need to be investigated.

PTB has been observed to translocate from nucleus to the cytoplasm with the treatment of doxorubicin, which induces DNA damage and upregulates p53 translation (19). *TP53*, encoding the tumor suppressor p53 protein is one of the best characterized genes that are required for the maintenance of the genome stability. P53 is expressed at low level under normal physiological conditions for its rapid degradation, while it is activated and triggers cell cycle arrest, senescence or apoptosis in stress conditions (20). Following DNA damage, *TP53* is not altered at the mRNA level, whereas the translation of p53 is promoted by enhanced association of polysomes (21,22). Several RNA binding proteins, including RPL26, Nucleolin, MAMX/MDM2, HuR, RBM38, Tia, CPEB1, Wig1, PTB and about 20 miRNAs are trans-acting factors that bind to the 5′UTR or the 3′UTR of *TP53*

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mRNA to regulate the translation or the stability of *TP53* (23). PTB is reported to promote the expression of p53 by interacting with the internal ribosome entry site (IRES) of *TP53* to enhance its translation. PTB has also been found to bind to the 3′UTR of *TP53* for maintaining its stability by competing with miR-1285 (24).

In the current study, interestingly, we found that nucleus localized PTB mostly exists as dimer, while cytoplasmic PTB predominately presents as monomer. By protein truncation and point mutations analysis, we confirmed Cysteine 23 as the critical amino acid that mediates PTB dimerization. We then screened the thioredoxin superfamily members and found that PDI is responsible for PTB dedimerization, which is further demonstrated by gene overexpression and small molecule mediated protein inhibition assay. Additionally, the a' domain of PDI was then identified to be critical for the de-dimerization of PTB. Furthermore, upon DNA damages generated by etoposide and doxorubicin treatment, we found the de-dimerization of PTB with cytoplasmic accumulation. Finally, exogenous cytoplasmic PTB was demonstrated to associate with ribosome and mitochondria, and to enhance the translation of p53. This study advances our understanding of the molecular mechanism of PTB dimerization, localization and function.

MATRERIALS AND METHODS

Antibodies

Antibodies used for immunoblotting and immunefluorescent staining were: Anti-Cyclin B1 (Santa Cruz, SC-245), anti-Cyclin E (Santa Cruz, SC-247), anti-GFP (Santa Cruz, SC-81045 or SC-9996), anti-PTB (Santa Cruz, SC-73391), anti-p53 (Santa Cruz, SC-126), Anti-PTB (Abcam, ab133734) and Anti-Histone H3 (Abcam, ab1791), Anti-PDI (CST, 3510S), Anti-Cyclin D (CST, 2922S), Anti-GAPDH (Huaxingbochuang Biotechnology, HX1828), Anti-β-Actin (Huaxingbochuang Biotechnology, HX1829), Alexa Fluor 488 donkey anti-rabbit IgG (Abcam, ab150073), Alexa Fluor 594 donkey anti-mouse IgG (Abcam, ab150115). RPS6 (Abcam, ab40820), Bip (CST, 3177T), eIF2α (CST, 3524T), Anti-mouse and rabbit HRP-conjugated secondary antibodies were from Huaxingbochuang Biotechnology.

Cell culture, transfections and biochemical reagents

HEK293 cells, A549 cells, MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (Gibco) and 100 U/ml penicillin/streptomycin. PTB KO cells were generated by co-transfection of hnRNP I CRISPR/Cas9 KO plasmid (SC-401435-KO2) and hnRNP I HDR plasmid (SC-401435), and selected by puromycin. Transfections of plasmids were performed with lipofectamine 2000 (Invitrogen) in antibiotic-free medium according to the manufacturer's instructions. NADP (ab146316) and NADPH (ab146317) were purchased from Abcam company. Etoposide (S1225) was purchased from Selleck company. CCF642 (SML1827) and MG132 (M8699) were purchased from Sigma-Aldrich company. KU-60019 (A10507) and ETP-46464(A13328) were purchased from ADOOQ Bioscience company. Tunicamycin (HZB2310-1) was purchased from Harvey company.

Cell cycle synchronization

For G1 phase block, cells were treated with lovastatin (25 μM) for 24 h. And for G2/M phase block, cells were treated with nocodazole (0.2 μg/ml) for 24 h.

Cell cycle analysis

The single-cell suspension was prepared in PBS, permeabilized by cold 70% ethanol for 30 min at 4°C, and then washed and resuspended in 500 μl PBS, followed by treatment with 5 μl RNase (DNase-free) at 37°C for 30 min, chilled on ice, and 1 μl PI (propidium iodide; Roche) treatment in the dark at room temperature for 1 h. DNA contents were acquired using Muse Cell Cycle analyzer.

Quantitative RT-PCR Analysis

Total RNA was prepared using TRIZOL reagent (Life Technologies) or RNA easy mini kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from total RNA using Super Script II Reverse Transcriptase (Life Technologies) with random hexamer. Quantitative real-time PCR was done with SYBR Select Master Mix on Step One Plus thermal cycler (Applied Biosystem). The threshold cycle (Ct) value for each gene was normalized to the Ct value for GAPDH. The relative mRNA expression was calculated using ΔΔCt method. Sequences of the primers were: *TP53*(ATTTGCGTGTGGAGTATTTG, AGTCTTCCAGTGTGATGATG) and *GAPDH* (GAAGGTGAAGGTCGGAGTC, GAAGATGGTGATGGGATTTC).

Immunofluorescence

Cells were seeded in Lab-Tek chamber slide, fixed for 30 min in 4% PFA in PBS, and permeabilized for 20 min with 0.5% Triton X-100 in PBS at room temperature. Immunofluorescent staining was done with standard procedures using the anti-PTB antibody (SC-73391; Santa Cruz) and anti-PDI PDI (3510S, Selleck company) as primary antibodies. Secondary antibodies are donkey anti-mouse Alexa Fluor 594 and donkey anti-rabbit Alexa Fluor 488. Cell nuclear was stained with Hoechst.

Immunoblotting

Cells were prepared using a lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate] or RIPA buffer (Santa Cruz Biotechnology), respectively, containing protease inhibitor mixture (Roche) and analyzed by SDS-PAGE under reducing (DTT) or non-reducing conditions. Western blot analysis was carried out according to standard methods. Reduced samples were heated at 95°C for 15 min and cooled prior to loading onto a 10% SDS-PAGE gel. Proteins were then transferred onto a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad) and immediately blocked with 5%

BSA/PBS with 0.1% Tween 20 overnight at 4°C. Primary antibodies were incubated with their targets overnight at 4°C or 2 h at room temperature. Following three washes with PBS with Tween 20 (0.1%), membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washes with PBS with 0.1% Tween 20, blots were developed using the ECL substrate (Thermo Fisher Scientific). Images were analyzed using ImageJ.

Immunoprecipitation and mass spectrum

HEK-293 cells were harvested and washed twice with PBS. Cell pellets were resuspended with Lysis Buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) containing protease inhibitor. Cell extracts was incubated with 20 μl Anti-GFP magnetic beads overnight and washed 3 times and boiled for 10 mins at 95°C. SDS-PAGE and Silver staining analysis were performed. Mass Spectrum was performed using Q Exactive Orbitrap Mass Spectrometers (MS) High-resolution accurate-mass LC–MS. And protein association networks were made using STRING (https://www.string-db.org).

Plasmids construction

Full length and truncated PTB with GFP tag were obtained from cDNA of HEK293 cells which were cloned into pEGFP-N1 expression vector. PTB point mutations were made by using Transgene fast mutation kit according to the manufacturer's instructions. Expression plasmid of PDI was purchased from Sino Biological. PDI point mutations were made by using Transgene fast mutation kit according to the manufacturer's instructions. Sequences of the primers for plasmid construction are listed in Supplementary Table S2.

RNA interference

For siRNA transfections, cells were transfected with 20 nmol/l siRNA (Tsingke Company) by Lipofectamine 2000 by standard protocol. The siRNA sequences are listed in Supplementary Table S3.

Statistical Analysis

Statistical analysis between groups was performed by two-tailed Student's *t* test by Graphpad Prism to determine significance when only two groups were compared. P values of less than 0.05 and 0.01 were considered significant. Error bars on all graphs are SD.

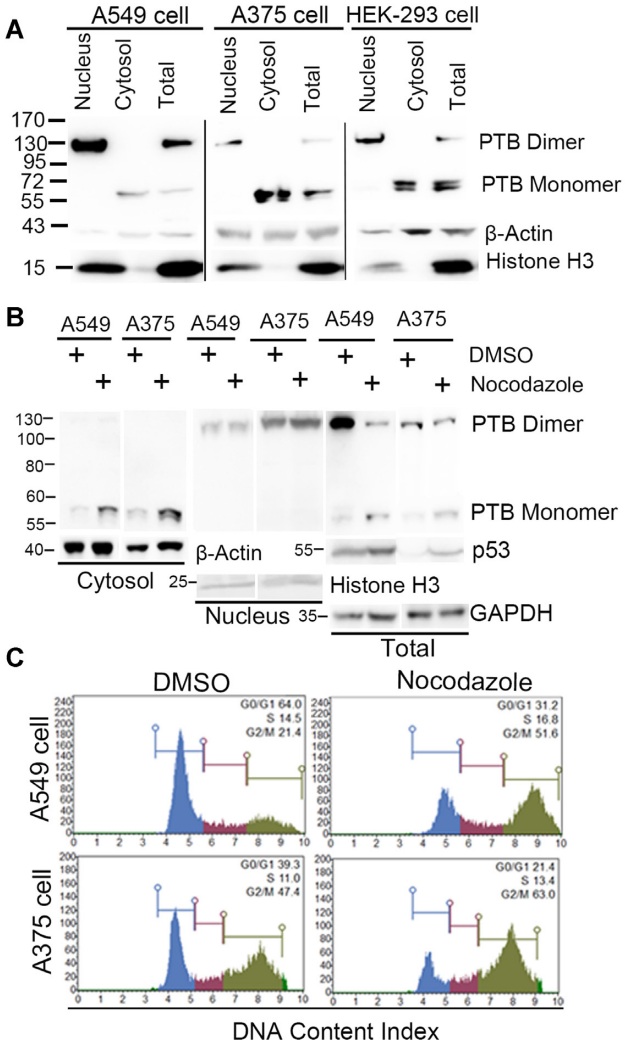

Figure 1. Cytoplasmic and nuclear PTB exist as monomer and dimer respectively. (**A**) Western blotting analysis of the expression of PTB of the cytoplasm, nuclear and total proteins under non-reducing conditions in indicated cells. Histone H3 and β-Actin were used as loading controls and indicators of the separation of nucleus protein and cytoplasm protein. (**B**) Western blotting analysis of PTB expression of cells treated with nocodazole (0.2 μg/ml) 24 hours for synchronization at G2/M phase and G1 phase under non-reducing conditions. Nucleus and cytosol proteins were separated, Histone H3 and β-Actin were used as loading controls and indicators of the separation of nucleus protein and cytoplasm protein. (**C**) Cell cycle distribution of the cells in (B) were analyzed by Muse cell cycle analyzer.

RESULTS

PTB exists as dimer and monomer in nucleus and cytoplasm respectively

PTB functions in both nucleus and cytoplasm. We found accidentally that nuclear PTB mainly exists as dimer while cytoplasmic PTB predominantly exists as monomer in three randomly selected cell lines under non-reducing SDS-PAGE (Figure 1A). Additionally, the cells synchronized at G2/M phase with nocodazole treatment expressed more PTB monomer and decreased level of PTB dimer in total proteins after cell lysis, which changes confirmed the distinct localization of PTB dimer and monomer (Figure 1B and C). The finding was also proved by increased level of PTB monomer in the cytoplasm, which was due to dedimerization of more PTB in the purified cytosol proteins for cells in mitosis. There was no significant difference in the expression level of PTB dimer in a similar amount of purified nucleus proteins from the complete nucleus in the gap phase (Figure 1B).

An intermolecular disulfide bond involving cysteine 23 mediates PTB dimerization

To understand the molecular mechanism of PTB dimerization, we divided PTB into four parts according to its domain structures, and fused each of them with GFP (Figure 2A). Each of the partial PTB-GFP is predicted to be about 40k Da in molecular weight. Under non-reducing conditions, a band about 80K Da was observed only in the PTB (1–130)-GFP group (Figure 2B), indicating the formation of dimer. Although the RRM3 and RRM4 were reported to interact with each other according to the crystal structure and suggested to mediate PTB dimerization (14), no band was observed at 80k Da when PTB (258–408)-GFP and PTB (409–526)-GFP were co-expressed (Figure 2B). Hence amino acids that are critical for PTB dimerization likely lie between amino acid aa. 1 and aa.130. Additionally, two bands about 40K Da were found in the PTB (1–130)-GFP group (Figure 2B). Phosphorylation and glycation are common modifications that would lead to the increase of protein size, therefore, ALP (alkaline phosphatase) and tunicamycin were used to remove phosphate groups and block glycosylation respectively. The ratio of these two bands did not change (Figure 2D), suggesting that the existence of the additional band was not due to protein phosphorylation or glycation. And nearly all PTB (1–526)-GFP and PTB (1–130)-GFP both harboring the nuclear localization sequence (12) are localized in the nucleus (Figure 2C). However, most PTB (131–257)-GFP and PTB (409–526)-GFP are also localized in the nucleus, which might be resulted from the interaction with other hnRNPs (hnRNP A1, C1, E2, K and L) through RRM2 (25) and from the strongest RNA-binding activity of the C-terminal part of PTB (26).

As phosphorylation of PTB at Serine 16 was reported to mediate its cytoplasmic exportation (11), we mutated Serine 16 to Alanine (A) to mimic Serine de-phosphorylation and Serine 16 to Asparate (D) or Glutamate (E) to mimic PTB constitutive phosphorylation (Figure 2A). However, no significant difference was observed in the dimerization of PTB (Figure 2E), indicating that the phosphorylation status of PTB Serine 16 does not influence its dimerization. As disulfide bonds formed between the sulfhydryl (SH) side chains of cysteine residues are frequently found in protein dimer, and Cysteine 23 is the only cysteine between aa. 1 and aa.130, we mutated Cysteine 23 to Serine to disrupt potential Cysteine 23 mediated disulfide bond formation (Figure 2A). To exclude the possibility that phosphorylation of S16 contributes to C23 mediated dimerization, double mutations of Serine 16 and Cysteine 23 were also made (Figure 2A). C23S mutation alone was sufficient to completely disrupt PTB dimerization (Figure 2F and G). Therefore, the disulfide bond involving Cysteine 23 is responsible for the dimerization of PTB.

PDI catalyzes de-dimerization of PTB

The formation of disulfide bonds between cysteine residues, which mostly takes place in the endoplasmic reticulum, is a rate-limiting step for the correct folding of proteins. The thioredoxin superfamily, which includes 21 members, catalyze the formation, breakage and rearrangement of most disulfide bonds (15). We used siRNAs to identify potential enzymes responsible for the dimerization of PTB and found that the silencing of PDI enhanced the dimerization of PTB at most (Supplementary Figure S1 and Figure 3A). Overexpression of PDI, however, did not significantly alter the ratio of PTB dimer and monomer (Figure 3B). As PDI may act as an oxidase, a reductase or an isomerase, requiring electron acceptor or electron donor depending on the reacting state (16). We then treated cells with NADP or NADPH with the over expression of PDI, and, found that the addition of NADPH but not NADP induced the de-dimerization of PTB (Figure 3D), suggesting that PDI is an enzyme that catalyzes the de-dimerization of PTB.

PDI structurally comprises four thioredoxin-like domains (a, b, b' and a') that are required for substrate binding and a C-terminal acidic tail (Figure 3C) (27,28). The a and a' domains, which contain a CGHC active site respectively, are responsible for the thiol–disulfide interchange (15). To identify the active sites responsible for PTB de-dimerization, three mutants including C53S/C56S, C397S/C400S, and C53S/C56S/C397S/C400S were constructed to inactive the active site of domain a, a' and both of them, respectively. And mutation of domain a' or domain a and a' but not domain a alone failed to de-dimerize PTB with the addition of NADPH (Figure 3E), indicating that the CGHC active site of the a' domain of PDI is responsible for the de-dimerization of PTB.

De-dimerization of PTB is accompanied with p53 upregulation upon DNA Damage

PTB was reported to translocate from nucleus to cytoplasm following DNA damage (19). To explore whether there are changes in the dimerization of PTB, we used etoposide, which induces DNA double stranded breaks (DSBs) by forming complex with topoisomerase II and DNA (29,30), to generate DNA damage. The nucleus was almost two times larger after etoposide treatment (Supplementary Figure S2A and S2B), and the cells were arrested at G2/M phase (Supplementary Figure S2C). And p53 and γ-H2X, indicators of DNA damages at DSBs, and Cyclin E and Cyclin B1 which activate G2/M transition or S phase entry, were up regulated, whereas Rb which inhibits G1/S transition was suppressed (Figure 4B and Supplementary Figure S2A). There was a significant shift of PTB protein from dimer to monomer which was accompanied with its accumulation in the cytoplasm and an enhanced expression of PDI (Figure 4A and B).

Doxorubicin, another topoisomerase inhibitor, also induced the de-dimerization of PTB and the upregulation of p53 and PDI (Figure 4C). And CCF642, which specially inhibits the reductase activity of PDI, showed to promote the dimerization of PTB with decreasing PTB monomer and p53 (Figure 4D), and to suppress the de-dimerization of PTB and the upregulation of p53 induced by doxorubicin treatment (Figure 4E).

Moreover, protein kinases ATM and ATR are key components that are recruited to damaged DNA sites and then activate DNA repair and cell cycle arrest (31). We used small

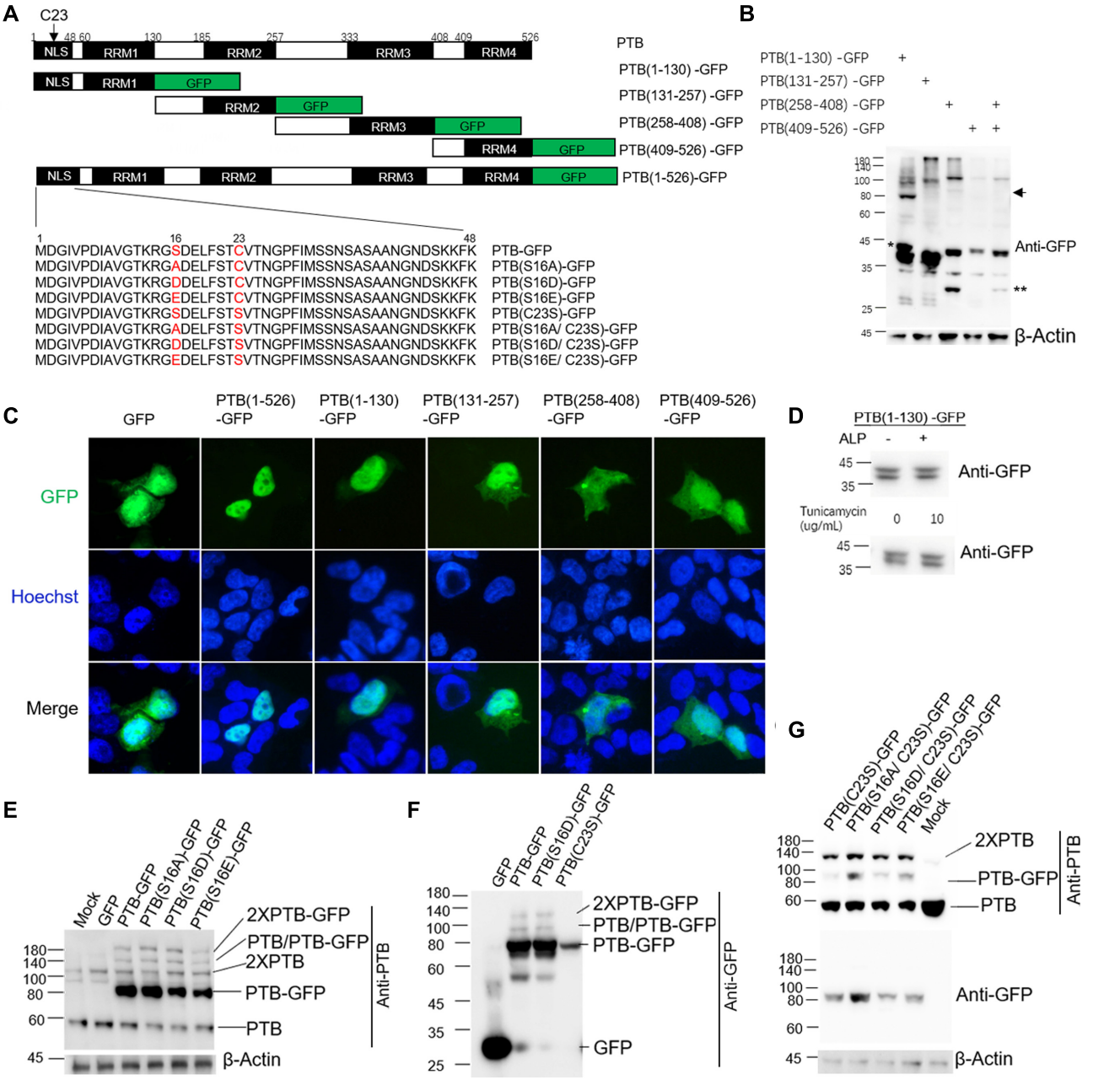

Figure 2. Cysteine 23 is involved in the dimerization of PTB. (**A**) Schematic representation of full-length PTB domain structure and truncated PTB proteins fused with GFP. Individual or combined mutations of Serine 16 and Cysteine 23 were indicated below. (**B**) Expression of indicated truncated proteins under non-reducing SDS-PAGE. The arrow indicates the dimer. The star indicates potential modified protein. Two stars indicate potential cleaved products. β-Actin is used as the loading control. (**C**) Immuno-localization (green signal) of truncated PTB protein is counterstained with Hoechst (blue signal). Hoechst is used for nuclear staining. (**D**) Expression of PTB (1–130)-GFP under non-reducing conditions without or with the treatment of tunicamycin. (**E**–**G**) Expression of individual or combined mutations of S16A, S16D and C23S of PTB and were analyzed by western blotting under non-reducing conditions. β-Actin was used as the loading control.

molecules, KU-60019 and ETP-46464, to inhibit the activation of ATM and ATR, and found that the de-dimerization of PTB and the up-regulation of p53 induced by etoposide were inhibited (Figure 4F). These results indicated that the de-dimerization of PTB is regulated by PDI and is involved in ATM/ATR/p53 signaling pathways upon DNA damage.

PTB monomer in the cytoplasm enhances p53 translation

P53 is critical for the DNA damage response after the injury of the genome (32), during which PTB is de-dimerized and accumulated in the cytoplasm as suggested by the above data. Previous reports showed that PTB physically interacts with the IRES and 3′UTR of *TP53* mRNA, which suggested the function to enhance the expression of p53

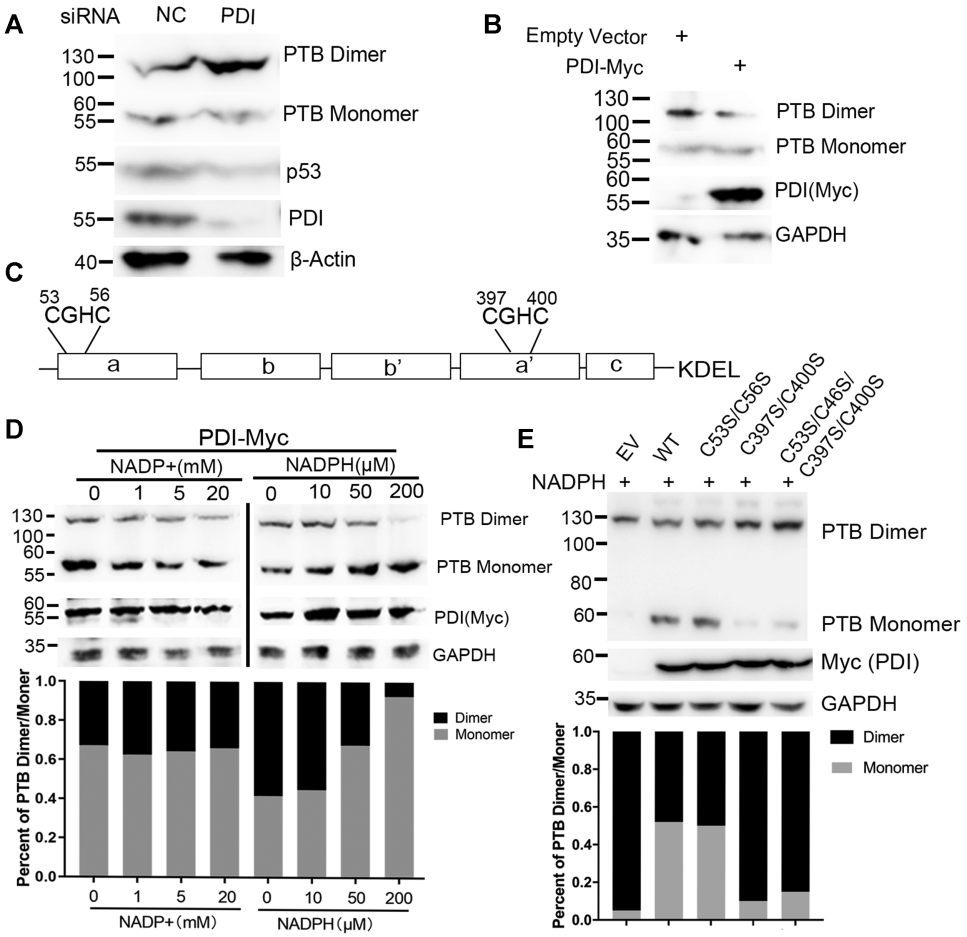

Figure 3. Regulation of PTB dimerization by PDI. (**A**) The expression level of indicated proteins treated with siRNAs against NC or PDI were analyzed by non-reducing SDS-PAGE. β-Actin was used as the loading control. (**B**) The expression level of indicated proteins with over expression of PDI were analyzed by non-reducing SDS-PAGE. β-Actin was used as the loading control. (**C**) Schematic illustration of PDI domain structures. The CGHC active sites were indicated above. (**D**) Expression of PTB dimer and monomer with over expression of PDI treated with NADP or NADPH at indicated concentrations for 12 h were analyzed by non-reducing SDS-PAGE and quantified below. GAPDH was used as the loading control. (**E**) Expression of PTB with over expression of PDI WT and PDI mutants were analyzed under non-reducing SDS-PAGE and quantified below. GAPDH was used as the loading control.

(19,24). The C23S mutant of PTB was found to localize in the nucleus as it harbors the nuclear localization signal at the N-terminal (Supplementary Figure S3A). To explore whether the dimerization status and localization of PTB are involved in the regulation of p53, we generated PTB ΔNLS-GFP by deleting the N-terminal nuclear localization signal (NLS), which formed granules outside of the nucleus (Supplementary Figure S3B). We overexpressed PTB, PTB C23S and PTB ΔNLS in HEK-293 cells, and found that only PTB ΔNLS slightly enhanced p53 expression (Figure 5A).

In line with previous reports (19,24), knocking down of PTB suppressed p53 expression (Figure 5C). Moreover, PTB was knocked out in HEK-293 cells using CRISPR–Cas9 to exclude the impact of endogenous PTB (Figure 5B). Re-expression of PTB ΔNLS-GFP but not PTB-GFP and PTB C23S-GFP rescued the suppression of p53 expression after the knocking out of endogenous PTB, suggesting that only PTB monomer in the cytoplasm enhanced p53 expression. As the knocking out and re-expression of PTB did not influence *TP53* mRNA expression (Figure 5D) and localization (Supplementary Figure S4), we reasoned that PTB ΔNLS-GFP might enhanced p53 translation. Furthermore, in the presence of MG-132 that blocks proteasome mediated protein degradation pathway, overexpression of PTB ΔNLS-GFP did promoted p53 expression (Figure 5E). These results indicate that PTB monomer in the cytoplasm enhances p53 translation.

Cytoplasmic PTB monomer interacts with ribosomal proteins

To identify the interacting proteins of cytoplasmic PTB monomer, we then performed the immunoprecipitation of GFP and PTB ΔNLS-GFP with magnetic beads against GFP, following SDS-PAGE and silver staining 8 specific regions were characterized with mass spectrometry (Figure 6A). A total of 86 proteins were identified (Supplementary Table S1). Proteomic network analysis recognized 24 ribosome proteins and another four proteins (EIF4A1, EIF4A3, EIF6 and EEF1A1) that are involved in the initiation and elongation of protein translation, and seven proteins are components of the mitochondria ribosome and one (TUFM) is responsible for the binding of aminoacyl-

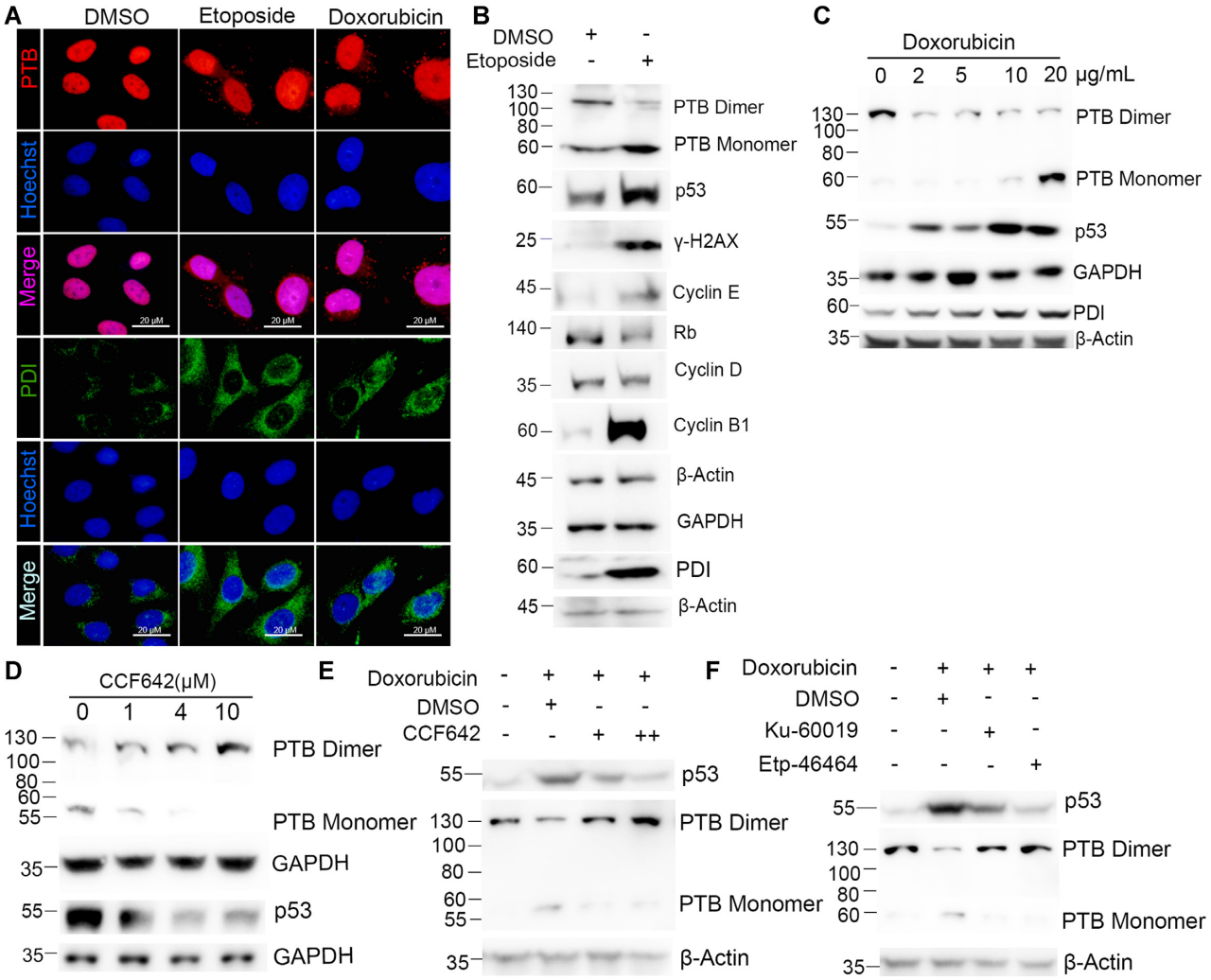

Figure 4. De-dimerization of PTB upon DNA damage. (**A**) HEK-293 cells were treated with etoposide (10 μg/ml) and doxorubicin (10 μg/ml) for 24 hours. Immuno-fluorescent staining of PTB and PDI were counterstained with Hoechst. (**B**) Expression of PTB dimer and monomer were analyzed by non-reducing SDS-PAGE with or without the treatment of etoposide. The expression of other proteins indicated were analyzed by reducing SDS-PAGE. β-Actin and GAPDH were used as loading controls. (**C**) Expression of PTB dimer and monomer with the treatment of indicated concentrations of doxorubicin for 8 hours were analyzed by non-reducing SDS-PAGE. The expression of p53 and PDI were analyzed by reducing SDS-PAGE. β-Actin and GAPDH were used as loading controls. (**D**) HEK-293 cells were treated with indicated concentrations of CCF642, and the expression of PTB dimer and monomer were analyzed by non-reducing SDS-PAGE and quantified on the right. (**E**) HEK-293 cells were treated with or without doxorubicin and CCF642, expression of PTB dimer/monomer and p53 were analyzed by non-reducing and reducing SDS-PAGE. β-Actin was used as the loading control. (**F**) HEK-293 cells were treated with or without doxorubicin, Ku-60019 (10 μM) and Etp-46464 (20 μM), expression of PTB dimer/monomer and p53 were analyzed by non-reducing and reducing SDS-PAGE. β-Actin was used as the loading control.

tRNA to the A-site of ribosomes during protein synthesis in mitochondria (Figure 6B). Importantly, RPS6, a component of the ribosome 40S subunit, was verified as an interacting partner of PTB ΔNLS (Figure 6C). These data indicate that cytoplasmic PTB monomer associated with ribosome and mitochondria ribosome plays an important function in protein translation.

DISCUSSION

PTB is well known as an RNA binding protein that exists not only as dimer but also as monomer, and regulates RNA metabolism in both nucleus and cytoplasm (1,10,12,13). However, how the differential localization and dimerization of PTB might contribute to the biological functions of PTB are overlooked. Here, we firstly showed that PTB localized at nucleus and cytoplasm exist as dimer and monomer respectively. Then by truncations and point mutations, we confirmed Cysteine 23 to be critical for the dimerization of PTB. Additionally, by screening the thioredoxin superfamily, we identified PDI to be the enzyme catalyzing the de-dimerization of PTB, which process is dependent on the CGHC active site of the a' domain of PDI. Furthermore, upon DNA damage, PTB accumulated in the cytoplasm and was accompanied with de-dimerization which required PDI. Finally, we demonstrated that exogenous cytoplasmic PTB associated with the ribosome and enhanced the translation of p53.

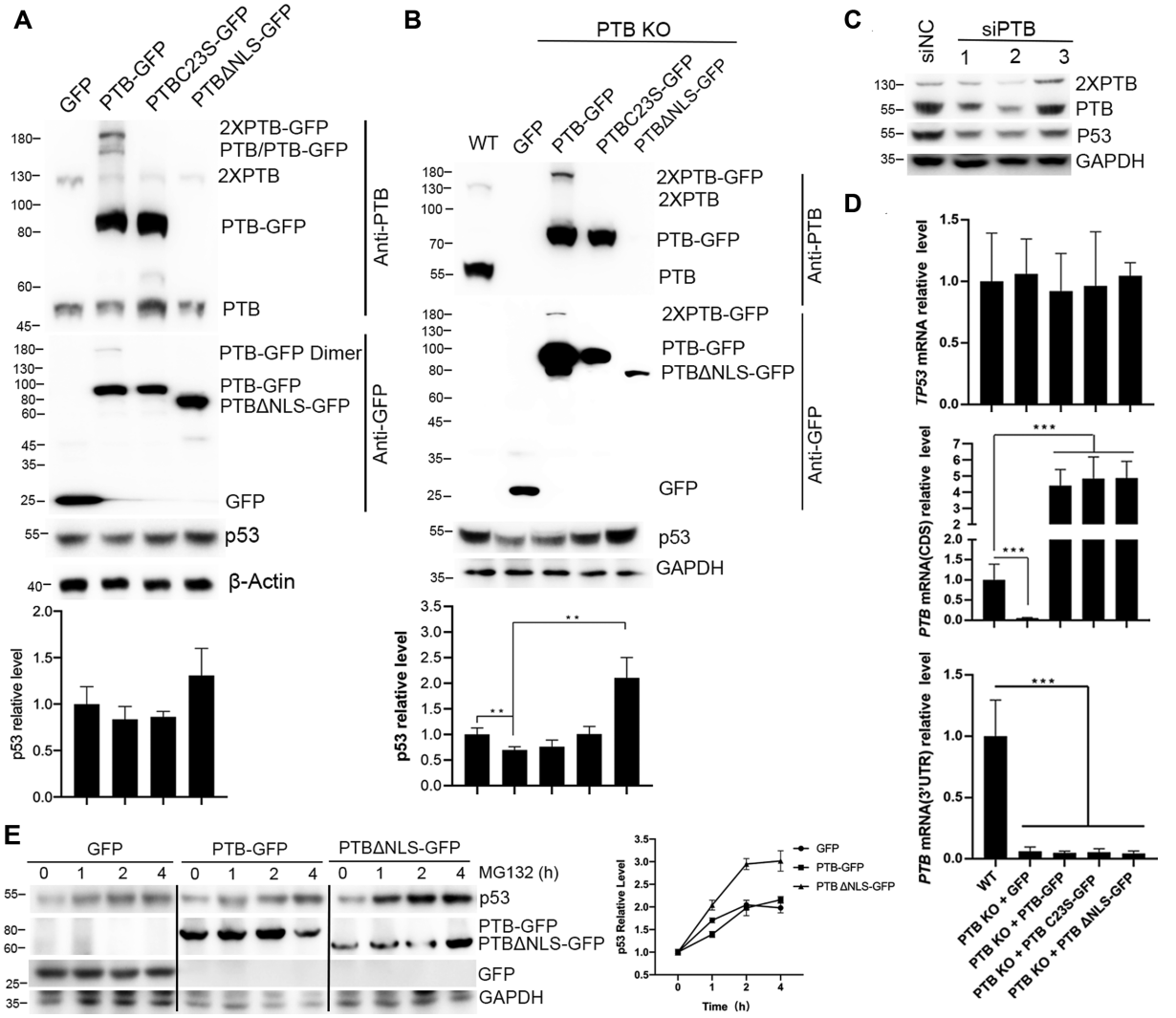

Figure 5. Cytoplasmic PTB monomer promotes p53 translation. (**A**) GFP, PTB-GFP and PTB C23S were over expressed in WT HEK-293 cells, and the expression level of indicated proteins were analyzed by western blotting. β-Actin was used as the loading control. The expression level of p53 is quantified and shown below. (**B**) PTB is knocked out in HEK293 cells using CRISPR-Cas9, PTB, PTB C23S mutant or PTB with N-terminal nuclear localization signal deleted (PTB ΔNLS) were fused with GFP and were expressed in PTB KO cells. The expression of p53, endogenous and exogenous PTB were detected by western blotting using antibodies against PTB or GFP. PTB ΔNLS was not recognized by the PTB antibody used but was recognized by the GFP antibody. The expression level of p53 was quantified and shown below. GAPDH was used as the loading control. Data were represented as mean ± SD as indicated from three independent experiments. **, $P < 0.01$. (**C**) Expression of p53 treated with siRNAs against NC or PTB was analyzed by western blotting. (**D**) The mRNA levels of *TP53* corresponding to (B) were analyzed by RT-qPCR. And primers for the amplification of PTB CDS and 3′UTR were used for RT-qPCR to confirm the efficiency of knocking out and re-expression of PTB. ***$P < 0.001$. (**E**) Expression of p53 with the treatment of MG132 (20 μM) for indicated times in PTB KO cells with overexpression of GFP, PTB -GFP or PTB ΔNLS-GFP were analyzed by western blotting and quantified as shown on the right.

Although the existence of PTB dimer was raised in 1997 (12), the relationship between PTB dimerization and localization is unclear. By non-reducing SDS-PAGE and western blotting analysis, we firstly demonstrated that nuclear localized PTB is dimer and cytoplasm localized PTB is monomer. To find out the critical elements that mediate PTB dimerization, we screened the four RRMs by truncation and narrowed the candidates to RRM1. The results were different from previous reports in which purified recombinant constructs of GST-PTB with deletions were analyzed and the primary determinant for dimerization was mapped to RRM2 (10,14,33). The discrepancy might be due to the different strategies used in our study and the above literatures. In addition, RRM1 primarily localized in the nucleus in our study, which is consistent with the finding that the first 60 amino acids of PTB encode the nuclear localization signal (12). Phosphorylation of PTB at Serine-16 by PKA was shown to block nuclear import and regulate stabilities and localizations of target mRNA (11,34). By mutation of Serine 16 we further showed that phosphorylation at Serine 16 did not significantly influence PTB dimerization.

Moreover, the molecule that catalyzes PTB dimerization or de-dimerization was unknown. Disulfide bonds are present in nearly one-third of all eukaryote proteins and are critical for correct protein folding and functions. PDI

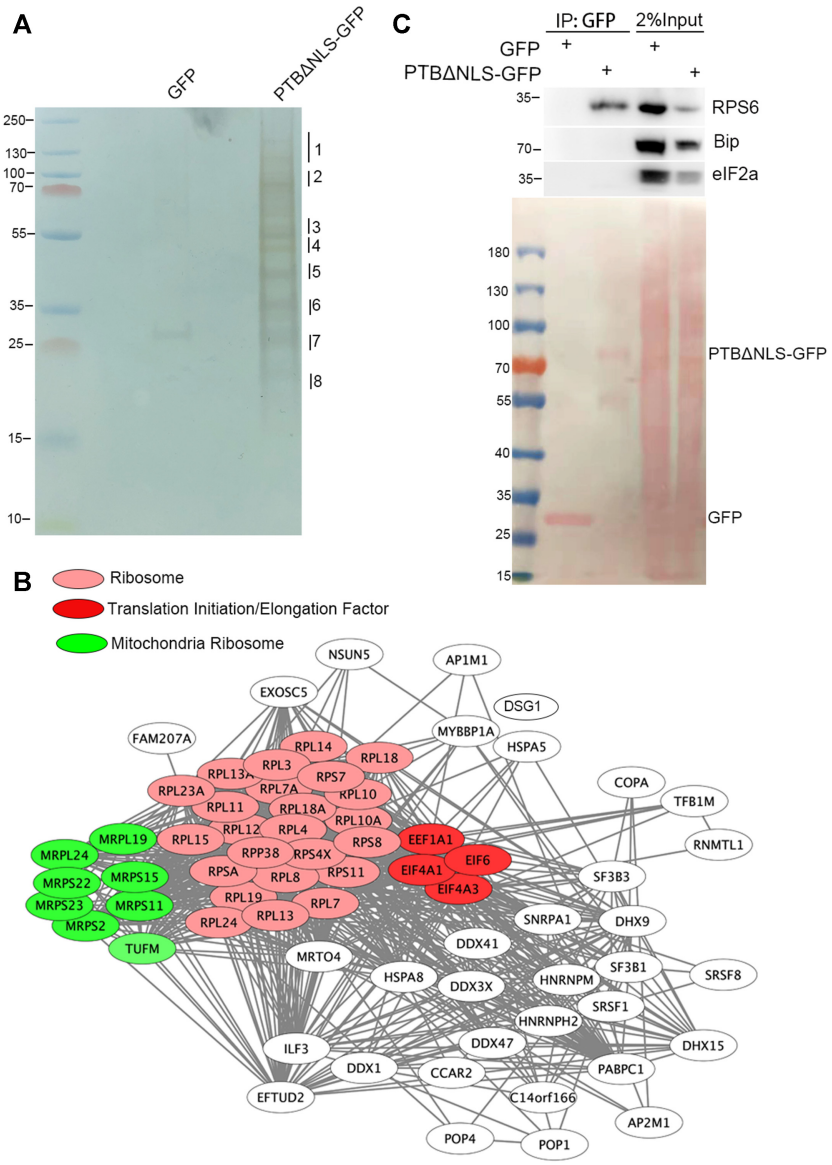

Figure 6. PTB ΔNLS-GFP associates with ribosome. (**A**) Silver staining of proteins immunoprecipitated by exogenous GFP and PTB ΔNLS-GFP in HEK-293 cells. Precipitated GFP was indicated with an arrow. Eight bands as indicated were performed with MS. (**B**) STRING network analysis of PTB ΔNLS-GFP interactome in HEK-293 cell. Rose Red Circles, Ribosome proteins; Red Circles, Translation initiation or elongation factors; Green circles, Mitochondria ribosome proteins. (**C**) Anti-GFP immunoprecipitates (IP) were prepared from cells expressing GFP or PTB ΔNLS-GFP. RPS6, Bip and eIFa were detected in IP and 2% of the total cell lysates by western blot analysis. RPS6 but not Bip (an endoplasmic protein) and eIF2α (involved in translation initiation) was identified in the IP-MS of PTB ΔNLS-GFP.

family of enzymes, predominately localizing at endoplasmic reticulum (ER), are essential for the formation and breakage of disulfide bonds (15). At least three members of the PDI family, including PDI, ERP57 and ERP72, have been found in non-ER locations, and PDI has been identified in mitochondria, cytosol, nucleus, cell surface and extracellular space (35). PDI can function both as an enzyme and as a molecular chaperone (36), and the non-ER located PDI are suggested to be mostly related to its reduction property (37). And both domain a and domain a' of ERP57 were able to oxidase TG2 for inactivation (35). Here we proved that a' domain required PDI is responsible for the de-dimerization of PTB.

PDI is highly expressed in many cancers, and the inhibition of PDI sensitizes radiotherapy of glioblastoma by inhibiting the expression of DNA repair and DNA damage response genes (37). We found that PDI expression was enhanced to catalyze the de-dimerization of PTB upon DNA damage. And when the reductase activity of PDI or the ATM/ATR pathway was chemically inhibited, the upregulation of p53 under the treatment of doxorubicin was inhibited, suggesting that the increased PDI is linked to the regulation of p53 by PTB.

Under DNA damage conditions, the *TP53* mRNA level is not changed but the 5′UTR-IRES mediated translation of p53 is enhanced by favored association with active polyribo-

somes (20). PTB is one of the RNA binding proteins that enhances p53 expression by interacting with the 5′UTR-IRES of *TP53* mRNA (19). In our study, the results of the inhibitor for PDI reductase activity and the overexpression of PTB ΔNLS-GFP in the presence of MG-132 demonstrated that PTB monomer in the cytoplasm enhances p53 translation under doxorubicin treatment. As IRES-mediated expression of p53 is high at mitosis (19,38), the data that cell cycle arrest at mitosis was accompanied with more PTB existing as monomer also support that PTB monomer contributes to the elevated expression of p53. Furthermore, the association between cytoplasmic PTB and ribosome proteins and translation initiation/elongation factors suggests that PTB might promote p53 translation by enhancing the interaction between *TP53* mRNA and the translational machinery.

Interestingly, when the nuclear localization signal of PTB was deleted and PTB ΔNLS-GFP was overexpressed, the cytoplasmic PTB was observed to form granules outside of the nucleus. The references report that mutations of hnRNP A1 and hnRNP A2/B1 in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) cause their sequestration in cytosolic stress granules (39,40). Therefore, our results suggest that cytoplasmic PTB might have the property of self-aggregation, which needs to be addressed in the future.

In summary, we describe here a PDI dependent mechanism that mediates the conversion of PTB dimer and monomer, and cytoplasmic PTB monomer is a functional bridge to link TP53 mRNA and the translational machinery.

DATA AVAILABILITY

Mass spectrometry proteomics data generated in this study have been deposited in the ProteomeXchange consortium under accession number: PXD026837

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Black,D.L. (2003) Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.*, **72**, 291–336.
2. Vuong,J.K., Lin,C.H., Zhang,M., Chen,L., Black,D.L. and Zheng,S. (2016) PTBP1 and PTBP2 serve both specific and redundant functions in neuronal pre-mRNA splicing. *Cell Rep.*, **17**, 2766–2775.
3. La Porta,J., Matus-Nicodemos,R., Valentín-Acevedo,A. and Covey,L.R. (2016) The RNA-binding protein, polypyrimidine tract-binding protein 1 (PTBP1) is a key regulator of CD4 T cell activation. *PLoS One*, **11**, e0158708.
4. Domingues,R.G., Lago-Baldaia,I., Pereira-Castro,I., Fachini,J.M., Oliveira,L., Drpic,D., Lopes,N., Henriques,T., Neilson,J.R., Carmo,A.M. *et al.* (2016) CD5 expression is regulated during human T-cell activation by alternative polyadenylation, PTBP1, and miR-204. *Eur. J. Immunol.*, **46**, 1490–1503.
5. Tang,S.J., Luo,S., Ho,J.X., Ly,P.T., Goh,E. and Roca,X. (2016) Characterization of the Regulation of CD46 RNA Alternative Splicing. *J. Biol. Chem.*, **291**, 14311–14323.
6. Sasanuma,H., Ozawa,M. and Yoshida,N. (2019) RNA-binding protein Ptbp1 is essential for BCR-mediated antibody production. *Int. Immunol.*, **31**, 157–166.
7. He,X., Arslan,A.D., Ho,T.T., Yuan,C., Stampfer,M.R. and Beck,W.T. (2014) Involvement of polypyrimidine tract-binding protein (PTBP1) in maintaining breast cancer cell growth and malignant properties. *Oncogenesis*, **3**, e84.
8. Cui,J. and Placzek,W.J. (2016) PTBP1 modulation of MCL1 expression regulates cellular apoptosis induced by antitubulin chemotherapeutics. *Cell Death Differ.*, **23**, 1681–1690.
9. Yang,B., Hu,P., Lin,X., Han,W., Zhu,L., Tan,X., Ye,F., Wang,G., Wu,F., Yin,B. *et al.* (2015) PTBP1 induces ADAR1 p110 isoform expression through IRES-like dependent translation control and influences cell proliferation in gliomas. *Cell. Mol. Life Sci.*, **72**, 4383–4397.
10. Oh,Y.L., Hahm,B., Kim,Y.K., Lee,H.K., Lee,J.W., Song,O., Tsukiyama-Kohara,K., Kohara,M., Nomoto,A. and Jang,S.K. (1998) Determination of functional domains in polypyrimidine-tract-binding protein. *Biochem. J.*, **331**, 169–175.
11. Xie,J., Lee,J.A., Kress,T.L., Mowry,K.L. and Black,D.L. (2003) Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 8776–8781.
12. Perez,I., McAfee,J.G. and Patton,J.G. (1997) Multiple RRMs contribute to RNA binding specificity and affinity for polypyrimidine tract binding protein. *Biochemistry*, **36**, 11881–11890.
13. Monie,T.P., Hernandez,H., Robinson,C.V., Simpson,P., Matthews,S. and Curry,S. (2005) The polypyrimidine tract binding protein is a monomer. *RNA*, **11**, 1803–1808.
14. Oberstrass,F.C., Auweter,S.D., Erat,M., Hargous,Y., Henning,A., Wenter,P., Reymond,L., Amir-Ahmady,B., Pitsch,S., Black,D.L. *et al.* (2005) Structure of PTB bound to RNA: specific binding and implications for splicing regulation. *Science*, **309**, 2054–2057.
15. Wilkinson,B. and Gilbert,H.F. (2004) Protein disulfide isomerase. *Biochim. Biophys. Acta*, **1699**, 35–44.
16. Xu,S., Sankar,S. and Neamati,N. (2014) Protein disulfide isomerase: a promising target for cancer therapy. *Drug Discov. Today*, **19**, 222–240.
17. Xu,S., Liu,Y., Yang,K., Wang,H., Shergalis,A., Kyani,A., Bankhead,A., Tamura,S., Yang,S., Wang,X. *et al.* (2019) Inhibition of protein disulfide isomerase in glioblastoma causes marked downregulation of DNA repair and DNA damage response genes. *Theranostics*, **9**, 2282–2298.
18. Liu,Y., Ji,W., Shergalis,A., Xu,J., Delaney,A.M., Calcaterra,A., Pal,A., Ljungman,M., Neamati,N. and Rehemtulla,A. (2019) Activation of the unfolded protein response via inhibition of protein disulfide isomerase decreases the capacity for DNA repair to sensitize glioblastoma to radiotherapy. *Cancer Res.*, **79**, 2923–2932.
19. Grover,R., Ray,P.S. and Das,S. (2008) Polypyrimidine tract binding protein regulates IRES-mediated translation of p53 isoforms. *Cell Cycle*, **7**, 2189–2198.
20. Yang,D.Q., Halaby,M.J. and Zhang,Y. (2006) The identification of an internal ribosomal entry site in the 5′-untranslated region of p53 mRNA provides a novel mechanism for the regulation of its translation following DNA damage. *Oncogene*, **25**, 4613–4619.
21. Kastan,M.B., Onyekwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.*, **51**, 6304–6311.
22. Fu,L. and Benchimol,S. (1997) Participation of the human p53 3′UTR in translational repression and activation following gamma-irradiation. *EMBO J.*, **16**, 4117–4125.

23. Haronikova,L., Olivares-Illana,V., Wang,L., Karakostis,K., Chen,S. and Fåhraeus,R. (2019) The p53 mRNA: an integral part of the cellular stress response. *Nucleic Acids Res.*, **47**, 3257–3271.
24. Katoch,A., George,B., Iyyappan,A., Khan,D. and Das,S. (2017) Interplay between PTB and miR-1285 at the p53 3′UTR modulates the levels of p53 and its isoform Delta40p53alpha. *Nucleic Acids Res.*, **45**, 10206–10217.
25. Kim,J.H., Hahm,B., Kim,Y.K., Choi,M. and Jang,S.K. (2000) Protein-protein interaction among hnRNPs shuttling between nucleus and cytoplasm. *J. Mol. Biol.*, **298**, 395–405.
26. Oh,Y.L., Hahm,B., Kim,Y.K., Lee,H.K., Lee,J.W., Song,O., Tsukiyama-Kohara,K., Kohara,M., Nomoto,A. and Jang,S.K. (1998) Determination of functional domains in polypyrimidine-tract-binding protein. *Biochem. J.*, **331**, 169–175.
27. Klappa,P., Ruddock,L.W., Darby,N.J. and Freedman,R.B. (1998) The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. *EMBO J.*, **17**, 927–935.
28. Pirneskoski,A., Klappa,P., Lobell,M., Williamson,R.A., Byrne,L., Alanen,H.I., Salo,K.E., Kivirikko,K.I., Freedman,R.B. and Ruddock,L.W. (2004) Molecular characterization of the principal substrate binding site of the ubiquitous folding catalyst protein disulfide isomerase. *J. Biol. Chem.*, **279**, 10374–10381.
29. Berger,N.A., Chatterjee,S., Schmotzer,J.A. and Helms,S.R. (1991) Etoposide (VP-16-213)-induced gene alterations: potential contribution to cell death. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 8740–8743.
30. Burden,D.A. and Osheroff,N. (1998) Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochim. Biophys. Acta*, **1400**, 139–154.
31. Matsuoka,S., Ballif,B.A., Smogorzewska,A., McDonald,E.R., Hurov,K.E., Luo,J., Bakalarski,C.E., Zhao,Z., Solimini,N., Lerenthal,Y. *et al.* (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science*, **316**, 1160–1166.
32. Giaccia,A.J. and Kastan,M.B. (1998) The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.*, **12**, 2973–2983.
33. Pérez,I., McAfee,J.G. and Patton,J.G. (1997) Multiple RRMs contribute to RNA binding specificity and affinity for polypyrimidine tract binding protein. *Biochemistry*, **36**, 11881–11890.
34. Knoch,K.P., Meisterfeld,R., Kersting,S., Bergert,H., Altkruger,A., Wegbrod,C., Jager,M., Saeger,H.D. and Solimena,M. (2006) cAMP-dependent phosphorylation of PTB1 promotes the expression of insulin secretory granule proteins in beta cells. *Cell Metab.*, **3**, 123–134.
35. Laurindo,F.R., Pescatore,L.A. and Fernandes Dde,C. (2012) Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radic. Biol. Med.*, **52**, 1954–1969.
36. Wang,C.C. and Tsou,C.L. (1993) Protein disulfide isomerase is both an enzyme and a chaperone. *FASEB J.*, **7**, 1515–1517.
37. Turano,C., Coppari,S., Altieri,F. and Ferraro,A. (2002) Proteins of the PDI family: unpredicted non-ER locations and functions. *J. Cell. Physiol.*, **193**, 154–163.
38. Grover,R., Candeias,M.M., Fåhraeus,R. and Das,S. (2009) p53 and little brother p53/47: linking IRES activities with protein functions. *Oncogene*, **28**, 2766–2772.
39. Kim,H.J., Kim,N.C., Wang,Y.D., Scarborough,E.A., Moore,J., Diaz,Z., MacLea,K.S., Freibaum,B., Li,S., Molliex,A. *et al.* (2013) Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature*, **495**, 467–473.
40. Geuens,T., Bouhy,D. and Timmerman,V. (2016) The hnRNP family: insights into their role in health and disease. *Hum. Genet.*, **135**, 851–867.