

# Ataxin-3 promotes genome integrity by stabilizing Chk1

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## ABSTRACT

**The Chk1 protein is essential for genome integrity maintenance and cell survival in eukaryotic cells. After prolonged replication stress, Chk1 can be targeted for proteasomal degradation to terminate checkpoint signaling after DNA repair finishes. To ensure proper activation of DNA damage checkpoint and DNA repair signaling, a steady-state level of Chk1 needs to be retained under physiological conditions. Here, we report a dynamic signaling pathway that tightly regulates Chk1 stability. Under unperturbed conditions and upon DNA damage, ataxin-3 (ATX3) interacts with Chk1 and protects it from DDB1/CUL4A- and FBXO6/CUL1-mediated polyubiquitination and subsequent degradation, thereby promoting DNA repair and checkpoint signaling. Under prolonged replication stress, ATX3 dissociates from Chk1, concomitant with a stronger binding between Chk1 and its E3 ligase, which causes Chk1 proteasomal degradation. ATX3 deficiency results in pronounced reduction of Chk1 abundance, compromised DNA damage response, G2/M checkpoint defect and decreased cell survival after replication stress, which can all be rescued by ectopic expression of ATX3. Taken together, these findings reveal ATX3 to be a novel deubiquitinase of Chk1, providing a new mechanism of Chk1 stabilization in genome integrity maintenance.**

## INTRODUCTION

The evolutionally conserved DNA damage response and checkpoint pathway guarantee genome stability. Four crucial protein kinases form two canonical signal axes: ATM-

Chk2 and ATR-Chk1. ATM-Chk2 pathway principally responds to double strand break (DSB), while ATR-Chk1 can be activated by various kinds of DNA damage insults, including replication stress, interstrand cross-link (ICL), virus infection and DSBs (1–6). Chk1, an important effector kinase in these genome surveillance pathways, is activated by DNA damage or replication stress. Activated Chk1 delays cell cycle progression to facilitate DNA repair or to induce cell death if the damage is too severe to be repaired (7–9). Furthermore, Chk1 also regulates mono-ubiquitination of proliferating cell nuclear antigen (PCNA) and Fanconi anemia complementation group D2 (FANCD2), and promotes homologous recombination (HR) repair (10–14). Besides, Chk1 is also active in unperturbed cell cycles and performs critical functions in gene transcription, embryo development and somatic cell viability (7,9,15–19).

To optimize cellular responses to DNA damage, Chk1 activity must be precisely regulated. So far, various mechanisms have been reported to modulate Chk1 activity, including protein post-translational modifications (9,20). In response to DNA damage or replicative stress, ATR-induced phosphorylation of Chk1 at S317 and S345 activates Chk1, thus regulating various signal pathways, such as DNA repair, cell cycle arrest and cell death in the case of excessive DNA damage (21), while dephosphorylation of activated Chk1 by PP1 and WIP1 promotes cell cycle recovery (22,23).

In addition to phosphorylation and dephosphorylation, ubiquitination of Chk1 has emerged as an important mechanism that modulates its overall activity. The Lys63-linked ubiquitination of Chk1 mediated by B-cell translocation gene 3 is reported to promote its chromatin localization and activation (24), while polyubiquitination and proteasomal degradation of Chk1 mediated by E3 ligase complexes SCF and CDT contributes to termination of Chk1 activity, allowing for essential control of checkpoint signaling

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(25–27). It has been demonstrated that ATR-mediated S345 phosphorylation of Chk1 not only activates Chk1 but also targets it for proteasomal destruction (25–28). Two E3 ligase complexes, CUL4A/DDB1 and CUL1/FBXO6, have shown to be responsible for Chk1 polyubiquitination and degradation; whereas deubiquitinases (DUB), USP1 and USP7, have been reported to promote Chk1 stabilization (29–31). However, whether the polyubiquitination and proteasomal degradation of Chk1 mediated by CUL4A/DDB1 and CUL1/FBXO6 can be reversed by deubiquitinases remains to be investigated.

ATX3 is a deubiquitinase which contains an N-terminal DUB activity domain, Josephin domain, followed by 2 or 3 ubiquitin-interacting motifs (UIMs) and variable length of polyglutamine (polyQ). The abnormal expansion of polyQ near the C-terminus of ataxin-3 (from 10–51 in normal individuals to 55–87 in affected population) causes a neurological disorder Machado-Joseph disease (MJD1, also known as spinocerebellar ataxia type 3, SCA3) characterized by progressive ataxia, spasticity, and ocular movement abnormalities (32–41). ATX3 is expressed ubiquitously in various tissues and cells (42,43). Two facts verify that ATX3 functions as a DUB *in vivo*: Inhibition of ATX3 catalytic activity by mutating its catalytic site cysteine14 leads to an obvious increase of polyubiquitinated proteins (44); ATX3 knockout mice display increased levels of ubiquitinated proteins (45). As a DUB, ATX3 is capable of binding polyubiquitin chains through its UIMs (46–49), preferring shortening polyubiquitin chains rather than complete disassembly (46,49–53). Nevertheless, the substrate(s) of ATX3 remains to be elucidated.

Here, we identified ATX3 as a novel deubiquitinase of Chk1. We have found that ATX3 exhibits dynamic interactions with Chk1 before/after prolonged replication stress, and that both the E3 ligase complexes SCF- and CDT-mediated polyubiquitin chains can be efficiently removed by ATX3 *in vitro* and *in vivo*. Consequently, ATX3 is essential for the maintenance of steady-state levels of Chk1, and ATX3-mediated deubiquitination of Chk1 significantly prolongs the half-life of Chk1, thus promoting checkpoint signaling and DNA repair upon genotoxic stress exposure. Moreover, ATX3 disassociates from Chk1 after prolonged replication stress, which allows Chk1 proteasomal degradation and thereby checkpoint termination. Our data demonstrate ATX3 to be a novel regulator of Chk1 stability, supporting a critical role of ATX3 in genome integrity maintenance via Chk1 stabilization.

## MATERIALS AND METHODS

### Tandem-affinity purification and mass spectrometry

For affinity purification, a total of five 10-cm dishes of 293T cells expressing ATX3-SBP-2 × Flag or control SBP-2 × Flag-vector were lysed in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% NP-40) containing protease inhibitors for 30 min at 4°C. Cell lysates were centrifuged, and the supernatants were incubated with 100  $\mu$ l M2 beads (SIGMA) for 2 h at 4°C. The beads were washed six times with NETN buffer, and the bound proteins were eluted with Flag peptide (in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4). The eluates were incubated with

50  $\mu$ l streptavidin Sepharose beads (GE Healthcare) for 2 h at 4°C, and the beads were washed six times with NETN buffer. The bound proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE, visualized by silver staining and subjected to mass spectrometric analysis.

### Immunoblotting and reagents

Proteins were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (Millipore). Membranes were probed with the specific primary antibodies, followed by peroxidase-conjugated secondary antibodies. The bands were visualized by Chemiluminescence (Millipore). Antibodies used in this study include: Chk1 (1:500, Santa Cruz Biotechnology, sc-8408 clone G-4), p-Chk1 (S345, 1:1000, Cell Signaling Technology, 2348 clone 133D3), Chk2 (1:10 000, Epitomics, 3428-1), p-Chk2 (T68, 1:1000, Cell Signaling Technology, 2661), ATX3 (1:10 000, Millipore, 5360), USP1 (1:1000, Cell Signaling Technology, D37B4), p-ATR (S428, 1:1000, Cell Signaling Technology, 2853), Myc (1:500, Santa Cruz Biotechnology, 9E10), Myc (1:1000, Covance, PRB-150P), M2 (1:2000, Sigma-Aldrich, F1804), polyclonal Flag (1:10 000, Sigma-Aldrich, 7425), HA (1:5000, Covance, PRB-101P), PCNA (1:500, Santa Cruz Biotechnology, PC10), H3 (1:10 000, Abmart, P30266), pH3 (S10, 1:1000, Cell Signaling Technology, D2C8), actin (1:10 000, proteintech, 00001-1), rabbit polyclonal ATX3 antibody was generated at Abmart. Camptothecin (CPT), hydroxyurea (HU), cycloheximide (CHX), nocodazole, reduced glutathione (rGTH), etoposide (ETO) and puromycin were from SIGMA, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was from ACROS ORGANICS, proteasome inhibitor MG132 was from Selleckchem, ATM and ATR inhibitor CGK733 was from Merck. Polyethylenimine, linear (23 966) was from Polysciences. Flag-DDB1 plasmid was from Addgene.

### Immunoprecipitation and pull down assays

Cells were lysed in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% NP-40) containing protease inhibitor (AMRESCO) and phosphatase inhibitors (NaF, NaVO<sub>3</sub>). After incubation for 30 min at 4°C, the total cell lysates were centrifuged. For endogenous immunoprecipitation, vehicle- or CPT-treated cell extracts were incubated with ATX3 pAb antibody overnight at 4°C and protein-A/G beads were added and incubated for 2 h at 4°C. For pulldown of Flag- or SFB-tagged proteins, cell extracts were coupled with M2 beads (Sigma-Aldrich) or streptavidin Sepharose beads (GE Healthcare) overnight at 4°C, followed by washing with NETN buffer. Samples were separated by SDS-PAGE and detected by immunoblotting with indicated antibodies. For the assay mapping the domain of Chk1 mediating its binding to ATX3, cells were treated with MG132 for 4 h before harvest as Chk1<sub>1–421</sub> truncation is unstable. For GST and His pulldown assays, bacterially purified GST-ATX3 or His-ATX3 immobilized on Glutathione Sepharose 4B beads (GE Healthcare) or Ni-NTA Agarose (QIAGEN) were incubated with 293T cell lysates. Endogenously bound Chk1 was analyzed by anti-Chk1 immunoblotting. Purified proteins were visualized by Ponceau S staining.

### Colony assay

A cell survival assay following genotoxic treatments was performed as described previously (54). Briefly, equal numbers of indicated cells were seeded into 6 cm dishes (500 cells per dish) in triplicate. Cells were incubated for 24 h before they were exposed to the indicated concentration of CPT or HU or different doses of UV. Cells were further cultured in complete medium for one week, and colonies were counted.

### Deubiquitination of Chk1 *in vivo* and *in vitro*

For the *in vivo* deubiquitination assay, transfected 293T cells were incubated with proteasome inhibitor MG132 (20  $\mu$ M) for 4 h before harvest. The cell extracts were subjected to immunoprecipitation and western blot analysis with the indicated antibodies. For preparation of ubiquitinated Chk1 as the substrate for the *in vitro* deubiquitination assay, 293T cells co-transfected with HA-ubiquitin, Flag-FBXO6/Flag-DDB1& Myc-CUL4A and PNTAP-Chk1 were treated with MG132 for 4 h before harvest. Ubiquitinated Chk1 was purified from the cell extracts with streptavidin Sepharose beads and followed by extensive washing with high salt NETN buffer (300 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% NP-40). *In vitro* deubiquitination reaction was performed as previously (55). In brief, ubiquitinated Chk1 was incubated with purified ATX3 in deubiquitination buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol and 5% glycerol) for 2 h at 37°C. And then, Chk1 was immunoprecipitated with SBP beads. The beads were washed with deubiquitination buffer, and the bound proteins were eluted by boiling in 2  $\times$  loading buffer and subjected to western blot analysis with the indicated antibodies. Alternatively, bacterially purified GST or GST-ATX3 or GST-ATX3 80Q immobilized on Sepharose beads were eluted with reduced glutathione (rGTH). And eluted GST fusion proteins were incubated with ubiquitinated Chk1 protein in deubiquitination buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) for 2 h at 37°C, and the bound proteins were eluted by boiling in 2  $\times$  loading buffer and subjected to western blot analysis with the indicated antibodies.

### Cell culture and shRNA

ATX3 WT and knockout (KO) mouse embryonic fibroblasts (MEFs) were from Drs Ina Schmitt and Ullrich Wüllner at University of Bonn (45). KO Cells stably expressing ATX3 or ATX3-C14A were selected by culturing in medium containing 1  $\mu$ g/ml puromycin and confirmed by immunoblotting. Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. Cell transfection was performed using PEI following the manufacturer's protocols.

ATX3 shRNAs were purchased from SIGMA.

ATX3 shRNA-1 (region: 3'UTR), sequence: CCGGCGTCGGTTGTAGGACTAAATACTCGA GTATTTAGTCCTACAACCGACGTTTTT ATX3 shRNA-2 (region: CDS) sequence: CCGGGCAGGGCTAT TCAGCTAAGTACTCGAGTACTTAGCTGAATAG CCCTGCTTTTT.

### Real-time PCR

Total RNA was extracted from WT and ATX3 KO MEF cells using the Trizol Reagent (Invitrogen), Purified RNA was then reversely transcribed to cDNA by using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed in triplicate on a Thermal Cycler using SYBR Green dye to measure amplification. GAPDH was chosen as an internal control for the normalization of the total cDNA. The primers used were as follows:

Chk1 Forward: GGATGCGGACAAATCTTACCA;  
Chk1 Reverse: CCTTAGAAAGTCGGAAGTCAACC;  
GAPDH Forward: GAGTCAACGGATTTGGTCGT;  
GAPDH Reverse: GACAAGCTTCCCGTTCTCAG.

### Cell cycle analysis

WT and ATX3 KO MEF cells treated with or without indicated DNA damage were harvested by trypsinization, washed twice with PBS, and then fixed in cold 70% ethanol overnight. Cells were collected by centrifugation and washed once with PBS, and then treated with RNase (suspended in PBS, containing 0.1% Triton X-100 and 1% BSA) for 20 min at room temperature, followed by incubation with 5  $\mu$ l of propidium iodide (PI) for 30 min in the dark. And finally, PBS was added to terminate the action of PI. Cell cycle distribution was analyzed by a FACSCalibur flow cytometer (BD Bioscience).

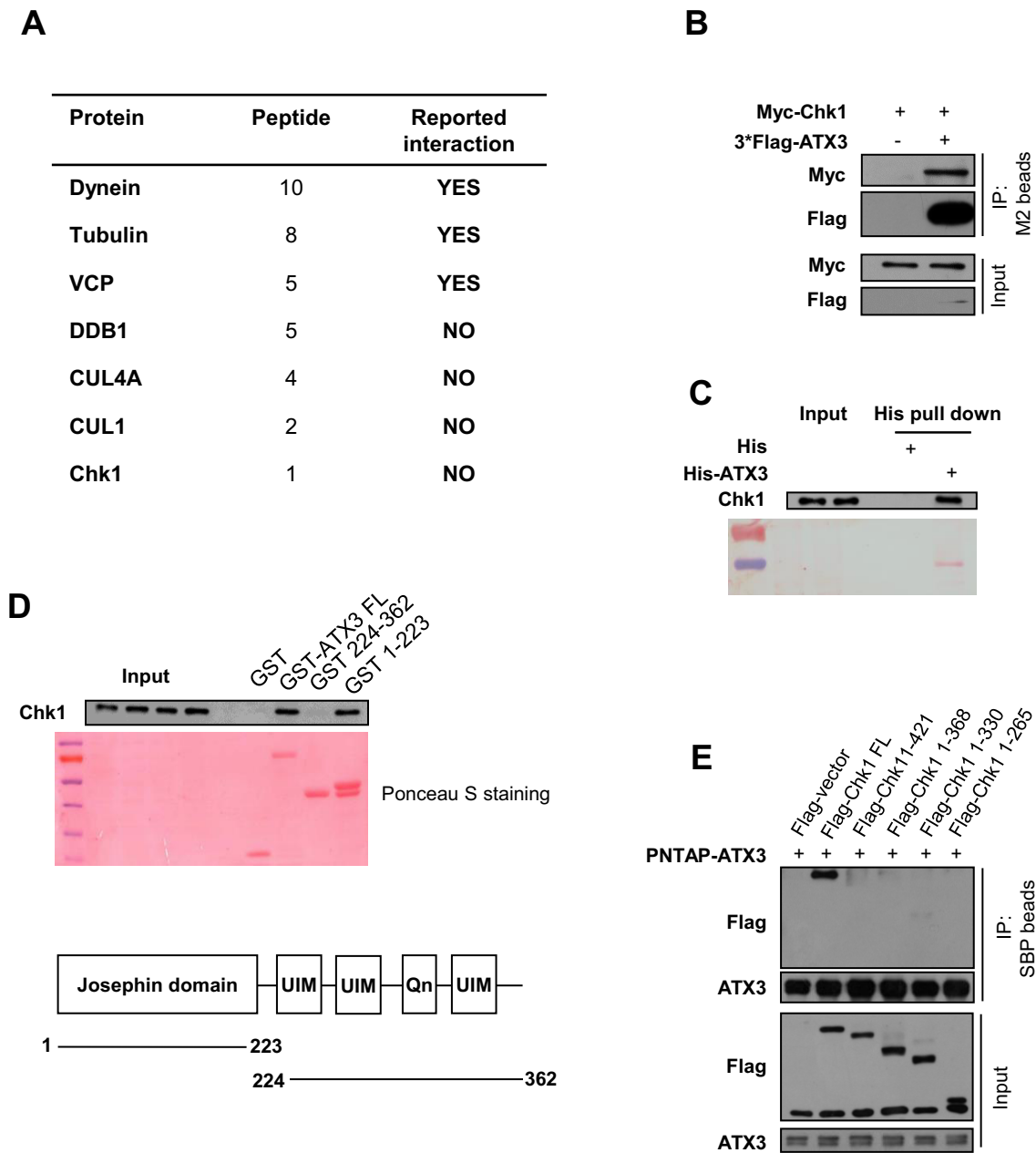
### Micronucleus assay

WT and KO cells were incubated for 12 h before they were treated with or without 100 nM CPT for 2 h, washed once with PBS, and cells were cultured in fresh DMEM containing 6  $\mu$ g/ml CB for another 24 h. Cells were washed once with PBS, trypsinized, washed again with PBS, followed by treatment with 0.075 mol/l KCl for 20 min. Cells were collected by centrifuge. Finally, acridine orange was added to stain the cells, and images were acquired using Leica.

## RESULTS

### Chk1 is a novel ATX3-interacting protein

To search for the potential substrates of ATX3 in DNA damage response, we performed immunoprecipitation experiments with SBP-2  $\times$  Flag-tagged ATX3 followed by mass spectrometry. Besides several previously reported ATX3-interacting proteins, such as VCP, dynein and tubulin (50,56,57), DDB1, CUL4A and Chk1 were identified in the immunoprecipitates (Figure 1A and Supplementary Figure S1A). The interactions between ATX3 and CUL4A or DDB1 were further confirmed by co-immunoprecipitation assays (Supplementary Figure S1B and S1C). E3 ligase complexes DDB1/CUL4A have been shown to be responsible for the polyubiquitination and degradation of Chk1 (25,27). Additionally, we also identified CUL1, an essential component of SCF E3 ligase complexes mediating Chk1 polyubiquitination and degradation, in our mass spectrometry experiments. These re-



**Figure 1.** Chk1 is a novel ATX3-interacting protein. (A) A partial list of proteins identified by mass spectrometry analysis. 293T cells transfected with control plasmid or plasmid encoding ATX3-SBP-2×Flag were subjected to tandem-affinity purification. Bound proteins were resolved by SDS-PAGE and stained with silver staining, followed by mass spectrometry. (B) Chk1 interacts with ATX3. 293T cells were transfected with Flag-ATX3 and Myc-Chk1, followed by immunoprecipitation with M2 beads and immunoblotting with antibodies against Myc and Flag. (C) His-pull down assay showing that ATX3 interacts with Chk1. 293T cell lysates were incubated with His-ATX3 immobilized on Ni-NTA beads, and bound Chk1 was detected by immunoblotting. Purified His-ATX3 was visualized by Ponceau S staining. (D) GST-pull-down experiments showing that Josephin domain of ATX3 is required for its association with Chk1. Purified GST, GST-tagged ATX3-full length, or its deletion mutants immobilized on Glutathione Sepharose beads were incubated with 293T cell lysates. Endogenous bound Chk1 was analyzed by anti-Chk1 antibody. Purified proteins were visualized by Ponceau S staining. Lower panel: truncations of ATX3 and numbers represent the amino acid residues in human ATX3. (E) Co-IP experiments showing that full length Chk1 is necessary for its interaction with ATX3. 293T cells were transfected with Flag-Chk1 fragments and PNTAP-ATX3, followed by immunoprecipitation with SBP beads and immunoblotting with indicated antibodies. Numbers represent the amino acid residues in Chk1.



sults raised the possibility that ATX3, an active deubiquitinase by virtue of the N-terminal Josephin domain and UIMs in its C-terminus, might share the same substrate with DDB1/CUL4A and CUL1, and play a role in Chk1 turnover.

To clarify whether Chk1 is a direct target of ATX3, we determined if the two proteins co-purify in immunoprecipitation experiments using ectopically expressed tagged proteins in human 293T cells. Indeed, Chk1 was detected in ATX3 immunoprecipitates (Figure 1B). His-pull down assay also verified their binding (Figure 1C). To further define the interaction between ATX3 and Chk1, we generated a series of truncations of ATX3 and Chk1, respectively. GST-pull down assay revealed that the Josephin domain, the catalytic domain of ATX3, was crucial for its association with Chk1 (Figure 1D). As for Chk1, full length of Chk1 was indispensable for its binding to ATX3 (Figure 1E). Collectively, these results revealed that Chk1 is a new ATX3-associating protein.

### ATX3 regulates Chk1 stability

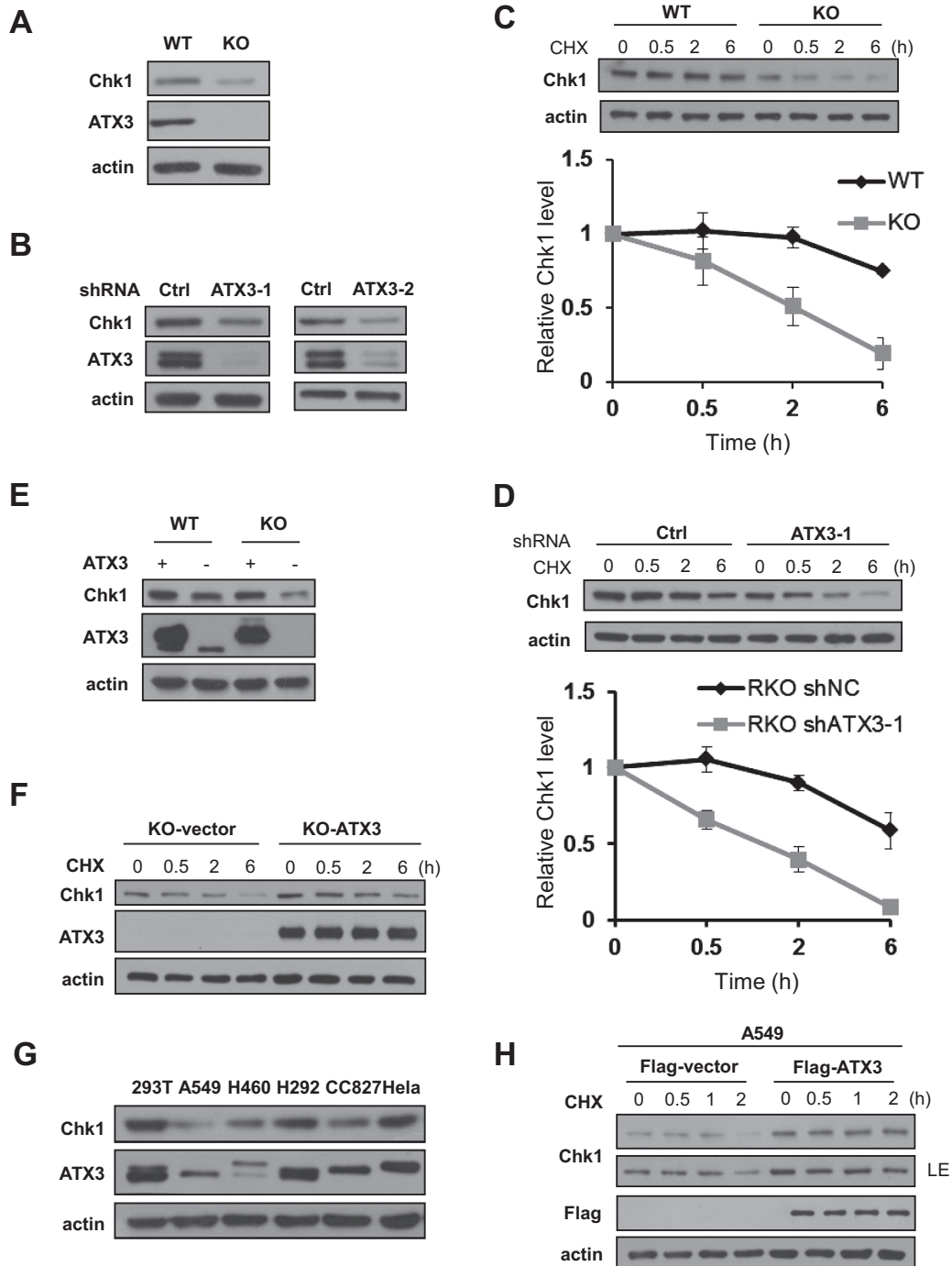
To test if ATX3 may promote Chk1 stabilization, we first evaluated whether ATX3 deficiency impairs Chk1 stability. We measured the Chk1 levels in ATX3 KO and two shRNA KD cells. ATX3 deficiency resulted in a significant reduction of Chk1 abundance (Figure 2A and B and supplementary Figure S2A). Chk1 protein level has been known to fluctuate throughout the cell cycle, peaking at S and G2 phase (58) when Chk1 exerts its function of regulating cell cycle progression and DNA damage checkpoint. To exclude the possibility of indirect effect of cell cycle changes, we analyzed the cell cycle distribution of WT and ATX3 KO cells. ATX3 depletion has no major effects on the G1 phase under normal conditions, indicating that the lower level of Chk1 in ATX3 KO cells is not a consequence of cell accumulation at G1 phase (Supplementary Figure S2B). We also examined the mRNA levels of Chk1 in WT and KO cells, and found that ATX3 depletion has no major effects on Chk1 transcription (Supplementary Figure S2C). In conclusion, reduction of Chk1 in ATX3 KO cells is not consequences of cell cycle distribution alteration or Chk1 transcription change.

Next, we measured the half-life of Chk1 in WT and ATX3 KO cells in the presence of CHX, an inhibitor of protein synthesis. We found that Chk1 in ATX3 KO cells was much less stable than that in WT cells, with a distinctly shortened half-life of about 2 h (Figure 2C). After shRNA-mediated depletion of ATX3, we also observed markedly reduced stability of Chk1 following CHX treatment (Figure 2D, Supplementary Figure S2D and S2E). Previous findings demonstrated that Chk1 is subjected to proteasomal degradation after periods of replicative stress (25–27,59). We also found that ATX3 KO cells exhibited an accelerated rate of destruction of Chk1 following replication stress (Supplementary Figure S2F). We then treated WT and KO cells with a proteasome inhibitor, MG132, to block proteasomal proteolysis of Chk1. We showed Chk1 protein level gradually increased in ATX3 KO cells when its proteasomal degradation was suppressed (Supplementary Figure S2G), indicating that ATX3 influences Chk1 stability through the

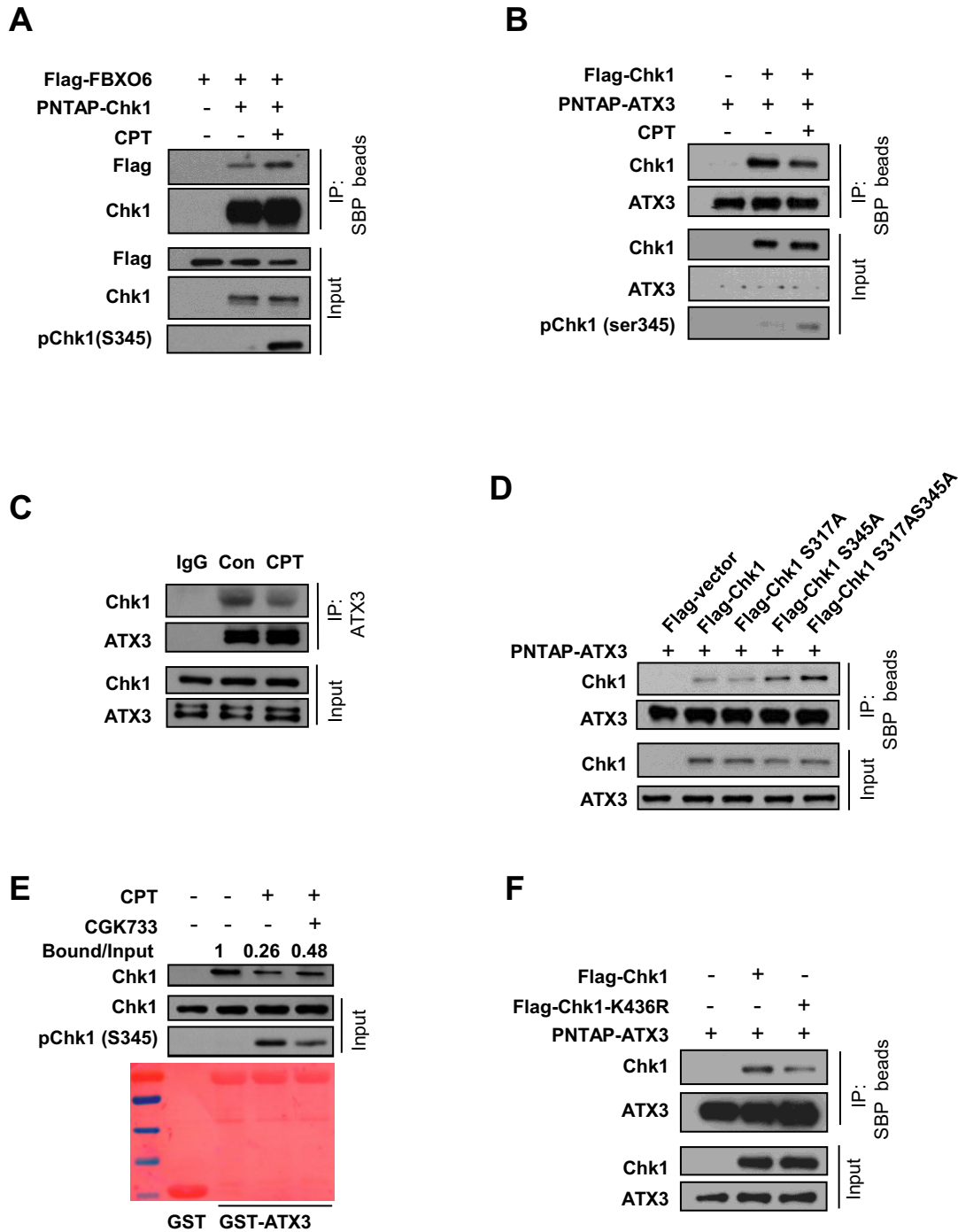
ubiquitin-proteasome system. In subsequent experiments, we investigated whether overexpression of ATX3 upregulates the steady-state Chk1 level. Indeed, transient lentiviral expressions of ATX3 in WT and KO cells caused higher levels of Chk1 than control cells transfected with vector (Figure 2E). Again, elevated turnover of Chk1 in ATX3 KO cells could be successfully rescued by re-introducing ATX3 into KO cells, while the catalytic mutant of ATX3 (ATX3-C14A) failed to do so (Figure 2F and Supplementary Figure S2H). To gain further insights into the regulation of Chk1 by ATX3, we examined the expression of Chk1 and ATX3 in several human cancer cell lines. Although ATX3 and Chk1 were variably expressed in these cell lines, there was a noticeable positive correlation between the expression levels of ATX3 and Chk1 (Figure 2G). The A549 cell line containing the lowest level of Chk1 was chosen for subsequent studies. If ATX3 stabilizes Chk1, then forced expression of ATX3 should upregulate the steady-state level and stability of Chk1. Indeed, ectopic expression of Flag-ATX3 in A549 cells nicely upregulated the endogenous level of Chk1, and promoted the stability of Chk1 after CHX treatment (Figure 2H). Taken together, our results demonstrated that ATX3 promoted Chk1 stability through the ubiquitin-proteasome pathway. Additionally, expression of USP1 which maintains total and phosphorylated Chk1 level through limiting DDB1-mediated degradation (29), was unaffected by loss of ATX3 (Supplementary Figure S2I).

### Interaction between ATX3 and Chk1 is affected by DNA damage

Consistent with previous evidences that Chk1 is subjected to proteasome-dependent degradation following prolonged replication stress (25,27,28,59,60), treatment with CPT for 4–6 h resulted in significant reduction of Chk1 level (Supplementary Figure S3A). It has been shown that the association between Chk1 and DDB1/CUL4A or CUL1, which targets Chk1 for proteasomal degradation, enhances under replicative stress (25,27). We checked the association between Chk1 and FBXO6, the targeting subunit of SCF E3 complexes responsible for Chk1 polyubiquitination and degradation. Similarly, Chk1 interacted with FBXO6 with a stronger extent under prolonged replication stress (Figure 3A). We have demonstrated the ATX3/Chk1 interaction under unperturbed conditions (Figure 1). We next sought to determine if DNA damage affects their interaction. Different types of DNA damage agents were used, including two agents that cause replication stress (CPT and HU), and ETO, which induces double strand break. As shown in Figure 3B and Supplementary Figure S3C, the association between Chk1 and ATX3 significantly decreased after long exposure (4 h) to CPT and HU. At early stage of replication stress (1 h after HU and CPT), ATX3 associated with Chk1 stronger than unstressed groups (Supplementary Figure S3C and E). In contrast, treatment with ETO, which has little effect on Chk1 expression, did not change the interaction between ATX3 and Chk1 significantly (Supplementary Figure S3B and D). Using an ATX3 specific antibody, we further verified the dynamic interaction change between endogenous ATX3 and Chk1 under unperturbed conditions and after persistent replication stress (Figure 3C). Further-



**Figure 2.** ATX3 regulates Chk1 stability. (A and B) ATX3 deficiency results in significant reduction of Chk1 protein level. Lysates of WT and ATX3 KO MEF cells were immunoblotted with the antibodies indicated (A). Lysates of RKO cells transfected with control shRNA or shATX3-1 and shATX3-2 were immunoblotted with the indicated antibodies (B). (C and D) ATX3 promotes the stability of Chk1. WT and ATX3 KO cells were treated with 50  $\mu$ g/ml cycloheximide (CHX). Cells were collected at indicated time points and immunoblotted with Chk1 and actin. The graph shows the levels of Chk1 protein quantified by densitometry using ImageJ software and normalized to actin in WT and ATX3 KO cells. Values are for each time point and are shown relative to the levels of Chk1 at the 0 h time point (C). RKO cells transfected with control shRNA or ATX3 shRNA (shATX3-1) were treated with 50  $\mu$ g/ml CHX. Cells were collected at indicated time points and immunoblotted with antibodies indicated. The graph shows the levels of Chk1 protein quantified by densitometry using Image J software and normalized to actin in RKO cells transfected with control shRNA or ATX3 shRNA. Values are for each time point and are shown relative to the levels of Chk1 at the 0 h time point (D). (E) Transient expression of ATX3 upregulates the level of Chk1. Lysates of WT and ATX3 KO cells overexpressed with human ATX3 or vector by lentiviral transfection were immunoblotted with indicated antibodies. (F) The decreased stability of Chk1 in ATX3 knockout cells can be rescued by re-introducing ATX3. KO cells stably expressing ATX3 or vector were treated with 50  $\mu$ g/ml CHX, collected at indicated time points, and were immunoblotted with Chk1, ATX3 and actin. (G) The positive correlation between the expression levels of ATX3 and Chk1. Cells derived from various tumor types were lysed and blotted with indicated antibodies. (H) Transient expression of ATX3 upregulates the level of Chk1 and promotes its stability in A549 cells. A549 cells transiently transfected with ATX3 or vector were treated with 50  $\mu$ g/ml CHX, collected at indicated time points. Cell lysates were immunoblotted with indicated antibodies. LE: long exposure.



**Figure 3.** Interaction between ATX3 and Chk1 is affected by DNA damage. (A) Co-IP experiments showing interaction between Chk1 and FBXO6 is increased after prolonged replication stress. 293T cells expressing indicated tagged proteins were cultured in the presence of 500 nM CPT and 20  $\mu$ M MG132 for 4 h before harvest. PNTAP-Chk1 was immunoprecipitated with SBP beads and precipitates were analyzed by western blotting. (B) Co-IP experiments showing interaction between Chk1 and ATX3 is decreased after prolonged replication stress. 293T cells expressing the indicated tagged proteins were cultured in the presence of 500 nM CPT and 20  $\mu$ M MG132 for 4 h. PNTAP-ATX3 was immunoprecipitated with SBP beads. Precipitates were resolved by SDS-PAGE and analyzed by western blotting. (C) Endogenous-IP suggests that interaction between Chk1 and ATX3 is decreased after prolonged replication stress. Endogenous ATX3 was immunoprecipitated (IP) from control cells or cells treated with 500 nM CPT for 4 h. Cells were also treated with 20  $\mu$ M MG132 for 4 h before harvest. Precipitates were resolved by SDS-PAGE and analyzed by western blotting. (D and E) Ser345 phosphorylation of Chk1 is involved in regulating the interaction between Chk1 and ATX3. 293T cells transfected with PNTAP-ATX3 and Flag-Chk1 or its phosphorylation mutants were treated with 500 nM CPT and 20  $\mu$ M MG132 for 4 h before harvest, immunoprecipitated with SBP beads and blotted with antibodies indicated (D). 293T cells were cultured in the presence of vehicle (DMSO) or 20  $\mu$ M CGK733 for 4 h. Cells were also exposed to 500 nM CPT and 20  $\mu$ M MG132 for 4 h before harvest. Lysates were incubated with purified GST or GST-tagged ATX3 immobilized on Glutathione Sepharose beads. Endogenous bound Chk1 was analyzed by anti-Chk1 antibody. The ratios of bound to input Chk1 proteins derived from a densitometric analysis of the blot were given on top of the panel. Purified proteins were visualized by Ponceau S staining (E). (F) The ubiquitination of Chk1 is involved in regulating the interaction between Chk1 and ATX3. PNTAP-ATX3 and Flag-Chk1 or its ubiquitination mutants were expressed in 293T cells, immunoprecipitated with SBP beads and blotted with antibodies against Chk1 and ATX3.

more, when we monitored the interaction between ATX3 and Chk1 at different time points after CPT exposure, we noticed that Chk1 dissociated from ATX3 after persistent replicative stress, which paralleled very well to a stronger association with FBXO6 (Supplementary Figure S3E and S3F).

Given that S345 phosphorylation of Chk1, mediated by ATR, targets Chk1 for degradation via the ubiquitin-proteasome pathway (25,27,28,59), we next assessed the impact of mutation of this phosphorylation site on the ATX3/Chk1 interaction. As seen in Figure 3D, mutant S345A and mutant S317AS345A bound to ATX3 with a stronger extent than WT Chk1 and mutant S317A, coincident with that S345A mutant of Chk1 is resistant to proteasome degradation (25). In support of our idea that S345 phosphorylation is involved in regulating the interaction between ATX3 and Chk1 after persistent replication stress, the decreased association between ATX3 and Chk1 under replicative stress was restrained when Chk1 S345 phosphorylation was inhibited by CGK733, an inhibitor of ATM and ATR (Figure 3E). K436 is a candidate ubiquitination site in Chk1 (26), and ATX3 is a deubiquitinase capable of binding polyubiquitin chain. We proposed that Chk1 ubiquitination may also be involved in regulating its interaction with ATX3. Consistent with this idea, association between Chk1 K436R mutant and ATX3 was significantly impaired comparing with WT Chk1 (Figure 3F). Collectively, our data revealed that ATX3 interacts with and stabilizes Chk1 under unchallenged conditions, and this interaction decreases remarkably after prolonged replication stress when Chk1 needs to be degraded by proteasome. Ser345 phosphorylation which targets Chk1 for polyubiquitination and degradation plays an essential role in regulating the interaction between Chk1 and ATX3.

### ATX3 deubiquitinates and thus stabilizes Chk1

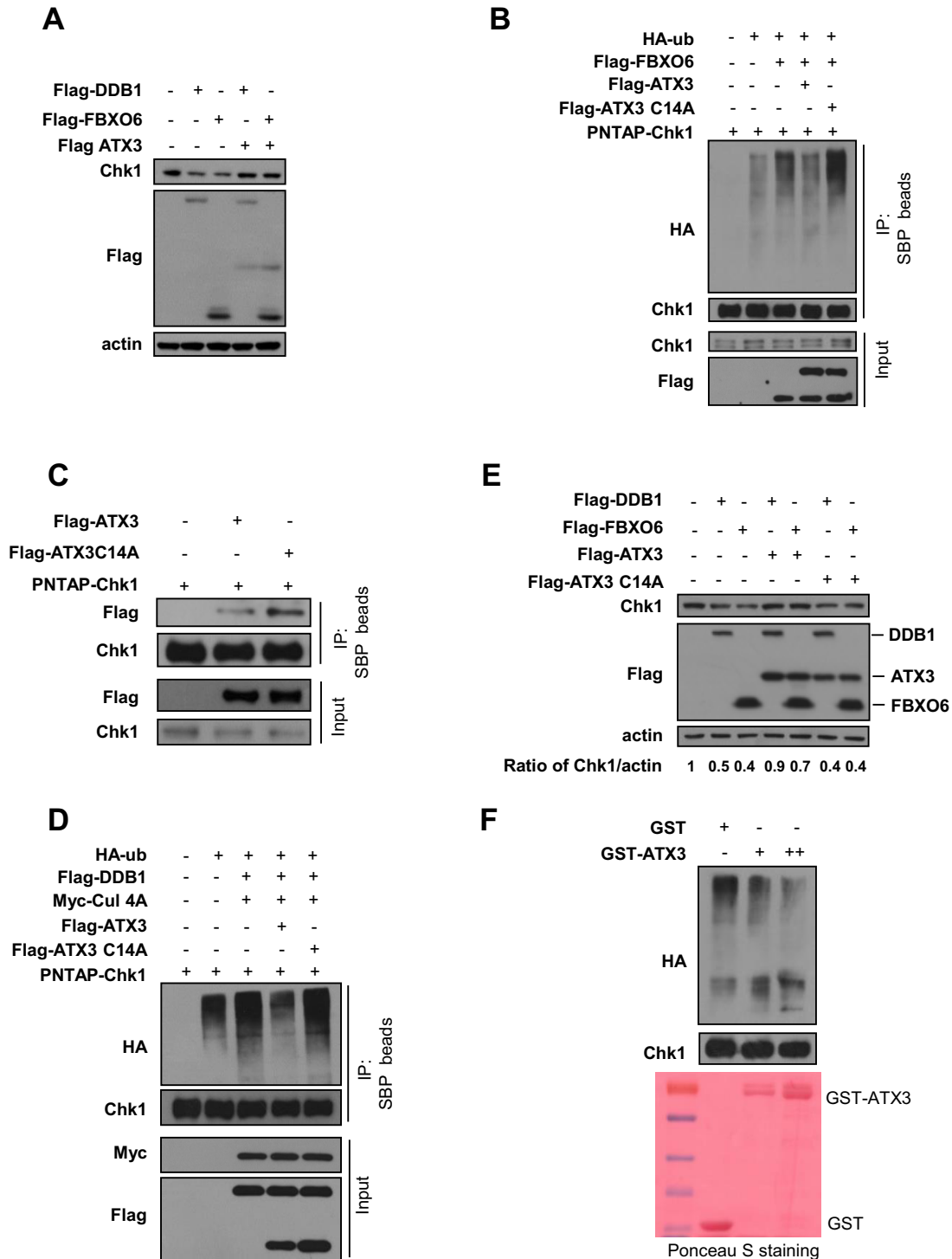
As mentioned above, ATX3 promotes Chk1 stability through inhibiting its proteasomal destruction. Therefore, it is plausible that ATX3, as a DUB, functions to deubiquitinate Chk1 to counteract the action of E3 ligases. Indeed, overexpression of ATX3 but not ATX3-C14A significantly reduced the polyubiquitination level of Chk1 (Supplementary Figure S4A). Chk1 is reported to be polyubiquitinated and degraded by two E3 ligase complexes including CDT and SCF, we therefore proceeded to determine whether ATX3 antagonizes this process. Indeed, overexpression of FBXO6 or DDB1 resulted in significant decrease of Chk1 protein level, whereas co-expression of ATX3 effectively restored Chk1 from E3 ligase-mediated degradation (Figure 4A). Next we test if ATX3 is able to affect Chk1 polyubiquitination *in vivo*. Expression of FBXO6 induced higher level of polyubiquitination in Chk1, which can be remarkably decreased by co-expression of ATX3 (Figure 4B and Supplementary Figure S4B). In contrast, although ATX3-C14A, the catalytic inactive mutant of ATX3 (Supplementary Figure S4A), had an even stronger binding affinity to Chk1 (Figure 4C), it lost the ability to restrain Chk1 polyubiquitination induced by FBXO6 (Figure 4B). Similarly, expression of WT ATX3 but not ATX3-C14A significantly suppressed the polyubiquitination of Chk1 mediated by E3

ligase complexes DDB1/CUL4A (Figure 4D). Based on inability of ATX3-C14A mutant to antagonize E3 ligase-mediated polyubiquitination, we hypothesized that it might also be defective in reversing E3 ligase-mediated degradation of Chk1. As anticipated, mutation of the catalytic site cysteine14 compromised the capability of ATX3 to protect Chk1 from E3 ligase-mediated degradation (Figure 4E). To directly examine the deubiquitination ability of ATX3 towards Chk1, we performed *in vitro* deubiquitination assay. To obtain ubiquitinated Chk1, we co-expressed PNTAP-Chk1, HA-ub and Flag-FBXO6 in 293T cells, and bacteria-expressed GST-ATX3 was purified to incubate with ubiquitinated Chk1 *in vitro*. Our result indicated that GST-ATX3 effectively decreased the polyubiquitination of Chk1 in a dose-dependent manner (Figure 4F). Further, consistent with results from *in vivo* deubiquitination assay, the enzymatically inactive mutant ATX3-C14A failed to deubiquitinate polyubiquitinated Chk1 *in vitro* (Supplementary Figure S4C). Taken together, ATX3 can deubiquitinate polyubiquitinated Chk1 both *in vitro* and *in vivo*.

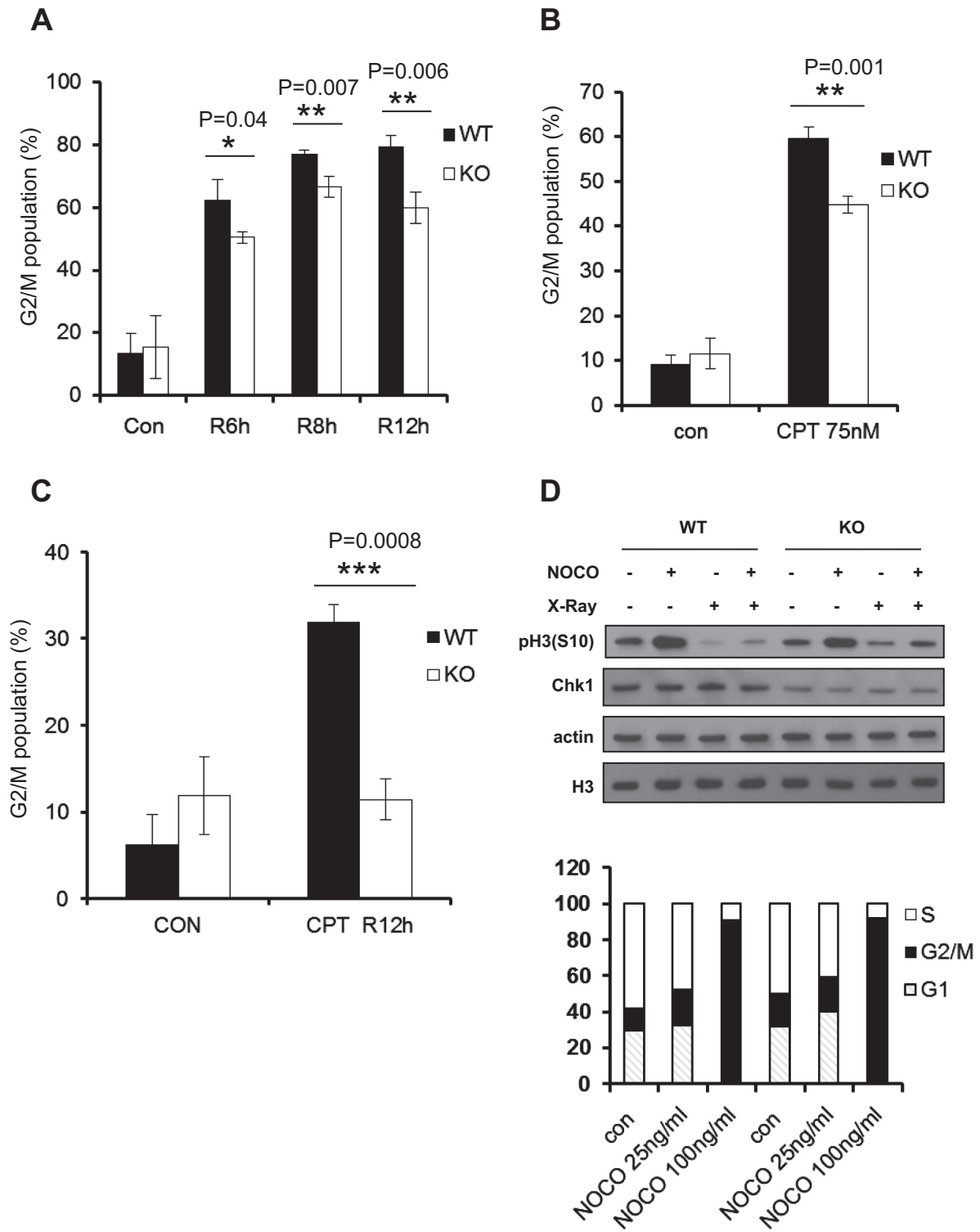
### ATX3 deficiency impairs Chk1-mediated G2/M DNA damage checkpoint

The discovery that ATX3 deficiency results in significant reduction of Chk1, normally activated by DNA damage and replication stress, prompted us to investigate the downstream consequences of the significant reduction of Chk1 abundance in ATX3 KO cells. In response to replication stress or DNA damage, Chk1 activates G2/M checkpoint to delay cell cycle progression and to facilitate DNA repair (61). We speculated that ATX3 KO cells, due to less Chk1 and declined Chk1 stability, might be compromised in their capacity to enforce G2/M DNA damage checkpoint following replication stress or DNA damage. As expected, X-Ray irradiation led to arrest of WT cells in G2/M phase, and ATX3 knockout resulted in a moderate but significant decrease in the G2/M population (Figure 5A). Moreover, ATX3-depleted cells re-entered the cell cycle more rapidly than WT cells did, implying that Chk1 reduction caused by ATX3 knockout can accelerate recovery from the G2/M damage checkpoint (Figure 5A), which is consistent with previous results from Chk1<sup>-/-</sup> ES cells (7). This observation was further corroborated when we treated cells with another DNA damage agent CPT. In line with X-Ray irradiation, ATX3 KO cells failed to arrest in G2/M phase as efficiently as WT cells and exhibited an accelerated rate of cell cycle resumption (Figure 5B and C). To determine whether ATX3 KO cells enter mitosis in the presence of DNA damage, WT and ATX3 KO cells were treated with X-Ray, followed by treatment with nocodazole to avoid progression of the cell cycle to the next G1. The levels of histone H3 phosphorylated at Ser10, an M-phase specific marker, were monitored to evaluate the integrity of IR-induced G2 checkpoint (27). As shown in Figure 5D (lower panel), 100 ng/ml nocodazole effectively arrested WT and KO cells at G2/M phase. H3 Ser10 phosphorylation was significantly diminished in WT cells pre-treated with X-Ray, indicating that WT cells effectively induce cell cycle arrest before mitosis. In contrast, pH3 was more apparent in the X-Ray-treated ATX3 KO cells, indicating that X-ray fails to induce efficient





**Figure 4.** ATX3 deubiquitinates and thus stabilizes Chk1. (A) ATX3 protects Chk1 from DDB1- and FBXO6-mediated degradation. 293T cells transfected with the indicated constructs were lysed and cell lysates were blotted with indicated antibodies. (B) Regulation of Chk1 ubiquitination levels *in vivo* by ATX3. 293T cells transfected with the indicated constructs were treated with 20  $\mu$ M MG132 for 4 h. PNTAP-Chk1 was immunoprecipitated with SBP beads and immunoblotted with HA antibody. (C) The catalytic inactive mutant of ATX3, ATX3-C14A, remains the capacity to interact with Chk1. PNTAP-Chk1 and Flag-ATX3 or its catalytic inactive mutant (ATX3-C14A) were expressed in 293T cells, immunoprecipitated with SBP beads and blotted with indicated antibodies. (D) Regulation of Chk1 ubiquitination levels *in vivo* by ATX3. 293T cells transfected with the indicated constructs were treated with 20  $\mu$ M MG132 for 4 h. PNTAP-Chk1 was immunoprecipitated with SBP beads and immunoblotted with HA antibody. (E) The catalytic inactive mutant of ATX3, ATX3-C14A, fails to restrain DDB1- and FBXO6-mediated degradation of Chk1. 293T cells transfected with the indicated constructs were lysed and then blotted with the indicated antibodies. The ratios of Chk1 to actin proteins derived from a densitometric analysis of the blots were given beneath the panel. (F) Deubiquitination of Chk1 *in vitro* by ATX3. 293T cells expressing HA-ub, Flag-FBXO6 and PNTAP-Chk1 were treated with 20  $\mu$ M MG132 for 4 h to obtain polyubiquitinated Chk1, immunoprecipitated with SBP beads, and incubated with purified GST or increasing amounts of GST-ATX3 *in vitro*, and then blotted with HA antibody.



**Figure 5.** ATX3 deficiency impairs Chk1-mediated G2/M DNA damage checkpoint. (A) ATX3 knockout cells fail to arrest at G2/M phase and re-enter the cell cycle more rapidly than WT cells after IR treatment. WT and KO cells were subjected to 10 Gy IR, harvested at indicated time. Cell cycle profiles were determined by FACS. (B) ATX3 knockout cells fail to arrest at G2/M phase after CPT treatment. WT and KO cells were treated with or without 75 nM CPT for 12 h and analyzed by flow cytometry. (C) ATX3 knockout cells re-enter the cell cycle more rapidly than WT cells after CPT treatment. WT and KO cells were treated with or without 75 nM CPT for 8 h, repaired for 12 h and analyzed by flow cytometry. Statistic significance of (A), (B) and (C) was determined by two-tailed, unpaired Student's *t*-test. The experiments were repeated three times. (D) ATX3 knockout cells exhibit deficient G2/M checkpoint. WT and KO cells were treated with or without indicated concentrations of nocodazole (NOCO) for 12 h and analyzed by flow cytometry (lower panel). WT and KO cells were treated with or without 10 Gy IR and nocodazole (100 ng/ml) for 12 h. Cell lysates were immunoblotted with the indicated antibodies.

G2/M arrest (Figure 5D). Nevertheless, KO cells can be arrested at G1 phase as efficient as WT cells following HU treatment (Supplementary Figure S5). In conclusion, as a consequence of the reduced Chk1 protein level, and therefore activity, ATX3 deficiency compromises Chk1-mediated G2/M DNA damage checkpoint.

### ATX3 deficiency compromises DNA repair and confers DNA damage sensitivity

The phosphorylation of Chk1 by ATR stimulates its kinase activity, and activated Chk1 in turn phosphorylates its downstream effectors, which influence cell cycle progression, DNA repair and cell survival. Then, we assessed whether Chk1 reduction arising from ATX3 deficiency impairs DNA damage response. Our results revealed that ATX3 KO cells failed to efficiently respond to DNA damage insults compared with WT cells, as measured by phosphorylation of Chk1 (Figure 6A). Similar results were obtained in ATX3 KD cells (Figure 6B and C and Supplementary Figure S6A and B), indicating that ATX3 is required for efficient response to DNA damage. We also checked the upstream of Chk1 phosphorylation, and found that ATR activation in ATX3 KO cells was comparable to that of WT cells after DNA damage assaults, as evidenced by intact ATR S428 phosphorylation after IR (Figure 6C). Accordingly, our further experiments manifested that ATX3 ablation resulted in a significant reduction of steady-state Chk1 in the presence or absence of DNA damage (Figure 6A and B and Supplementary Figure S6A and B). However, Chk2 phosphorylation at Thr68 and Chk2 basal level remained unaffected in ATX3 KD cells (Figure 6B and Supplementary Figure S6A). We confirmed our results in WT and ATX3 KO cells by using another damage agent CPT (Figure 6D). Notably, the phosphorylation defect of Chk1 in ATX3-depleted cells is caused by reduction of Chk1, as indicated by comparable ratio of pChk1/Chk1 in ATX3-WT and ATX3-deficient cells (Figure 6B and D). Further, decreased Chk1 level and Chk1 phosphorylation defect in ATX3 KO cells after DNA damage treatment could be rescued by re-introducing ATX3 (Figure 6E). Therefore, we conclude from these experiments that ATX3 deficiency impairs DNA damage response through regulating Chk1 specifically.

Next, we tested whether lack of ATX3 also affects downstream targets of Chk1. It has been shown that Chk1 regulates DNA damage-induced ubiquitination of PCNA, which does not depend on its kinase activity (12). We first analyzed PCNA ubiquitination in WT and ATX3 KO cells, and found that ubiquitination of PCNA in ATX3 deficient cells was notably declined after DNA damage assaults (Figure 6F and Supplementary Figure S6C). Next, we wonder whether the compromised PCNA ubiquitination in ATX3 KO cells is attributable to the reduction of Chk1. To this end, we transiently expressed Chk1 in ATX3 KO cells and measured the level of PCNA ubiquitination. Indeed, transient overexpression of Flag-Chk1 corrected PCNA ubiquitination defect in ATX3 KO cells, indicating that ATX3 plays an essential role in DNA damage response by stabilizing Chk1 (Figure 6F). In subsequent studies, we checked the effect of ATX3 depletion on DNA damage sensitiv-

ity. WT and ATX3 KO cells were exposed to HU (an inhibitor of ribonucleotide reductase), CPT (an inhibitor of topoisomerase) or UV, and then released into fresh medium and grown for one week to allow colony formation. The colony assay results showed that ATX3 depletion sensitized cells to these DNA damage insults, exhibiting much lower cell survival rate than WT cells after damage stress (Figure 6G). Moreover, the hypersensitivity to replication stress conferred by depletion of endogenous ATX3 could be rescued by stably expressing WT ATX3 but not the catalytic inactive mutant ATX3-C14A, suggesting that the deubiquitinase activity of ATX3 is essential for its role in promoting cell survival in response to replication stress (Figure 6G and Supplementary Figure S6D). Micronucleus assay also supported our idea that ATX3 exerts its important protective function in DNA damage response and DNA repair (Figure 6H). Based on these findings, we concluded that the significant reduction of Chk1 abundance due to ATX3 deficiency results in compromised DNA damage response and confers DNA damage sensitivity.

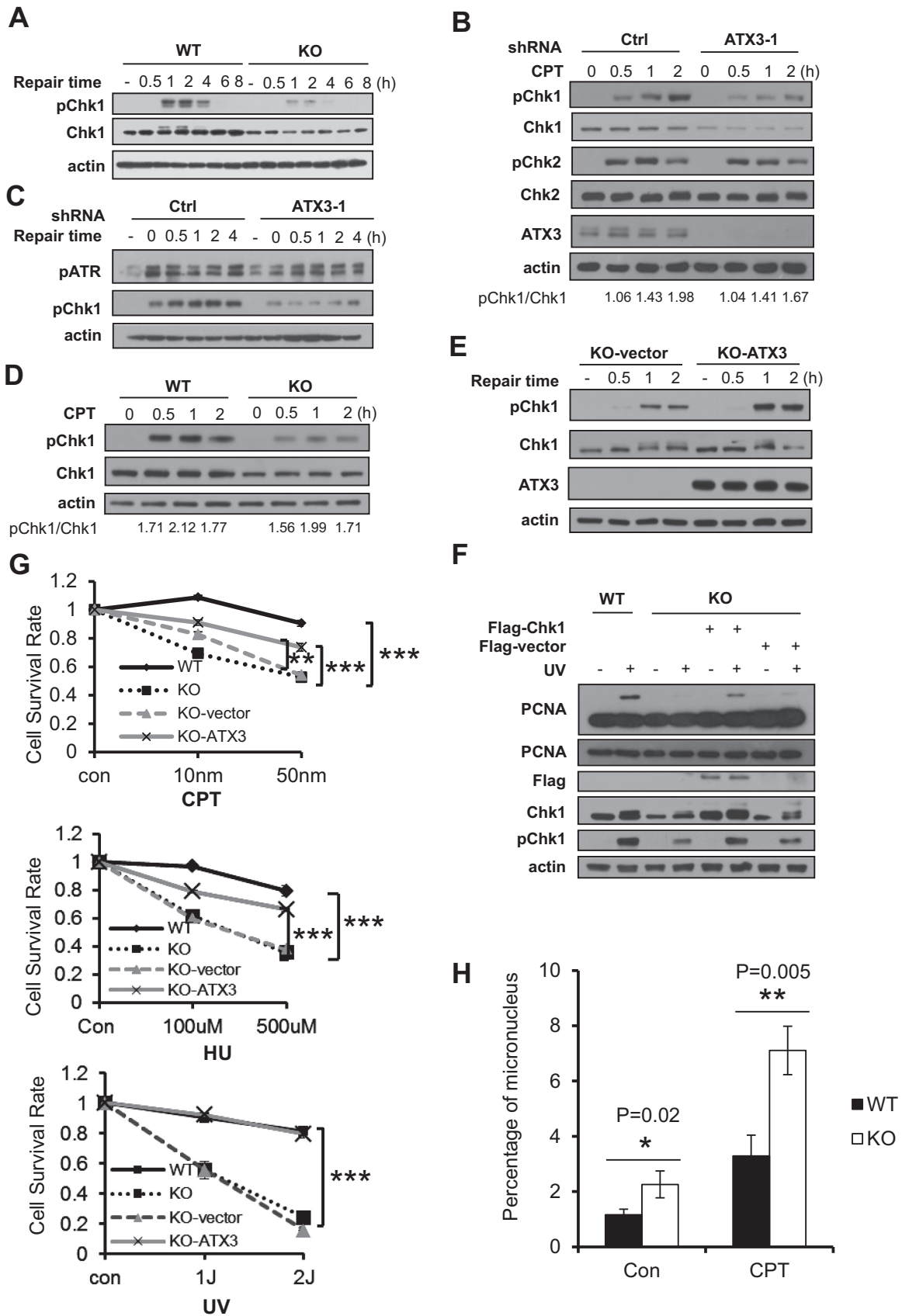
### PolyQ-expanded ATX3 remains the capacity to deubiquitinate and to stabilize Chk1

SCA3 is considered to be caused by an abnormal polyQ expansion in ATX3, which may impair the functions of ATX3-interacting proteins. We wondered whether polyQ expansion compromises the interaction between ATX3 and Chk1. Expanded ATX3 (80Q) and WT ATX3 were found to bind to Chk1 with a similar affinity (Figure 7A). GST-pull down assay further confirmed this result (Figure 7B). Moreover, analogous to WT ATX3, polyQ-expanded ATX3 remained the capacity to oppose E3 ligase-mediated degradation (Figure 7C) and polyubiquitination (Figure 7D and E) of Chk1. Collectively, our data indicate that polyQ expansion does not impair the protease activity of ATX3 toward Chk1.

## DISCUSSION

Ubiquitin-proteasome pathway (UPP) is one of main mechanisms for the turnover of short-lived proteins or damaged proteins. Deubiquitinases are implicated in reversing protein ubiquitination mediated by E3 ligase, therefore regulating stability or activity of targeted proteins (55,62). Although the importance of ATX3 in UPP is supported by increased polyubiquitinated proteins after ATX3 knockout or inhibition its catalytic activity (44,45,63), its exact substrate(s) remains to be investigated. Here, we identify Chk1, a key factor in DNA damage response, as one such substrate of ATX3. We find that ATX3 facilitates genome stability maintenance by regulating Chk1 ubiquitination and degradation under unperturbed conditions and also during replication stress.

The Chk1 turnover is essential during normal cell cycle and during replication stress. The expression of Chk1 fluctuates throughout the cell cycle, peaking at S and G2 phases at both mRNA and protein levels, whose pattern correlates well with its activity (58). It has also been reported that resumption of DNA synthesis and S phase progression after replication stress induced by CPT is specifically dependent



**Figure 6.** ATX3 deficiency compromises DNA repair and confers DNA damage sensitivity. (A) DDR activation in WT and KO cells after H<sub>2</sub>O<sub>2</sub> treatment. Cells were treated with 5 mM H<sub>2</sub>O<sub>2</sub> for 10 min and repaired for the indicated time. Cells were lysed and processed for Western blotting. The phosphorylation



on continued expression of Chk1 (25). Post-translational modifications such as phosphorylation/dephosphorylation and ubiquitination also play a vital role in regulating Chk1 activity (7–9,22–27,64–67). Recently, chaperone-mediated autophagy is described to play a crucial role in genome integrity control through regulated degradation of activated Chk1 after genotoxic exposure, promoting checkpoint termination (68). In addition, proteasomal degradation of Claspin, a key mediator during ATR-mediated Chk1 phosphorylation (69–72), can also restrain Chk1 activity (73–75).

Since the Chk1 turnover is relevant during normal cell cycle progression and during replication stress, a series of studies have focused on identification of ubiquitin E3 ligases and DUBs involved in this process. CUL1- and CUL4-containing E3 ligase complexes are responsible for polyubiquitination and degradation of Chk1 after periods of replication stress, which promotes checkpoint termination after completion of DNA repair, and plays a vital role in the cytotoxic effects of anti-cancer drugs (25–27). It has been previously reported that deubiquitinase USP1 maintains both total and phosphorylated Chk1 level and that USP1 deletion promotes degradation of activated Chk1 in a DDB1-dependent manner (29). However, whether USP1 promotes Chk1 stability by directly reversing DDB1-mediated polyubiquitination and degradation of Chk1 remains unclear. Additionally, deubiquitinase USP7 stabilizes Chk1 by cleavage of polyubiquitin chain (30,31), but whether USP7 specifically restrains SCF- or CDT-mediated polyubiquitination or just functions as a general deubiquitinase remains to be elucidated. In this study, we report that ATX3 associates with Chk1 and antagonizes both FBXO6- and DDB1/CUL4A-mediated polyubiquitination and subsequent degradation of Chk1. Additionally, deubiquitinating enzyme activity of ATX3 is indispensable for its role in stabilizing Chk1, as catalytic inactive mutant of ATX3, ATX3-C14A, fails to reverse these two E3 ligase-mediated polyubiquitination and degradation of Chk1, indicating a direct involvement of ATX3 in Chk1 turnover.

The expression level of Chk1 is critical for its function in cell viability and tissue development. Previous studies have shown that reducing Chk1 level, via gene disruption or chemical inhibition, leads to increased spontaneous cell death and development defects (25,76–79). Chk1 haploinsufficiency can result in anemia and defective erythropoiesis in mice (80). Moreover, Chk1 is observed to be overex-

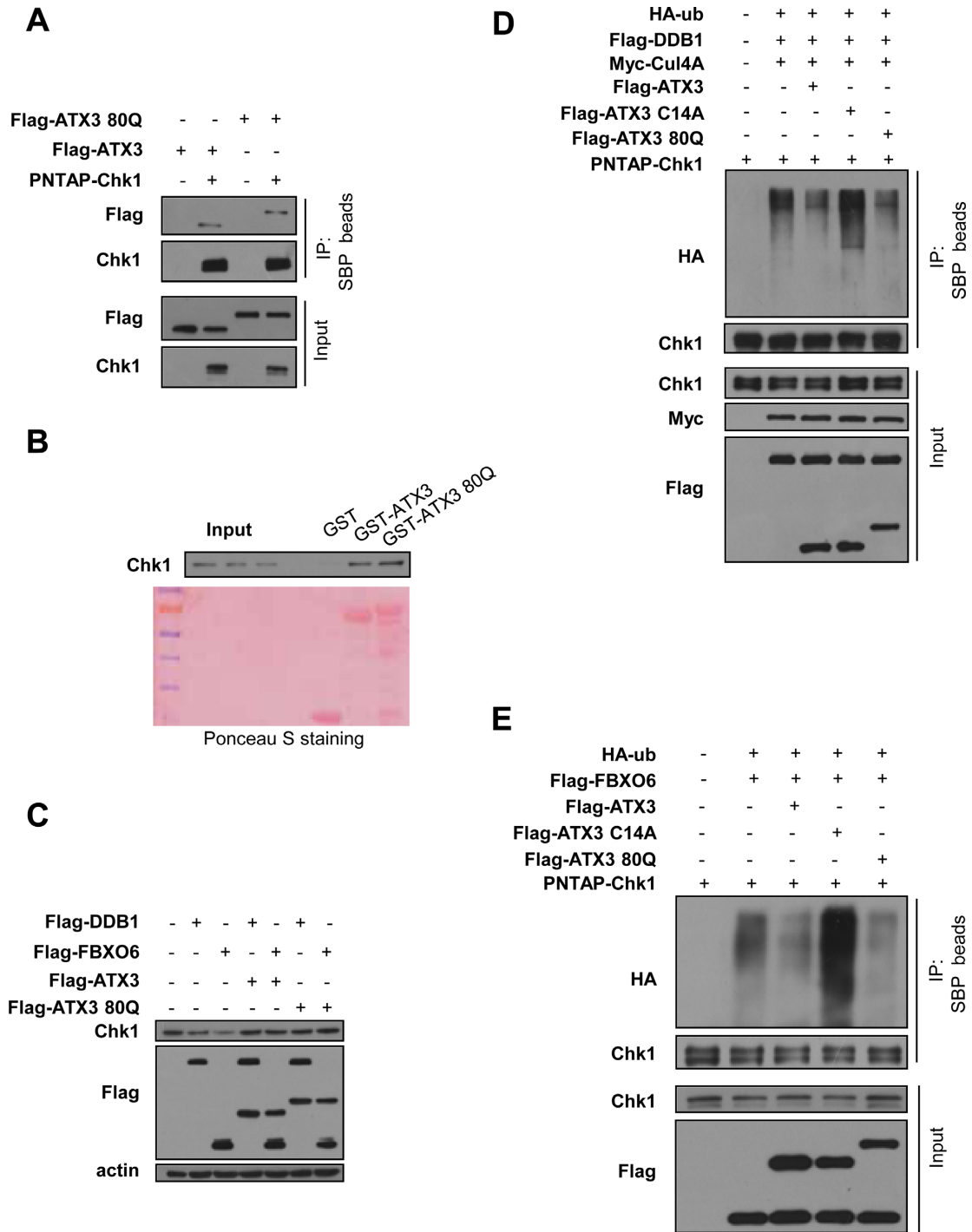
pressed in a variety of human tumors, and there is a positive correlation between Chk1 level and tumor grade (81–88). Consequently, Chk1 abundance and activity must be finely-tuned both under unperturbed conditions and in response to replication stress. The finding that treatment of cells with proteasome inhibitor leads to both the appearance of ubiquitinated Chk1 and a noticeable upregulation of the basal level of Chk1 indicates that Chk1 is subjected to continuous ubiquitination and degradation during the normal cycling cells (25). We discover that, under unperturbed conditions and upon DNA damage, deubiquitination of Chk1 by ATX3 limits its polyubiquitination and subsequent degradation mediated by CUL1- and CUL4A-containing E3 ligase complexes, thus promoting Chk1 stabilization and further checkpoint signaling and DNA repair. Therefore, ATX3 is required to maintain the steady-state level of Chk1 and genome integrity (Figure 8, left panel). In line with it, ATX3 deficiency results in a pronounced reduction of Chk1, which compromises Chk1-mediated G2/M checkpoint and DNA damage response, as evidenced by defective phosphorylation of Chk1, reduced PCNA mono-ubiquitination and increased DNA damage sensitivity.

Under prolonged replicative stress, ATX3 dissociates from Chk1, parallel nicely to a stronger association between Chk1 and its E3 ligase, followed by polyubiquitination and proteasomal destruction of Chk1 (Figure 8, right panel), which contributes to restricting the duration of Chk1 signaling. Given that Chk1 is undergoing posttranslational modification after DNA damage treatment, the dynamic interaction between ATX3 and Chk1 before and after prolonged replication stress suggests that inducible modification of Chk1 and/or ATX3 might be implicated in regulating their association. In support of that, ser345 phosphorylation of Chk1 is found to play an essential role in this prolonged replication stress-induced dissociation between ATX3 and Chk1 (Figure 3D and E). It is possible that other unidentified factors might also modulate the dynamic interaction, which deserves further investigation.

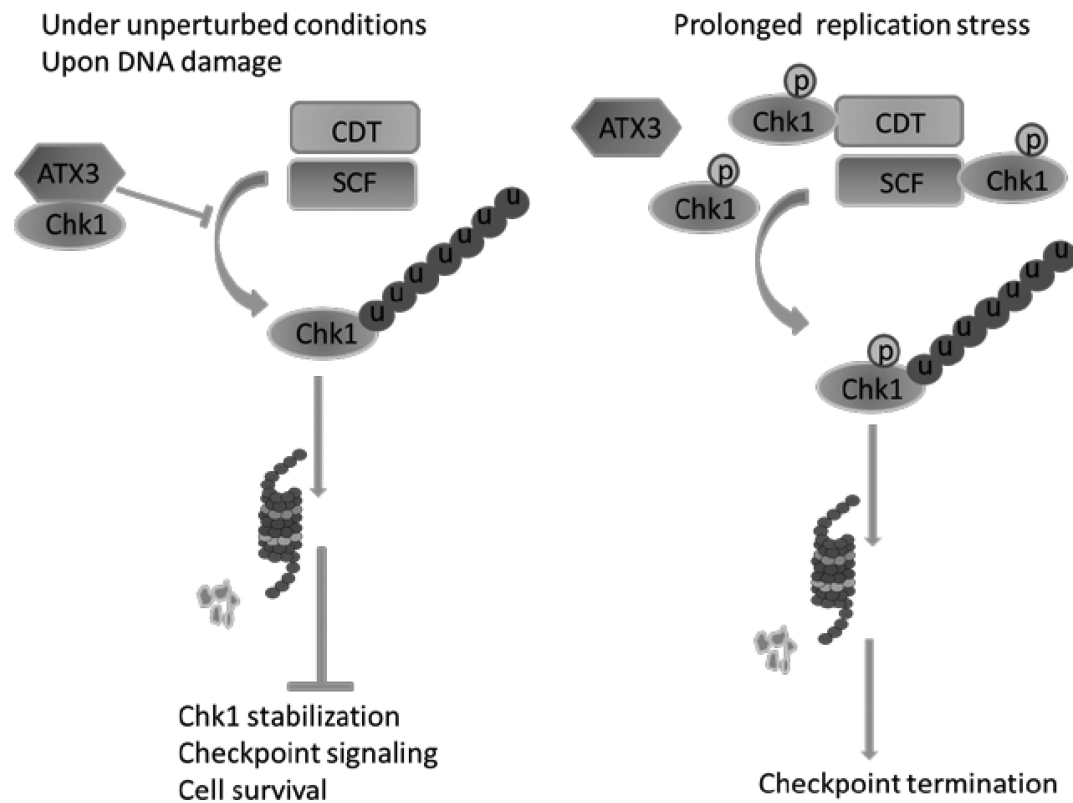
The dynamic regulation of Chk1 stability by ATX3 before and after prolonged replication stress has significant biologic significances. First, cells containing enough Chk1 can efficiently ensure DNA damage checkpoint signaling and facilitate DNA repair when DNA is attacked exogenously and endogenously. Second, proteasomal destruction of Chk1 after periods of replicative stress contributes to checkpoint termination, cell cycle resumption and cell sur-

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status of Chk1 was analyzed, and actin was used as the loading control. (B) The phosphorylation of Chk1 and the steady-state level of Chk1 are decreased in ATX3 knockdown cells. RKO cells transfected with control shRNA or ATX3 shRNA (shATX3-1) were treated with 500 nM CPT, collected at indicated time points. Cell lysates were immunoblotted with the antibodies indicated. (C) ATR activation remains intact in ATX3 knockdown cells. HeLa cells transfected with control shRNA or ATX3 shRNA (shATX3-1) were treated with 10 Gy X-ray, collected at indicated time points. Cell lysates were immunoblotted with the indicated antibodies. (D) The phosphorylation of Chk1 and the steady-state level of Chk1 are decreased in ATX3 knockout cells. WT and KO cells were treated with 500 nM CPT, harvested at different time points and immunoblotted with pChk1, Chk1 and actin. (E) Both the decreased phosphorylation of Chk1 after DNA damage and the steady-state level of Chk1 in ATX3 knockout cells can be rescued by re-introducing ATX3. ATX3 KO cells stably expressing ATX3 (vector as the negative control) were treated with 5 mM H<sub>2</sub>O<sub>2</sub> for 10 min, harvested at different time points, immunoblotted with indicated antibodies. (F) The compromised mono-ubiquitination of PCNA in ATX3 knockout cells can be rescued by Chk1. WT, KO and KO cells transfected with Flag-Chk1 or Flag-Vector were exposed to 20 J UV, lysed 3 h later. Lysates were analyzed by western blot with the indicated antibodies. (G) ATX3 knockout confers DNA damage sensitivity, which can be rescued by re-introducing ATX3. WT, KO and KO cells stably expressing ATX3 (vector as control) were subjected to CPT (up), HU (middle) and UV (bottom), respectively. Data are the mean of biological replicates from a representative experiment. Error bars: SD, *n* = 3. Statistic significance was determined by two-tailed, unpaired Student's *t*-test. (H) Percentage of micronucleus after DNA damage is increased in ATX3 KO cells. WT and KO cells were treated with or without 100 nM CPT for 2 h, and then subjected to micronucleus assay. Data are from three independent experiments. Statistic significance was determined by two-tailed, unpaired Student's *t*-test.



**Figure 7.** PolyQ-expanded ATX3 remains the capacity to deubiquitinate and to stabilize Chk1. (A and B) PolyQ-expanded ATX3 remains the capacity to interact with Chk1. Flag-tagged ATX3 or Flag-tagged ATX3-80Q and PNTAP-Chk1 were expressed in 293T cells, immunoprecipitated with SBP beads. Immunoblotting was performed using indicated antibodies (A). Purified GST, GST-tagged ATX3-full length, or its expanded mutant GST-ATX3 80Q immobilized on Glutathione Sepharose beads were incubated with 293T cell lysates. Endogenous bound Chk1 was analyzed by anti-Chk1 immunoblotting. Purified proteins were visualized by Ponceau S staining (B). (C) PolyQ-expanded ATX3 remains the capacity to protect Chk1 from DDB1- and FBXO6-mediated degradation. 293T cells transfected with the indicated constructs were lysed and lysates were blotted with the indicated antibodies. (D and E) Regulation of Chk1 ubiquitination levels *in vivo* by ATX3 and expanded ATX3. 293T cells transfected with indicated constructs were treated with 20  $\mu$ M MG132 for 4 h before harvest. PNTAP-Chk1 was immunoprecipitated with SBP beads and immunoblotted with HA antibody.



**Figure 8.** The working model of how ataxin-3 promotes genome integrity by stabilizing Chk1. Under unperturbed conditions and upon DNA damage, ATX3 interacts with Chk1 and promotes Chk1 stability by antagonizing DDB1/CUL4A- and FBOX6-mediated polyubiquitination and degradation of Chk1, thus facilitating DNA repair, checkpoint signaling and cell survival when cells are attacked by genotoxic or replicative stress. Under prolonged replicative stress, phosphorylation of Chk1 at Ser345 results in dissociation between Chk1 and ATX3, concomitant with a stronger binding between Chk1 and its E3 ligase, which mediates Chk1 proteasomal degradation, contributing to the checkpoint termination.

vival. Upon DNA damage or replicative stress, Chk1 delays cell cycle progression to facilitate DNA repair. However, checkpoint termination, which is essential for resumption of cell cycle after completion of DNA repair, is also necessary. Proteolytic destruction of Chk1 has emerged as an important mechanism that functions to terminate Chk1 signaling. In this study, we have demonstrated ATX3 to be a new important regulator in checkpoint initiation and termination. We find that ATX3 associates with Chk1 and maintains the stability of Chk1 under unperturbed conditions, and that ATX3 dissociates from Chk1 after prolonged replication stress, and the released Chk1 binds to its E3 ligase which mediates polyubiquitination and degradation of Chk1 to terminate the checkpoint, suggesting the existence of a dynamic molecular interaction switch that tightly regulates Chk1 stability and function.

In this work, we have identified ATX3 as a novel regulator of Chk1 stability, providing a new perspective of the functional relationship between ATX3 and genome protection. Despite significant progress in establishing normal functions of ATX3 and etiology of SCA3, the underlying molecular mechanism of SCA3 pathogenesis remains enigmatic. It is suspected that loss of functions of ATX3-interacting proteins, sequestered in the polyQ aggregates, contributes to cellular toxicity and neurodegeneration in SCA3 (89–96). Nevertheless, it seems that polyQ expansion does not impair the protease activity of ATX3 towards Chk1, which remains

the capacity to oppose E3 ligase-mediated polyubiquitination and degradation of Chk1. Our data is in line with a previous report which suggests that polyQ stretch does not affect the binding affinity and cleavage efficiency of ATX3 in an *in vitro* assay (46). Recently, two research teams reported the interaction between ATX3 and PNKP, and discovered that polyQ-expanded ATX3 inactivates PNKP phosphatase activity, causing persistent DNA damage and chronic activation of pro-apoptotic signaling, which leads to SCA3 pathogenesis (97,98). It has been reported that ATX3 interacts with HHR23A and HHR23B, homolog of DNA repair protein RAD23 (99), and that ATX3 can be recruited to the DNA damage sites induced by laser microirradiation (100). All these data imply a function of ATX3 in DNA damage response and repair pathway. The exact functional significance of ATX3 in DNA damage response warrants further investigation.

#### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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