

The ATM-E6AP-MASTL axis mediates DNA damage checkpoint recovery

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Abstract

Checkpoint activation after DNA damage causes a transient cell cycle arrest by suppressing CDKs. However, it remains largely elusive how cell cycle recovery is initiated after DNA damage. In this study, we discovered the upregulated protein level of MASTL kinase hours after DNA damage. MASTL promotes cell cycle progression by preventing PP2A/B55-catalyzed dephosphorylation of CDK substrates. DNA damage-induced MASTL upregulation was caused by decreased protein degradation, and was unique among mitotic kinases. We identified E6AP as the E3 ubiquitin ligase that mediated MASTL degradation. MASTL degradation was inhibited upon DNA damage as a result of the dissociation of E6AP from MASTL. E6AP depletion promoted cell cycle recovery from the DNA damage checkpoint, in a MASTL-dependent manner. Furthermore, we found that E6AP was phosphorylated at Ser-218 by ATM after DNA damage and that this phosphorylation was required for its dissociation from MASTL, the stabilization of MASTL, and the timely recovery of cell cycle progression. Together, our data revealed that ATM/ATR-dependent signaling, while activating the DNA damage checkpoint, also initiates cell cycle recovery from the arrest. Consequently, this results in a timer-like mechanism that ensures the transient nature of the DNA damage checkpoint.

eLife assessment

This interesting study builds on previous work by the PI implicating Greatwall/MASTL kinase in recovery from DNA damage in cultured human cells. This study identifies a ubiquitin ligase that may regulate the stability of Greatwall/MASTL protein stability, and the authors propose that this constitutes a molecular "timer" that controls recovery from DNA damage. Should this be validated it would be an **important** advance for the field, but the data presented are currently **incomplete** and do not yet fully support this claim.

Introduction

DNA damage activates a wide range of cellular responses, including DNA repair, cell cycle checkpoints, and cell death. Collectively defined as the DNA damage response (DDR), these

mechanisms ensure genomic integrity and prevent progression of cancer, aging, and other diseases (Ciccio and Elledge, 2010; Jackson and Bartek, 2009; Lou and Chen, 2005). Among these DDR pathways, the DNA damage checkpoint temporally halts the progression of the cell cycle, to facilitate DNA repair and avoid detrimental accumulation of DNA damage. The DNA damage checkpoint can act at multiple stages of the cell cycle to impede DNA replication and block mitotic entry. Initiation of the DNA damage checkpoint signaling relies on two phosphoinositide 3 kinase-related protein kinases (PI3KK), ataxia-telangiectasia mutated (ATM), ATM and RAD3-related (ATR). Upon DNA damage, ATM and ATR phosphorylate and activate checkpoint kinases CHK1 and CHK2, which, in turn, inhibit CDC25 to prevent activation of cyclin-dependent kinases (CDK) (Shiloh, 2003; Zhou and Elledge, 2000).

How does the cell initiate cell cycle recovery after DNA damage is an important, yet largely unanswered, question. Distinct from the simplistic view that the cell cycle resumes passively following the repair of DNA damage and decay of checkpoint signaling, recovery from the DNA damage checkpoint is likely an active and regulated process (Bartek and Lukas, 2007; Clemenson and Marsolier-Kergoat, 2009). For example, Polo-like kinase 1 (PLK1) and other cell cycle kinases can facilitate the deactivation of DNA damage signaling and resumption of cell cycle progression (Peng, 2013; van Vugt et al., 2004). Furthermore, it has been observed in yeast, frog, and mammalian cells that the cell cycle can be restarted without completion of DNA repair, a phenomenon defined as adaptation (Bartek and Lukas, 2007; Clemenson and Marsolier-Kergoat, 2009; Syljuasen, 2007; Toczyski et al., 1997; van Vugt and Medema, 2004; Yoo et al., 2004). Aside from senescence and other types of permanent cell cycle withdrawal, the DNA damage checkpoint-mediated cell cycle arrest is transient, raising the question about mechanisms that mediate the timely initiation of DNA damage checkpoint recovery.

Microtubule-associated serine/threonine kinase like (MASTL, also known as Greatwall) has been characterized as an important regulator of mitosis. Like CDK1 and other mitotic kinases, MASTL is activated during mitotic entry, and the kinase activity of MASTL promotes mitosis, although mitotic entry is still permitted in mammalian cells depleted of MASTL (Archambault et al., 2007; Blake-Hodek et al., 2012; Castilho et al., 2009; Peng and Maller, 2010; Vigneron et al., 2011; Voets, 2010; Wang et al., 2016; Yu et al., 2004; Yu et al., 2006). Upon activation, MASTL phosphorylates α -endosulfine (ENSA) and cyclic AMP-regulated 19 kDa phosphoprotein (ARPP19) which then inhibit PP2A/B55 (protein phosphatase 2A/B55 targeting subunit). Because PP2A/B55 functions as the principal phosphatase catalyzing the dephosphorylation of CDK substrates, the coordination between the kinase activity of MASTL with that of CDK1 enables substrate phosphorylation and mitotic progression (Castilho et al., 2009; Gharbi-Ayachi et al., 2010; Mochida et al., 2009; Mochida et al., 2010; Vigneron et al., 2009).

Interestingly, in addition to its mitotic function, MASTL is also required for cell cycle recovery from the DNA damage checkpoint in *Xenopus* egg extracts (Medema, 2010; Peng et al., 2011; Peng et al., 2010). Addition of MASTL in extracts facilitated, and depletion of MASTL hindered, DNA damage checkpoint recovery, as evidenced by both mitotic phosphorylation of CDK substrates and de-activation of checkpoint signaling (Peng et al., 2011; Peng et al., 2010). Consistently, ectopic expression of MASTL in human cells promoted cell proliferation under DNA damage (Wang et al., 2014; Wong et al., 2016). In this study, we discovered the protein stabilization of MASTL post DNA damage, identified E6 associated protein (E6AP) as the underlying E3 ubiquitin ligase mediating MASTL proteolysis, and delineated ATM-mediated E6AP phosphorylation as a mechanism to initiate DNA damage checkpoint recovery.

Results

MASTL expression is upregulated after DNA damage

While investigating the role of MASTL in cell cycle regulation and DNA damage responses, we observed unexpected upregulation of MASTL protein levels in cells treated with DNA damage. As shown in [Fig. 1A-C](#), MASTL upregulation was evident in HEK293 cells treated with doxorubicin (DOX), hydroxyurea (HU), or camptothecin (CPT), generally within hours post treatment. MASTL accumulation was also confirmed in SCC38 cells treated with HU or ionized radiation (IR, [Fig. 1 Supplemental 1A&B](#)), and HeLa cells treated with DOX or HU ([Fig. 1 Supplemental 1C&D](#)). DNA damage-induced MASTL upregulation was consistent with the activation of ATM/ATR signaling ([Fig. 1D&E](#)), and was not likely to be caused by the completion of DNA repair, as judged by the phosphorylation of replication protein A (RPA, [Fig. 1A-D](#)), H2AX and ATM/ATR substrates ([Fig. 1D&E](#)). In contrast to MASTL, DNA damage did not induce upregulation of other cell cycle kinases that mediate mitotic progression, including CDK1/cyclin B, Aurora A, Aurora B, and PLK1, in HeLa cells treated with DOX ([Fig. 1F](#)), HU ([Fig 1. Supplemental 2A](#)), or SCC38 cells with HU ([Fig 1. Supplemental 2B](#)). Furthermore, HU-induced MASTL upregulation was abrogated when cells were treated with caffeine ([Fig. 1G](#)), indicating that DNA damage-induced ATM/ATR activation acted upstream of MASTL upregulation.

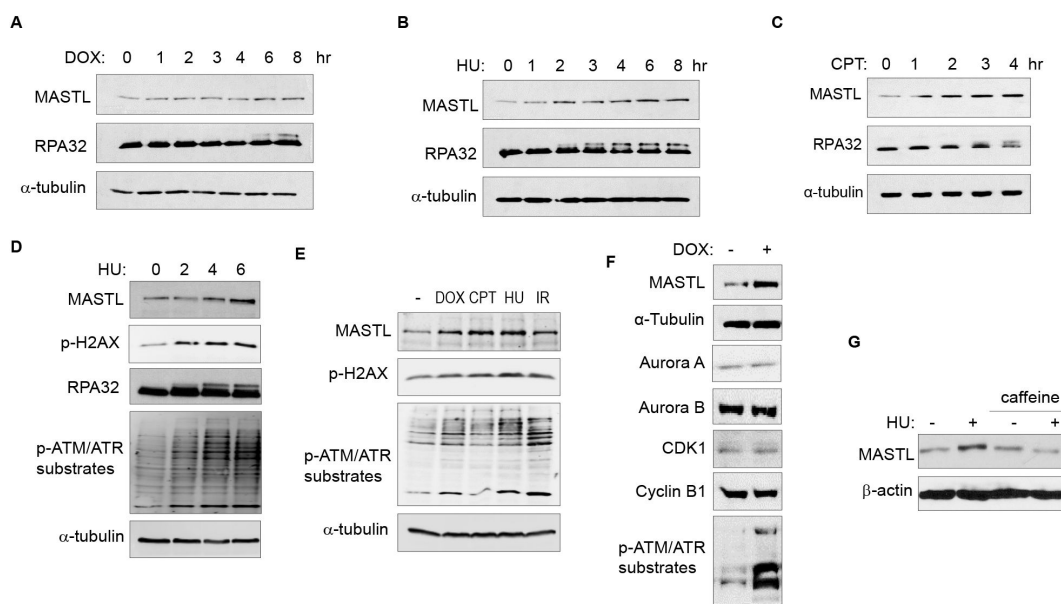


Figure 1.

The protein level of MASTL is upregulated after DNA damage.

(A) HEK293 cells were treated with 0.5 μ M doxorubicin (DOX) as indicated, cell lysates were collected and analyzed by immunoblotting for MASTL, RPA32 and α -tubulin. (B) HEK293 cells were treated with 10 mM hydroxyurea (HU) as indicated, cell lysates were collected and analyzed by immunoblotting for MASTL, RPA32 and α -tubulin. (C) HEK293 cells were treated with 10 nM camptothecin (CPT) as indicated, cell lysates were collected and analyzed by immunoblotting for MASTL, RPA32 and α -tubulin. (D) HeLa cells were treated with 10 mM HU, and incubated as indicated. Cell lysates were collected and analyzed by immunoblotting for MASTL, phospho-H2AX Ser-139, RPA32, phospho-ATM/ATR substrates, and α -tubulin. (E) HeLa cells were treated with or without 0.5 μ M DOX, 10 nM CPT, 10 mM HU and 20 Gy Ionizing Radiation (IR) for 4 hours. Cell lysates were collected and analyzed by immunoblotting for MASTL, phospho-H2AX Ser-139, phospho-ATM/ATR substrates, and α -tubulin. (F) HeLa cells were treated with 0.5 μ M DOX, and analyzed by immunoblotting for MASTL, α -tubulin, Aurora A, Aurora B, CDK1, Cyclin B1, and phospho-ATM/ATR substrates. (G) SCC38 cells were incubated with or without HU and caffeine, as indicated, and analyzed by immunoblotting for MASTL and β -actin.

MASTL upregulation after DNA damage is caused by protein stabilization

We did not observe a substantial increase in MASTL RNA transcripts, suggesting MASTL regulation at the post-translational level (Fig. 2A). Along this line, exogenous MASTL expressed from a different promoter underwent a similar pattern of upregulation after HU (Fig. 2B). These observations prompted us to examine the possibility that DNA damage increased the protein stability of MASTL. Indeed, CPT treatment in HeLa cells increased the protein stability of MASTL, as evaluated by the rate of protein degradation in the presence of cycloheximide (CHX), a protein synthesis inhibitor (Fig. 2C). Consistently, HU treatment

prolonged the half-life of MASTL protein in SCC38 and HEK293 cells (Fig. 2D-F, Fig. 2 Supplemental 1A&B).

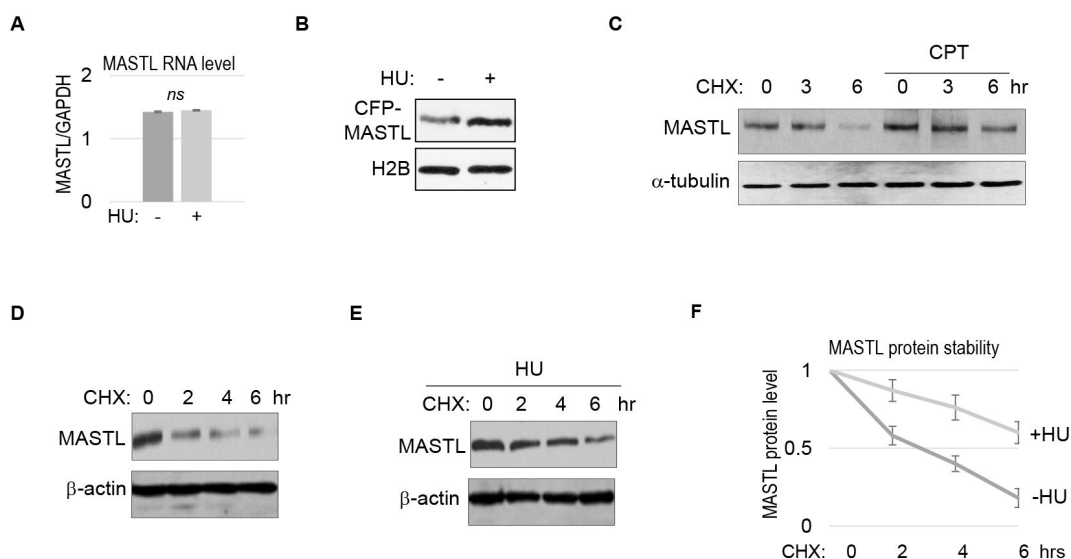


Figure 2.

MASTL upregulation after DNA damage is mediated by protein stabilization.

(A) HeLa cells were treated with 10 mM HU for 2 hours, and harvested for gene expression analysis. Quantitative RT-PCR was performed to detect the RNA levels of MASTL and GAPDH. The ratio of MASTL to GAPDH expression is shown. The mean values were calculated from three experiments, and statistical significance was evaluated using an unpaired 2-tailed Student's t test. A p-value more than 0.05 was considered non-significant (ns). (B) CFP-tagged MASTL was expressed in SCC38 cells. Cells were treated with or without 10 mM HU for 3 hours and analyzed by immunoblotting for CFP-MASTL and H2B. (C) HeLa cells were treated with or without 10 nM CPT for 1 hour. These cells were then treated with cycloheximide (CHX, 20 μ g/ml) at time 0 to block protein synthesis, and analyzed by immunoblotting for the protein stability of MASTL and α -tubulin. (D-F) SCC38 cells were treated without (D) or with (E) 10 mM HU for 2 hours. These cells were then treated with CHX (20 μ g/ml) at time 0 to block protein synthesis, and analyzed by immunoblotting for the protein stability of MASTL and β -actin. In panel F, the band signals were quantified using ImageJ, and the mean values and standard deviations of MASTL/ β -actin were calculated based on results of three experiments.

E6AP associates with MASTL

To elucidate MASTL regulation via protein stability, we sought to identify the ubiquitin ligase that mediates MASTL proteolysis. We previously performed a proteomic analysis of proteins associated with MASTL (Ren et al., 2017), and revealed E6AP as a potential binding pattern of MASTL. E6AP is encoded by gene ubiquitin-protein ligase E3A (UBE3A), and is the founding member of the HECT (homologous to E6AP C-terminus)- domain ubiquitin ligase family (Bernassola et al., 2008; Scheffner and Kumar, 2014). The association between E6AP and MASTL was confirmed by co-immunoprecipitation (Fig. 3A), and by pull-down of MASTL in HeLa cell lysate using recombinant E6AP (Fig. 3B). A direct interaction between purified

E6AP and MASTL proteins was also evident (Fig. 3 Supplemental 1A). Further analyses using various segments of MASTL showed that the N-terminal region of MASTL mediated E6AP-interaction in HeLa cells and *Xenopus* egg extracts (Fig. 3 Supplemental 1B, Fig. 3C). On the other hand, the N-terminus of E6AP co-immunoprecipitated MASTL (Fig. 3D); the MASTL-binding region was further mapped to aa 208-280 within the N-terminus of E6AP (Fig. 3 Supplemental 1C).

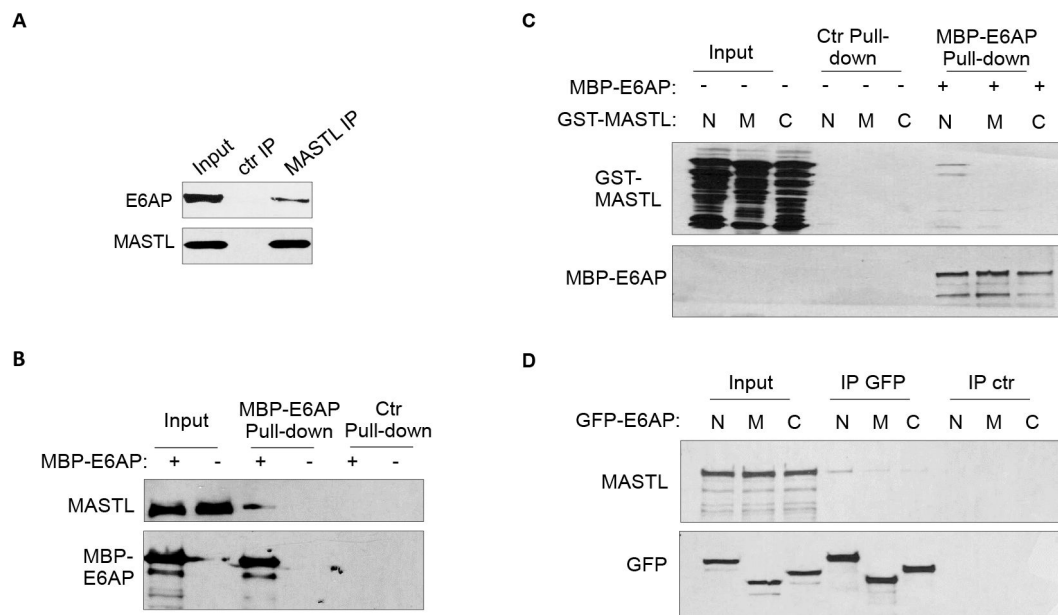


Figure 3.

E6AP associates with MASTL.

(A) Immunoprecipitation was performed in HeLa cell lysates, as described in Materials and Methods. The lysate input, MASTL IP, and control (ctr) IP samples were analyzed by immunoblotting for E6AP and MASTL. (B) A pull-down assay was performed in HeLa cell lysates using MBP-tagged E6AP, as described in Materials and Methods. The pull-down product, cell lysis input, and a control (-) pull-down (using empty beads) were analyzed by immunoblotting for E6AP, MASTL, and α -tubulin. (C) A pull-down assay was performed using MBP-tagged full length E6AP in *Xenopus* egg extract. Purified segments of MASTL, including N (aa 1-340), M (aa 335-660) and C (aa 656-887), were supplemented in the extracts. The pull-down products, egg extract inputs, and a control (-) pull-down (using empty beads) were analyzed by immunoblotting for GST and MBP. (D) Segments of E6AP, including N (aa 1-280), M (aa 280-497), and C (aa 497-770), were tagged with GFP, and transfected into HeLa cells for expression. 24 hours after transfection, cell lysates were harvested for GFP IP. The input, GFP IP, and control (ctr) IP using blank beads were analyzed by immunoblotting for MASTL and GFP.

E6AP mediates MASTL ubiquitination and degradation

To determine the potential role of E6AP in MASTL regulation, we depleted E6AP in cells using a siRNA and assessed the mRNA and protein levels of MASTL. As shown in Fig. 4A and Fig. 4. Supplemental 1A, E6AP depletion led to an increased level of MASTL protein, without significant change in the MASTL mRNA level. Ectopic expression of E6AP reduced MASTL

expression, as shown by immunoblotting (Fig. 4B) and immunofluorescence (Fig. 4C). Consistently, CRISPR-Cas9-mediated gene deletion of E6AP also augmented MASTL level, in a manner that was reversed by re-expression of exogenous E6AP (Fig. 4D). E6AP depletion increased, and overexpression reduced, the protein stability of MASTL (Fig. 4E&F). Furthermore, E6AP depletion disrupted MASTL ubiquitination in HeLa cells (Fig. 4G), indicating that E6AP mediated the ubiquitination of MASTL. An *in vitro* ubiquitination assay also confirmed MASTL as an effective substrate of E6AP (Fig. 4H). MASTL ubiquitination was specifically mediated by E6AP in the assay (Fig. 4 Supplemental 1B); the MASTL mutant deleted of the E6AP-binding motif was not ubiquitinated (Fig. 4 Supplemental 1C). Together, these data established E6AP as a key modulator of MASTL stability.

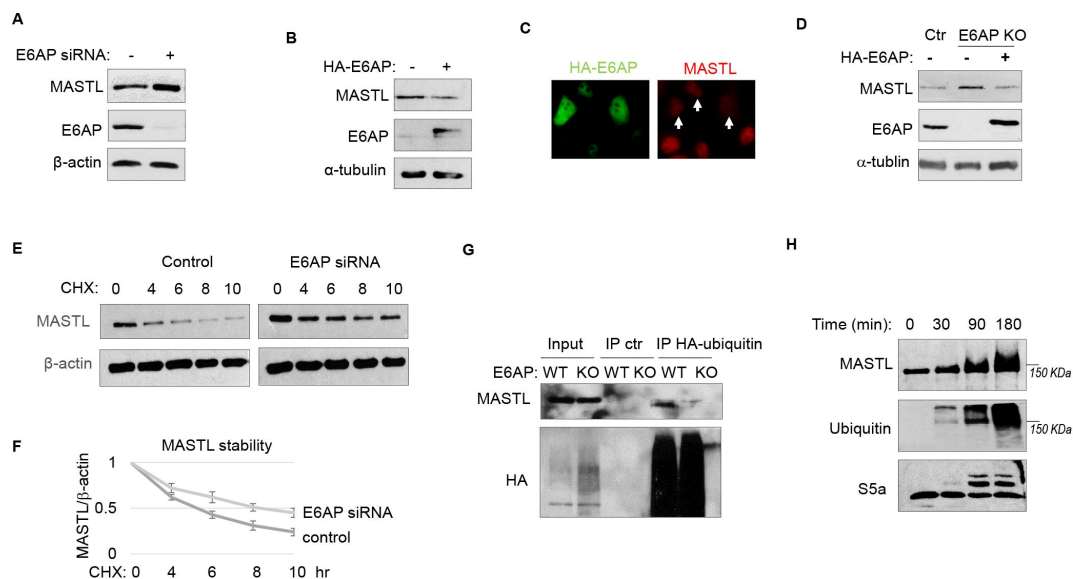


Figure 4.

E6AP mediates MASTL degradation.

(A) HeLa cells were transfected with control or E6AP-targeting siRNA E6AP for 24 hours. Cells were analyzed by immunoblotting for E6AP, MASTL and β -actin. (B) HeLa cells were transfected with HA-tagged E6AP. 24 hours after transfection, cells were analyzed by immunoblotting for E6AP, MASTL and α -tubulin. (C) As in panel B, HeLa cells were transfected with HA-E6AP, and analyzed by immunofluorescence (IF) for HA (green) and MASTL (red). Cells with HA-E6AP expression, as denoted by arrowheads, exhibited lower MASTL expression. (D) E6AP gene knockout (KO) was performed in HeLa cells, as described in Materials and Methods. HA-E6AP was expressed in E6AP KO cells, as indicated. Cells were analyzed by immunoblotting for MASTL, E6AP and α -tubulin. (E) HeLa cells transfected with control or E6AP siRNA were treated with 20 μ g/ml CHX, as indicated. Cells were harvested and analyzed by immunoblotting for MASTL and β -actin. (F) HeLa cells were treated as in panel E, MASTL and β -actin protein levels were quantified, and the ratio is shown for the indicated time points after CHX treatment, after normalized to that of time 0. The mean values and standard deviations were calculated from three experiments. (G) WT or E6AP knockout HeLa cells were transfected with HA-tagged ubiquitin for 12 hours, followed 50 μ M MG132 treatment for 4 hours. Cell lysates were harvested for HA IP or ctr IP using blank beads. The input and IP products were analyzed by immunoblotting for MASTL and HA. (H) *In vitro* ubiquitination assay was performed using E6AP as E3 ligase, and MASTL as substrate, as described in Materials and Methods. S5a was added as a control substrate. The reactions were incubated as indicated, as analyzed by immunoblotting for MASTL, ubiquitination, and S5a.

E6AP depletion facilitates DNA damage recovery via MASTL

A potential consequence of MASTL accumulation after DNA damage is cell cycle recovery, given the established role of MASTL in promoting cell cycle progression. To analyze cell cycle progression following ETO-treatment and release, we quantified mitotic cells that exhibited chromosome condensation and activation of Aurora A/B/C kinases (Fig. 5A). MASTL depletion substantially hindered DNA damage recovery, whereas E6AP knockout accelerated mitotic

entry after DNA damage (Fig. 5A). The recovery of mitotic entry in E6AP knockout cells was disrupted by MASTL downregulation, indicating its dependence on MASTL (Fig. 5A). To further study DNA damage recovery, we profiled the cell cycle progression of cells released from HU treatment (Fig. 5B). MASTL depletion caused prolonged cell cycle arrest; loss of E6AP promoted cell cycle recovery; and suppression of MASTL in E6AP-deleted cells abrogated cell cycle progression (Fig. 5B).

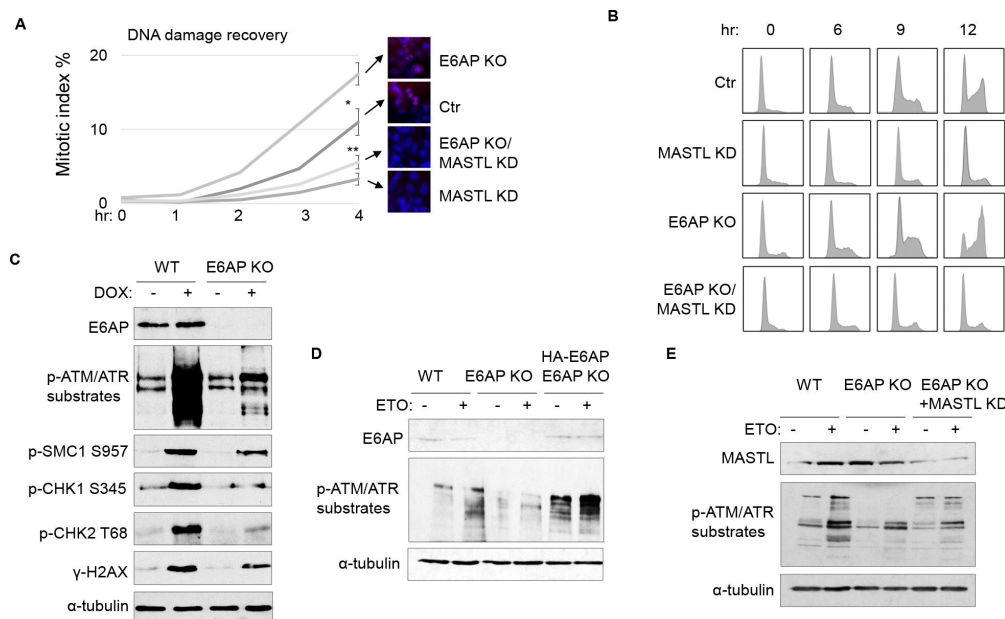


Figure 5.

E6AP depletion promotes DNA damage checkpoint recovery via MASTL.

(A) WT or E6AP KO HeLa cells were treated with or without MASTL siRNA. The cells were incubated in 0.1 μ M ETO for 18 hours, and released in fresh medium for recovery. Cells were harvested at the indicated time points (after the removal of ETO) for IF using an anti-phospho-Aurora A/B/C antibody. The activation of

Aurora phosphorylation (shown in red) and chromosome condensation (in blue) indicated mitosis. The percentages of cells in mitosis were quantified and shown. The mean values and standard deviations were calculated from three experiments. An unpaired 2-tailed Student's t test was used to determine the statistical significance (* $p < 0.05$, ** $p < 0.01$). MASTL knockdown by siRNA was shown by immunoblotting in the panel E. (B) WT or E6AP KO HeLa cells with or without MASTL siRNA, as in panel A, were treated with 2 mM HU for 18 hours. Cells were then released in fresh medium, and incubated as indicated, for recovery. The cell cycle progression was analyzed by Fluorescence-Activated Cell Sorting (FACS), as described in Materials and Methods. (C) WT or E6AP KO HeLa cells were treated with or without 0.5 μ M DOX for 4 hours. Cells were then analyzed by immunoblotting for E6AP, phospho-ATM/ATR substrates, phospho-SMC1 Ser-957, phospho-Chk1 Ser-345, phospho-Chk2 Thr-68, γ -H2AX and α -tubulin. (D) WT, E6AP KO, or E6AP KO with expression of HA-E6AP HeLa cells were treated with or without 0.1 μ M ETO, and analyzed by immunoblotting for E6AP, phospho-ATM/ATR substrates and α -tubulin. (E) WT, E6AP KO, or E6AP KO with transfection of MASTL siRNA HeLa cells were treated with or without 0.1 μ M ETO, and analyzed by immunoblotting for E6AP, phospho-ATM/ATR substrates and α -tubulin.

Interestingly, knockout of E6AP in HEK293 cells reduced DNA damage checkpoint signaling, measured by phosphorylation of SMC1, CHK1, CHK2, H2AX, and pan-ATM/ATR substrates after DOX treatment (Fig. 5C), and by phosphorylation of pan-ATM/ATR substrates after ETO (Fig. 5 Supplemental 1). A similar deficiency of ATM/ATR-mediated phosphorylation was observed in HeLa cells with E6AP gene deletion, which was rescued by E6AP re-expression (Fig. 5D). Interestingly, ATM/ATR-mediated substrate phosphorylation in E6AP knockout cells was also restored by MASTL depletion, indicating that E6AP promoted DNA damage checkpoint signaling by counteracting MASTL (Fig. 5E).

E6AP and MASTL association is regulated by ATM/ATR-mediated DNA damage signaling

Prompted by the findings that E6AP mediated the proteolysis of MASTL and that MASTL protein accumulated following DNA damage, we asked if the association between E6AP and MASTL was impacted by DNA damage. Interestingly, co-immunoprecipitation of E6AP with CFP-MASTL was profoundly disrupted by HU-treatment (Fig. 6A). This loss of E6AP and MASTL association was a result of active DNA damage signaling, as inhibition of ATM/ATR by caffeine preserved this protein association in the presence of HU (Fig. 6B). These lines of evidence pointed to a model that DNA damage-induced ATM/ATR activation disrupts E6AP association with MASTL, subsequently leading to reduced MASTL degradation and increased MASTL protein accumulation.

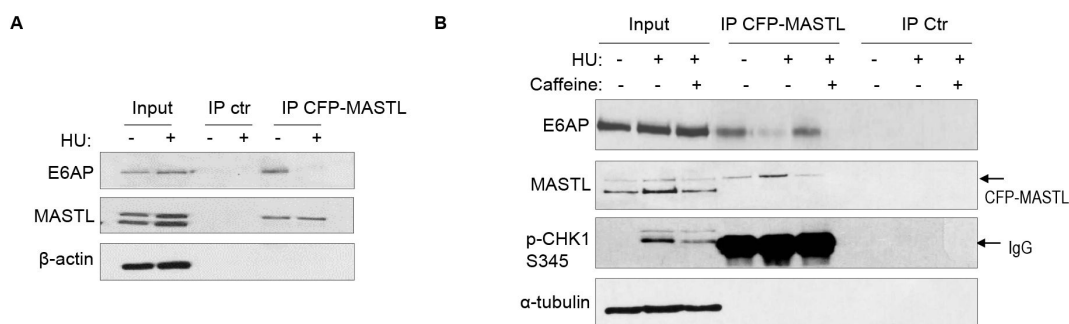


Figure 6.

The E6AP and MASTL association is disrupted by DNA damage-induced ATM/ATR signaling.

(A) HeLa cells expressing CFP-MASTL were treated without or with 10 mM HU. CFP-MASTL IP was performed using a GFP antibody. The input, GFP IP, and control (ctr) IP using blank beads were analyzed by immunoblotting for E6AP, MASTL, and α-tubulin. (B) HeLa cells expressing CFP-MASTL were treated without or with 10 mM HU and 4 mM caffeine, as indicated. CFP-MASTL IP was performed using a GFP antibody. The input, GFP IP, and control (ctr) IP using blank beads were analyzed by immunoblotting for E6AP, MASTL, phospho-CHK1 Ser-345, and α-tubulin.

ATM/ATR mediates E6AP S218 phosphorylation to modulate E6AP and MASTL association

ATM/ATR phosphorylates numerous substrates to regulate their functions after DNA damage. These kinases typically target serine or threonine residues followed by glutamine (S/TQ). E6AP possesses a single evolutionally-conserved serine, Ser-218 in humans, as a potential consensus site of ATM/ATR-mediated phosphorylation (Fig. 7A). E6AP Ser-218 phosphorylation was also documented in multiple proteomic databases (Beli et al., 2012; Franz-Wachtel et al., 2012; Kettenbach et al., 2011; Klammer et al., 2012; Matsuoka et al., 2007; Olsen et al., 2010; Schweppe et al., 2013; Weber et al., 2012). We generated a phospho-specific antibody for this residue, and observed the induction of Ser-218 phosphorylation after HU (Fig. 7B), or DOX treatment (Fig. 7C). This phospho-signal was absent in E6AP KO cells (Fig. 7B, Fig. 7 supplemental 1A), and was diminished with S218A mutation (Fig. 7E), confirming

its specificity. Inhibition of ATM using a selective kinase inhibitor reduced E6AP Ser-216 phosphorylation after DOX treatment in both HeLa and HEK293 cells (Fig. 7C, Fig. 7 supplemental 1B). ATM was also the primary kinase to mediate E6AP Ser-218 phosphorylation in response to HU (Fig. 7 supplemental 1C).

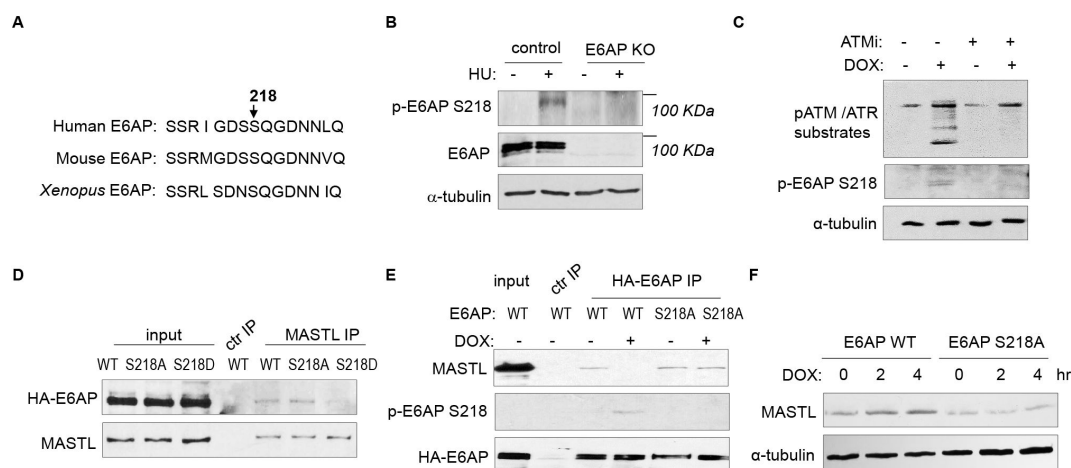


Figure 7.

ATM/ATR mediates E6AP S218 phosphorylation to disrupt MASTL association and proteolysis.

(A) The sequence alignment of the conserved E6AP Ser-218 motif in human, mouse and *Xenopus*. (B) A phospho-specific antibody recognizing E6AP Ser-218 was generated, as described in Materials and Methods. WT or E6AP KO HEK293 cells were treated without or with 10 mM HU, and analyzed by immunoblotting for phospho-E6AP Ser-218, E6AP, and α -tubulin. (C) HeLa cells were treated without or with 0.5 μ M DOX and 5 μ M KU55933 (ATMi), as indicated, and analyzed by immunoblotting for E6AP, phospho-E6AP Ser-218 and α -tubulin. (D) HeLa cells were transfected with HA-tagged WT, S218A or S218D E6AP. Cell lysates were harvest for IP assays. The input, MASTL IP, and a control IP using empty beads products were analyzed by immunoblotting for MASTL and HA. (E) HeLa cells were transfected with HA-tagged WT or S218A E6AP, as in panel D. Cells were treated with or without 0.5 μ M DOX for 3 hours, and harvested for IP assays. The input, HA IP, and a control IP using empty beads products were analyzed by immunoblotting for MASTL, phospho-E6AP Ser-218, and HA. (F) E6AP KO HeLa cells were transfected with HA-tagged WT or S218A E6AP, as in panel D. Cells were treated with or without 0.5 μ M DOX, incubated as indicated, and harvested for immunoblotting for MASTL and α -tubulin.

As we showed E6AP-MASTL dissociation in the DNA damage-induced and ATM/ATR-dependent manner, we hypothesized that E6AP phosphorylation modulated its protein association with MASTL. Indeed, the phospho-mimetic mutant form of E6AP, E6AP S218D, exhibited significantly reduced association with MASTL, compared to WT or phospho-deficient S218A E6AP (Fig. 7D). Furthermore, DNA damage disrupted MASTL association with WT E6AP, but not E6AP S218A (Fig. 7E). Consistent with the persistent MASTL association, S218A mutation also prevented MASTL protein accumulation after DNA damage (Fig. 7F).

E6AP S218 phosphorylation promotes DNA damage checkpoint recovery

As we established E6AP S218 phosphorylation in response to DNA damage, we sought to investigate the functional consequence of E6AP Ser-218 phosphorylation after DNA damage. Cell cycle recovery was examined in E6AP KO cells reconstituted with either WT or S218A E6AP. Interestingly, using both mitotic index measurement after ETO release, or cell cycle profiling after HU, we noted defective DNA damage checkpoint recovery in cells harboring E6AP S218A mutation (Fig. 8A&B). The resumption of cell cycle progression post DNA damage was also assessed biochemically, by phosphorylation of CDK substrates, and a similar pattern of deficiency was seen with S218A mutation (Fig. 8C). Furthermore, cells expressing S218A E6AP, compared to those expressing WT E6AP, exhibited elevated levels of DNA damage signaling (Fig. 8D).

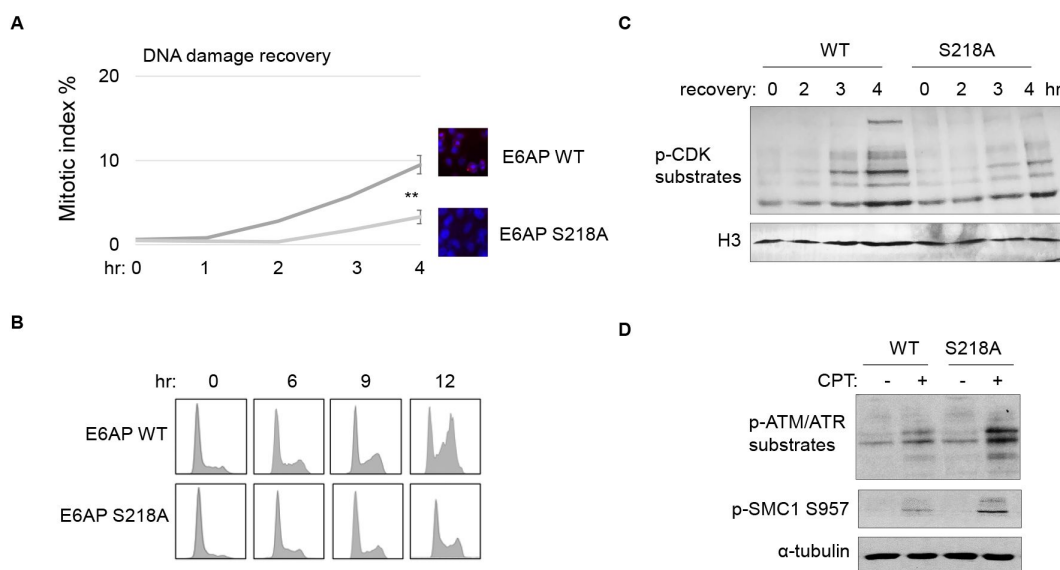


Figure 8.

E6AP S218 phosphorylation is required for DNA damage recovery.

(A) E6AP KO HeLa cells were transfected with HA-tagged WT or S218A E6AP, as in Fig. 7. Cells were treated with 0.1 μM ETO for 18 hours, and released in fresh medium for recovery. Cells were then harvested at the indicated time points (after the removal of ETO) for IF using an anti-phospho-Aurora A/B/C antibody. The activation of Aurora phosphorylation (shown in red) and chromosome condensation (in blue) indicated mitosis. The percentages of cells in mitosis were quantified and shown. The mean values and standard deviations were calculated from three experiments. An unpaired 2-tailed Student's t test was used to determine the statistical significance (** $p < 0.01$). (B) E6AP KO HeLa cells expressing HA-tagged WT or S218A E6AP, as in panel A, were treated with 2 mM HU for 18 hours. Cells were then released in fresh medium, and incubated as indicated, for recovery. Cell cycle progression was analyzed by FACS. (C) WT or S218A E6AP was expressed in E6AP KO HEK293 cells. Cells were treated without or with 0.1 μM ETO for 18 hours, released in fresh medium for recovery, and incubated as indicated. Cells were analyzed by immunoblotting for phospho-CDK substrates and histone H3. (D) WT or S218A E6AP was expressed in E6AP KO HEK293 cells, as in panel C. Cells were treated without or with 1 μM CPT for 90 minutes, and analyzed by immunoblotting for phospho-ATM/ATR substrates, phospho-SMC1 Ser-957, and α-tubulin.

Discussion

In this study, we identified E6AP as the underlying E3 ubiquitin ligase that mediated the ubiquitination and degradation of MASTL. Compared to the function of MASTL, regulation of MASTL is relatively under-investigated. Previous studies illustrated mechanisms that modulated the phosphorylation and subcellular localization of MASTL during cell cycle progression (Castro and Lorca, 2018). We reported here that DNA damage disrupted E6AP and MASTL association, leading to increased MASTL protein stability and accumulation of MASTL protein. Because MASTL promotes the phosphorylation of CDK substrates by inhibiting the counteracting phosphatase PP2A/B55, this fashion of MASTL upregulation can re-activate cell cycle progression while CDK activities are restrained by the DNA damage

checkpoint (Fig. 9). By comparison, other cell cycle kinases that promote mitotic progression, including CDK1/Cyclin B, Aurora A/B and PLK1, did not exhibit this pattern of DNA damage-induced upregulation.

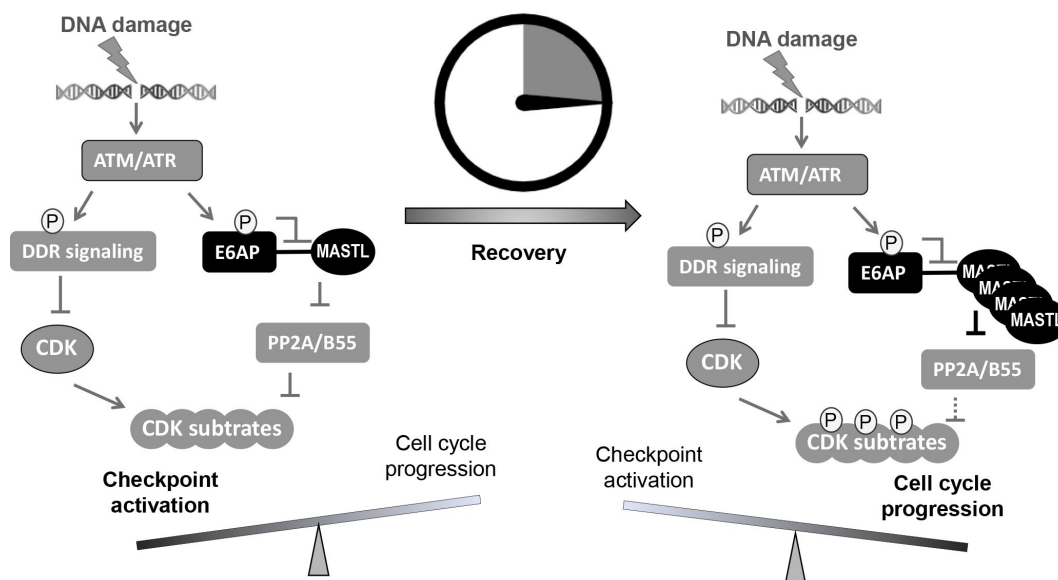


Figure 9.

A “timer” model for the role of the ATM-E6AP-MASTL axis in cell cycle arrest and recovery after DNA damage.

DNA damage induces ATM/ATR activation and checkpoint signaling. Activated ATM/ATR also phosphorylates E6AP Ser-218, leading to the dissociation of E6AP from MASTL and reduced MASTL degradation. The subsequent accumulation of MASTL promotes de-activation of the DNA damage checkpoint and initiates cell cycle resumption by inhibiting dephosphorylation of CDK substrates.

E6AP, also known as UBE3A, is the prototype of the E3 ligase subfamily containing a C-terminal HECT domain. Mutations of E6AP cause Angelman syndrome, a debilitating neurological disorder in humans (Bernassola et al., 2008; Buiting et al., 2016; Levav-Cohen et al., 2012; Sell and Margolis, 2015; Wolyniec et al., 2013). E6AP and other HECT-containing E3 ligases are emerging as potential etiological factors and drug targets in cancer (Bernassola et al., 2008; Scheffner and Kumar, 2014; Yu et al., 2020). Interestingly, mouse embryo fibroblasts lacking E6AP escaped replicative senescence, proliferated under stress conditions, supported anchorage-independent growth, and enhanced tumor growth *in vivo* (Levav-Cohen et al., 2012; Wolyniec et al., 2013). These phenotypes are not well explained in the context of known substrates of E6AP (Sell and Margolis, 2015), but can be potentially attributed to MASTL upregulation, as characterized in our current study. E6AP binds MASTL via its N-terminal domain, and mediates MASTL ubiquitination and degradation. The function of E6AP in the DNA damage checkpoint via MASTL modulation suggests E6AP as a DDR factor and a potential tumor suppressor.

ATM/ATR phosphorylates a myriad of substrates to promote DNA repair and activate the cell cycle checkpoints (Flynn and Zou, 2011; Shiloh, 2003). We found that MASTL accumulation after DNA damage was disrupted by inhibition of ATM/ATR, suggesting that ATM/ATR modulated MASTL proteolysis. Our study further revealed that ATM phosphorylated E6AP at

Ser-218, leading to E6AP dissociation from MASTL and the subsequent MASTL stabilization. Our functional characterization of E6AP Ser-218 is interesting, as phosphorylation of this residue has been detected in numerous proteomic studies as a modification induced by DNA damage (Beli et al., 2012; Matsuoka et al., 2007), mitosis (Franz-Wachtel et al., 2012; Kettenbach et al., 2011; Olsen et al., 2010), or cancer progression (Klammer et al., 2012; Schweppe et al., 2013; Weber et al., 2012). Our results suggest that ATM/ATR, while important for the activation of the DNA damage checkpoint, also engages a mechanism to initiate cell cycle recovery (Fig. 9). This mechanism at least partially answers the puzzling question of how cell cycle recovery is initiated from the state of the DNA damage checkpoint. The DNA damage checkpoint targets cell cycle kinases to halt the cell cycle; on the other hand, the reactivation of CDK, PLK1, MASTL, and other cell cycle kinases promotes the deactivation of DNA damage checkpoint signaling and cell cycle resumption (Peng, 2013). Cell cycle kinases are known to coordinate with each other in positive feedback reactions to achieve full activation and bring about mitosis. We speculate that, in the case of DNA damage recovery, ATM-mediated MASTL upregulation may provide an initial signal to trigger these positive feedback reactions, ultimately shifting the balance from cell cycle arrest to recovery. Of note, this timer-like mechanism can contribute to the transient nature of the DNA damage checkpoint, as observed in yeast, frog, and mammalian cells (Bartek and Lukas, 2007; Clemenson and Marsolier-Kergoat, 2009; Syljuasen, 2007; van Vugt and Medema, 2004; Yoo et al., 2004). Finally, the resumption of the cell cycle after DNA damage is a potentially vital process that enables tumor cell progression and treatment evasion. MASTL kinase has emerged as an important factor of tumorigenesis and treatment resistance in multiple types of cancer (Fatima et al., 2020; Marzec and Burgess, 2018; Wang et al., 2014). Thus, future studies built upon our current findings may shed new light on cancer resistance and identify new anti-cancer drug targets to enhance treatment outcome.

Materials and Methods

Antibodies and chemicals

Mouse antibody to MASTL (clone 4F9, Millipore MABT372) was described previously (Wang et al., 2011). Phospho-specific E6AP Ser-218 antibody was generated using a synthesized peptide (SSRIGDS phospho-S QGDNNLQ). Other antibodies include α -tubulin (Santa Cruz Biotechnology, #sc-5286), E6AP (Bethyl Laboratories, A300-351), HA (Cell signaling technology #3724), γ -H2AX Ser-139 (Cell signaling technology #9718S), phospho-ATM/ATR substrate motif (Cell signaling technology, #6966S), phospho-SMC1 Ser-957 (Cell signaling technology, #58052), phospho-CHK1 Ser-345 (Cell signaling technology, #2348), phospho-CHK2 Thr-68 (Cell signaling technology, #2197), Phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (Cell signaling technology, #2914), Aurora A (Cell signaling technology, #14475), Aurora B (Cell Signaling technology, #3094), CDK1 (Cell signaling technology, #9112), Cyclin B (Cell signaling technology, #4138), phosphor-CDK substrates (Cell signaling technology, #2325), RPA32 (Thermo Fisher Scientific, # PA5-22256), S5a (Boston Biochem, #SP-400), ubiquitin (Cell Signaling Technology, #3936), and GFP (Cell Signaling Technology, #2555).

The following chemicals were used: Hydroxyurea (HU, MP Biomedicals, #102023), doxorubicin (DOX, MilliporeSigma, #25316-40-9), caffeine (Sigma-Aldrich, #C0750), ATM inhibitor (KU55933, Selleckchem, #S1092), ATR inhibitor (VE-821, Selleckchem, #S8007), cycloheximide (CHX, Fluka analytical, #01810), etoposide (Sigma-Aldrich, #E1383), camptothecin (Sigma-Aldrich, #C9911), G418 sulfate (Thermo Fisher Scientific, #10131035), MG132 (Calbiochem, #133407-82-6), cisplatin (R&D systems, #15663-27-1), propidium iodide

(PI, Thermo Fisher Scientific, #P1304MP), and isopropyl-beta-D-thiogalactopyranoside (IPTG, RPI research products international, # 367-93-1).

Cell culture and treatment

Human cervix carcinoma (HeLa) and human embryonic kidney 293 (HEK293) cell lines were obtained and authenticated by ATCC, and maintained in Dulbecco's modified Eagle medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS, Hyclone). Human head and neck squamous cell carcinoma UM-SCC-38 cells, as characterized in (Wang et al., 2014), was maintained in DMEM (HyClone) with 10% FBS(HyClone). As described previously (Li et al., 2021), transfection of plasmid vectors was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. siRNA targeting human UBE3A or human MASTL (Integrated DNA Technologies) was transfected into cells using Lipofectamine RNAi MAX (Invitrogen). A non-targeting control siRNA was used as a control. UBE3A siRNA sequence: 5'-3' AGGAAUUGUCAUCUUU; 5'-3' UCAGAAUAAAGAUUGACA. MASTL siRNA sequence: 5'-3' GUCUACUUGGUAUGGAA; 5'-3' UAAGAUAUCCAUAUACCA. For fluorescence-activated cell sorting, cells were fixed in 70% cold ethanol, washed with cold PBS and stained with propidium iodide (20 ug/ml propidium iodide and 200 ug/ml RNase A diluted in PBS with 0.1% Triton X-100) at 37°C for 15 mins before analysis using BD FACSArray.

Generation of E6AP knockout cells

To generate E6AP-KO HeLa cells, a pCRSIPrv2-sgRNA construct expressing both Cas9 and a sgRNA targeting human E6AP were transfected into HeLa cells. Twenty-four hours after transfection, cells were selected with puromycin (1.5 µg/ml) for 2 days. Single cells were grown in 96-well plates for amplification. Individual clones were verified by immunoblotting for E6AP expression. The following sgRNA sequence was used for gene knockout: 5' CTACTACCACCAGTAACTG 3'. E6AP KO was also carried out in HEK293 cells using a CRISPR revolution sgRNA EZ Kit (Synthego), following the manufacturer's protocol. The following sgRNA sequence was used for gene knockout: 5' GCAAGCTGACACAGGTGCTG 3'.

Immunoblotting, immunofluorescence, and immunoprecipitation

Immunoblotting, immunofluorescence, and immunoprecipitation were performed as previously described (Wang et al., 2019). Briefly, for immunoblotting, samples were harvested in 1X Laemmli sample buffer (Bio-Rad) and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electro-transfer, PVDF membranes (Millipore, Billerica, MA) were blocked in 1× TBST (10mM Tris-HCl, pH7.5, 150mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk. Membranes were incubated in primary antibodies in a primary antibody dilution buffer (1X TBS, 0.1% Tween-20 with 5% BSA), and then horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) in 1× TBST. Detection was performed using an enhanced chemiluminescence (ECL) substrate kit (Thermo Scientific Pierce).

For immunofluorescence (IF), cells on microscope cover glasses were washed with PBS, fixed in 3% formaldehyde with 0.1% Triton X-100, permeabilized in 0.05% Saponin, and blocked with 5% goat serum. Primary antibodies were diluted in the blocking buffer and incubated with the cells for 2 hr. The cells were then incubated with Alexa Fluor secondary antibodies (Invitrogen, 1: 2,000) for 1 hr at room temperature. The nuclei of cells were stained with 4',6-diamidino-2-phenylindole (DAPI). Imaging was performed using a Zeiss Axiovert 200M inverted fluorescence microscope at the UNMC Advanced Microscopy Core Facility.

For immunoprecipitation (IP), cells were harvested in lysis 150 buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1mM DTT, and 0.5% Tween 20). Anti-rabbit or anti-mouse magnetic beads (Thermo Fisher) were conjugated to antibodies, and incubated in cell lysates for IP.

***In vitro* ubiquitination assay**

The *in vitro* ubiquitination assay was performed using an ubiquitin kit (Boston Biochem, #K-230). His-tagged S5a protein (provided in the kit as positive control) or GST-tagged MASTL protein (purified as above) was added in the ubiquitin reactions as substrate. After incubation at 37°C for up to 180 minutes, the reactions were terminated by the addition of Laemmli buffer and boiling.

Plasmid construction and protein expression

A vector expressing HA-tagged human E6AP was obtained from Addgene (Plasmid #8658), E6AP mutants were generated using site-directed mutagenesis (Agilent) following the protocol recommended by the manufacturer. Segments of E6AP, including N (aa 1-280), M (aa 280-497), C (aa 497-770), were inserted to a pEGFP vector for IP, and pMBP vector for pull-down. Additional segments, including N1 (aa 1-99), N2 (aa 100-207), and N3 (108-280) were cloned to pEGFP for IP. Expression vectors for MASTL were previously characterized (Yamamoto et al., 2014). Additionally, three segments of MASTL (N: aa 1-340; M: aa 335-660; C: aa 656-887) were inserted into pGEX 4T-1 (GE Healthcare). The resulting expression vectors were transfected into BL21 bacteria cells for protein expression and purification. For the pulldown experiments, GST-tagged proteins were purified on glutathione-Sepharose beads (New England Biolabs), and MBP-tagged proteins were purified on Amylose resin (New England Biolabs).

***Xenopus* egg extracts**

Cytostatic factor (CSF) extracts were prepared as described previously (Zhu and Peng, 2016). Eggs were incubated in 2% cysteine and washed in 1× XB (1M KCl, 11mM MgCl₂, 100mM HEPES (pH 7.7), and 500mM sucrose). Egg extracts were generated by centrifugation at 10,000 × g. Extracts were released into interphase by supplementation with 0.4 mM CaCl₂, and incubated for 30 min at room temperature.

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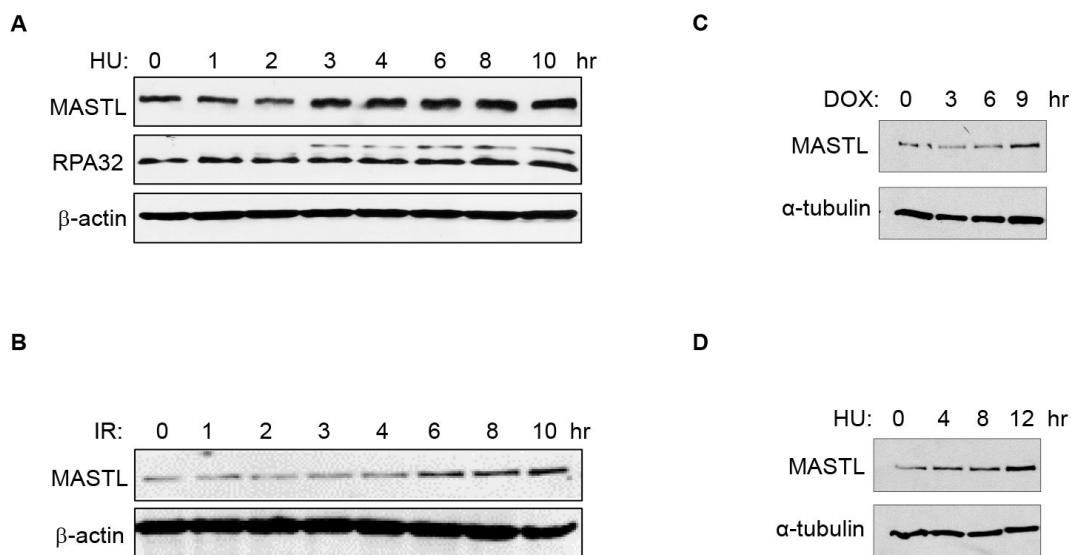


Figure 1 Supplemental 1.

MASTL upregulation after DNA damage.

(A, B) SCC38 cells were treated with 10 mM HU (A) or 10 Gy IR (B), and incubated for 0-10 hours, as indicated. The expression levels of MASTL, RPA, and α -tubulin were analyzed by immunoblotting. (C, D) HeLa cells were treated with 0.5 μ M DOX (C) or 10 mM HU (D) as indicated, cell lysates were collected and analyzed by immunoblotting for MASTL and α -tubulin.

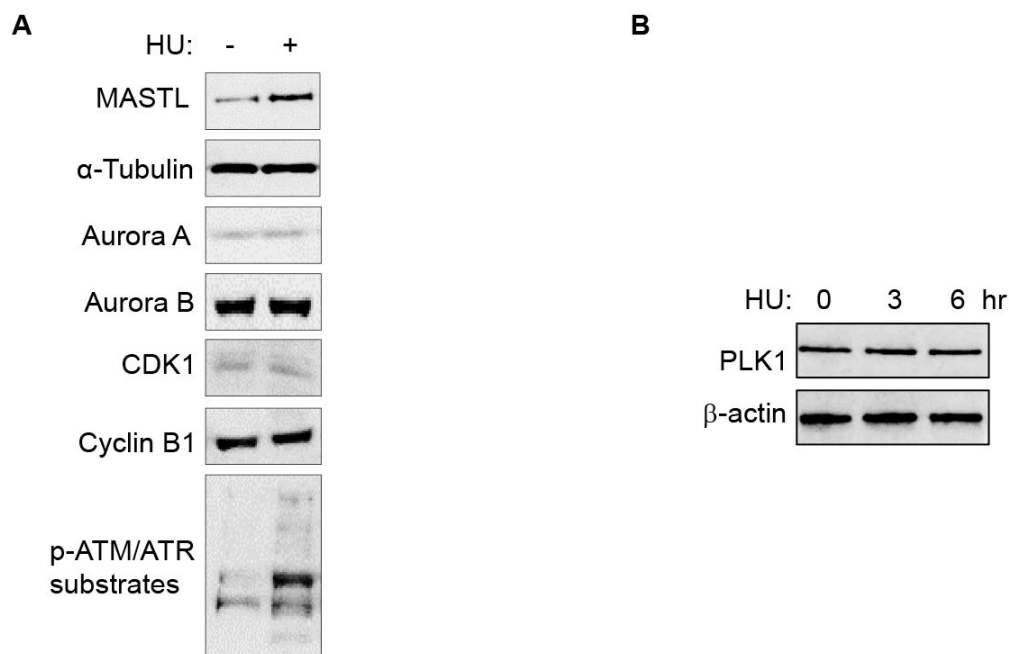


Figure 1 Supplemental 2.

The expression levels of cell cycle kinases after DNA damage.

HeLa (A) or SCC38 (B) cells were treated without or with 2 mM of hydroxyurea (HU) for 8 hours (A), or 3-6 hours (B). Cell lysates were harvested for immunoblotting.

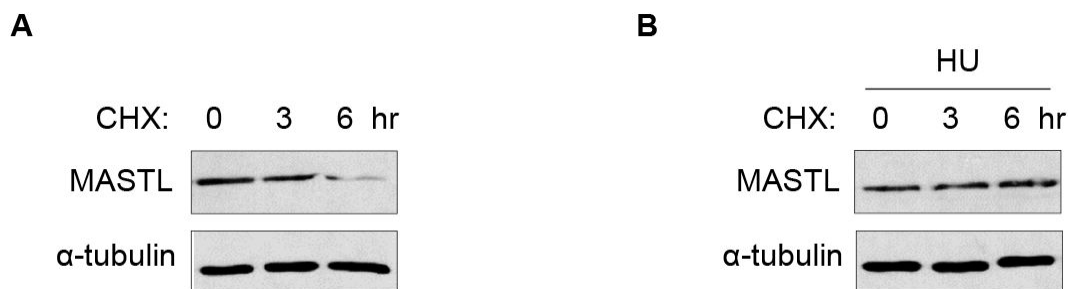


Figure 2 Supplemental 1.

Increased protein stability of MASTL after DNA damage.

HEK293 cells were treated without (A) or with (B) 10 mM HU for 2 hours. These cells were then treated with cycloheximide (CHX, 20 µg/ml) at time 0 to block protein synthesis, and analyzed by immunoblotting for the protein stability of MASTL and α-tubulin.

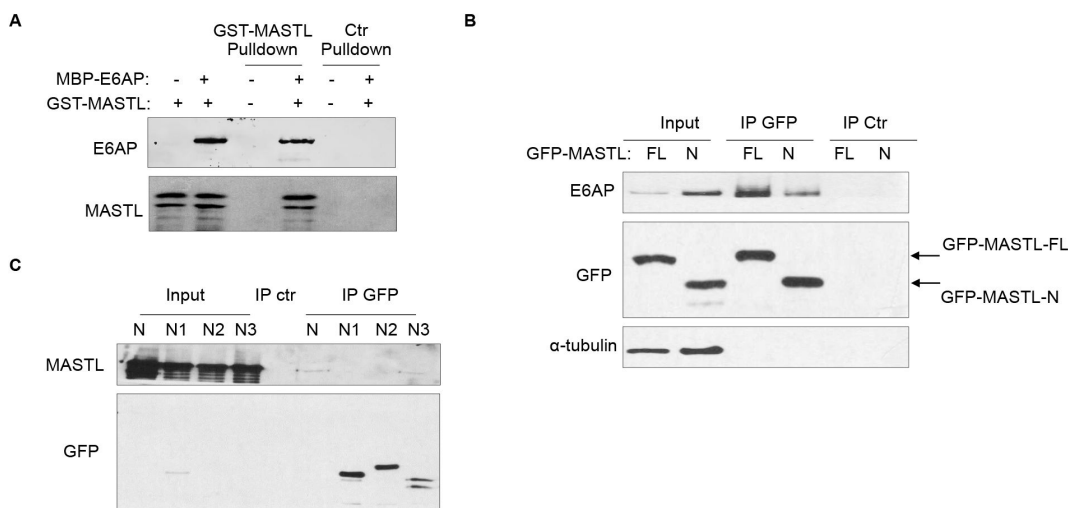


Figure 3 Supplemental 1.

E6AP and MASTL associate via their N-terminal motifs.

(A) MBP-E6AP and GST-MASTL were expressed and purified, as described in Materials and Methods. GST-MASTL on glutathione beads, or control glutathione beads, were incubated with MBP-E6AP, followed by pulldown, as described in Materials and Methods. The input, GST-MASTL pulldown, and control pull down samples were analyzed by immunoblotting for MASTL and E6AP. (B) GFP-MASTL, FL or N-terminus (aa 1-340), was expressed in HeLa cells. Immunoprecipitation (IP) was performed using a GFP antibody, and the presence of E6AP in the IP products was examined by immunoblotting. (C) IP was performed using GFP-tagged segments of E6AP expressed in HeLa cells. The IP product, cell lysate input, and a control IP (using empty beads) were analyzed by immunoblotting for MASTL and GFP. N: aa 1-280; N1: aa 1-99; N2: aa 100-207; N3: aa 108-280.

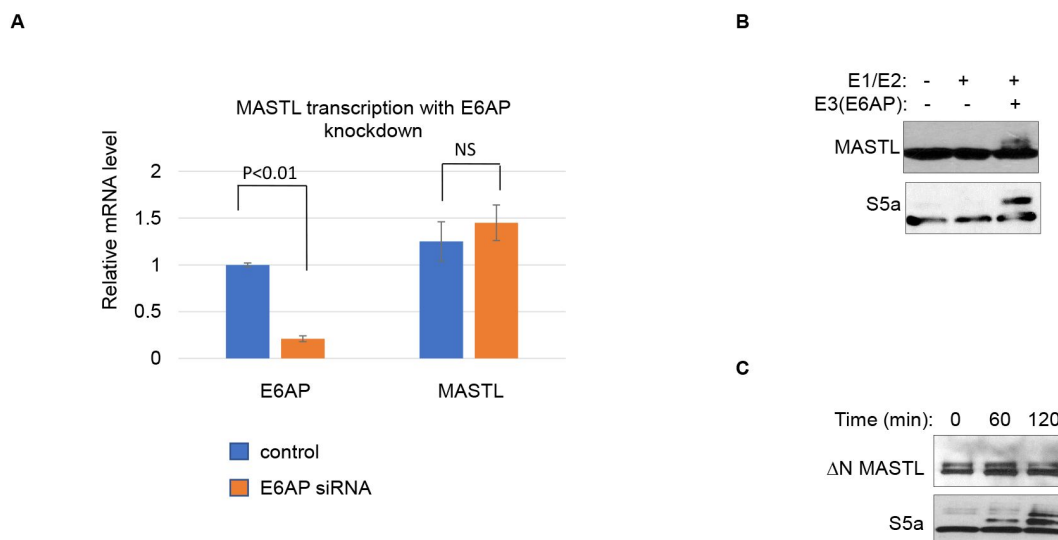


Figure 4 Supplemental 1.

E6AP mediates MASTL ubiquitination and degradation.

(A) HeLa cells were treated without or with E6AP siRNA. The RNA levels of E6AP and MASTL were quantified by real-time PCR. The mean values were calculated from three experiments. Statistical significance was determined using an unpaired 2-tailed Student's t test. A p-value more than 0.05 was considered non-significant (ns). (B) The E6AP *in vitro* ubiquitination assay was performed using MASTL as substrates, as in Fig. 4H. S5a was added as a control substrate. E1/2 and E3 (E6AP) enzymes were added in the reactions, as indicated. Samples were analyzed by immunoblotting for MASTL and S5a after 90 min incubation. (C) *In vitro* ubiquitination assay was performed using E6AP as E3 ligase, and Δ N MASTL (aa 335-887) as substrate. S5a was added as a control substrate. The reactions were incubated as indicated, as analyzed by immunoblotting for MASTL and S5a.

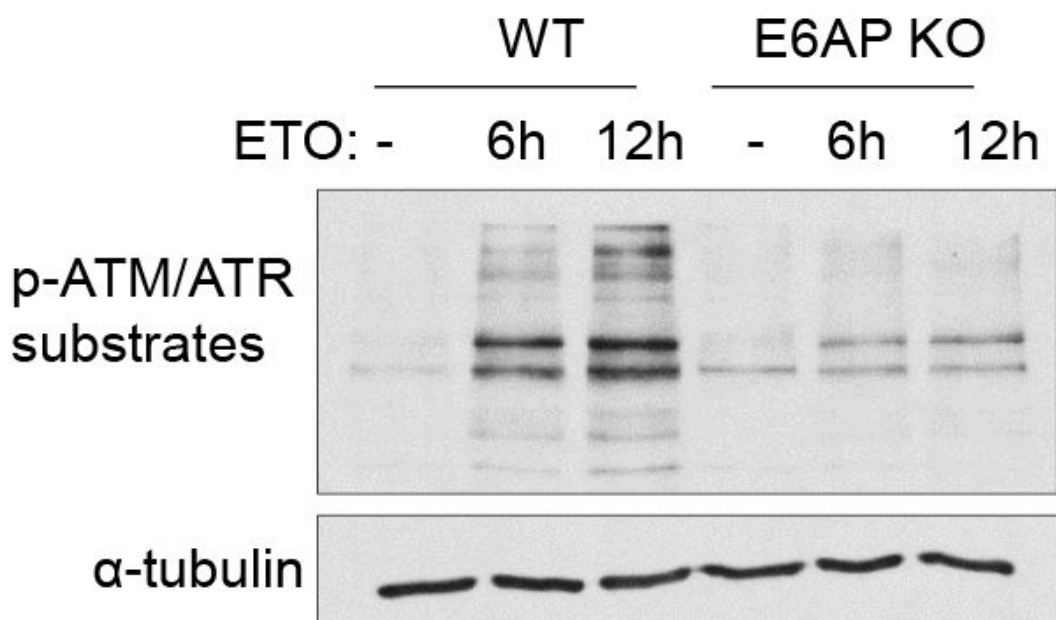


Figure 5 Supplemental 1.

Impaired DNA damage checkpoint signaling in E6AP-null cells.

WT or E6AP knockout (KO) HeLa cells were treated with 0.1 μ M etoposide (ETO) for the indicated hours. Cells were analyzed by immunoblotting for phospho-ATM/ATR substrates and α -tubulin.

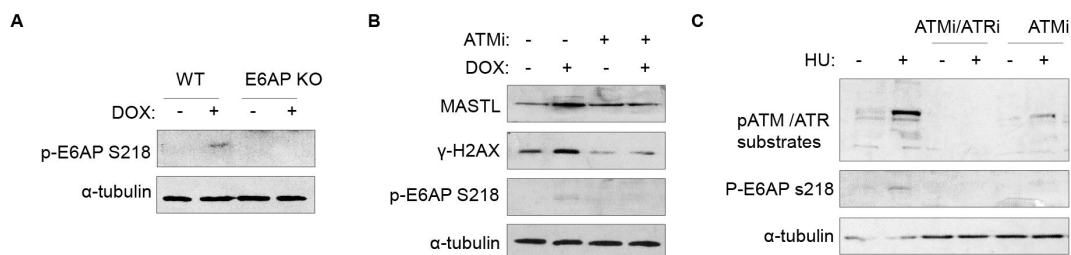


Figure 7 Supplemental 1.

DNA damage-induced E6AP Ser-218 phosphorylation is mediated by ATM.

(A) WT or E6AP knockout HEK293 cells were treated without or with doxorubicin (DOX, 0.5 μ M) for 4 hours. Cells were harvested and analyzed by immunoblotting for phospho-E6AP Ser-218 and α -tubulin. (B) HEK293 cells were treated without or with DOX (0.5 μ M), or ATM inhibitor (KU55933, 10 μ M), and analyzed by immunoblotting. (C) HeLa cells were treated with hydroxyurea (HU, 10 mM) combined with ATM/ATR inhibitor (caffeine, 4 mM) or ATM inhibitor (KU55933, 10 μ M), for 12 hours, as indicated. Cells were harvested and analyzed by immunoblotting.

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Reviewer #1 (Public Review):

In principle a very interesting story, in which the authors attempt to shed light on an intriguing and poorly understood phenomenon; the link between damage repair and cell cycle re-entry once a cell has suffered from DNA damage. The issue is highly relevant to our understanding of how genome stability is maintained or compromised when our genome is damaged. The authors present the intriguing conclusion that this is based on a timer, implying that the outcome of a damaging insult is somewhat of a lottery; if a cell can fix the damage within the allocated time provided by the "timer" it will maintain stability, if not then stability is compromised. If this conclusion can be supported by solid data, the paper would make a very important contribution to the field.

However, the story in its present form suffers from a number of major gaps that will need to be addressed before we can conclude that MASTL is the "timer" that is proposed here. The primary concern being that altered MASTL regulation seems to be doing much more than simply acting as a timer in control of recovery after DNA damage. There is data presented to suggest that MASTL directly controls checkpoint activation, which is very different from acting as a timer. The authors conclude on page 8 "E6AP promoted DNA damage checkpoint signaling by counteracting MASTL", but in the abstract the conclusion is "E6AP depletion promoted cell cycle recovery from the DNA damage checkpoint, in a MASTL-dependent manner". These 2 conclusions are definitely not in alignment. Do E6AP/MASTL control checkpoint signaling or do they control recovery, which is it?

Also, there is data presented that suggest that MASTL does more than just controlling mitotic entry after DNA damage, while the conclusions of the paper are entirely based on the assumption that MASTL merely acts as a driver of mitotic entry, with E6AP in control of its levels. This issue will need to be resolved.

Finally, the authors have shown some very compelling data on the phosphorylation of E6AP by ATM/ATR, and its role in the DNA damage response. But the time resolution of these effects in relation to arrest and recovery have not been addressed.

Reviewer #2 (Public Review):

This is an interesting study from Admin Peng's laboratory that builds on previous work by the PI implicating Greatwall Kinase (the mammalian gene is called MASTL) in checkpoint recovery.

The main claims of this study are:

1. Greatwall stability is regulated by the E6-AP ubiquitin ligase and this is inhibited following DNA damage in an ATM dependent manner.
2. Greatwall directly interacts with E6-AP and this interaction is suppressed by ATM dependent phosphorylation of E6-AP on S218
3. E6-AP mediates Greatwall stability directly via ubiquitylation
4. E6-AP knock out cells show reduced ATM/ATR activation and quicker checkpoint recovery following ETO and HU treatment
5. Greatwall mediated checkpoint recovery via increased phosphorylation of Cdk substrates

In my opinion, there are several interesting findings presented here but the overall model for a role of the E6-AP -Greatwall axis is not fully supported by the current data and will require further work. Moreover, there are a number of technical issues making it difficult to assess and interpret the presented data.

Major points:

1. The notion that Greatwall is indeed required for checkpoint recovery hinges on two experiments shown in Figures 5A and B where Greatwall depletion blocks the accumulation of HELA cells in mitosis following recovery from ETO treatment and in G2/M following release from HU. An alternative possibility to the direct involvement of Greatwall in checkpoint recovery could be that Greatwall in HeLa cells is required for S-phase progression (as for example Charrasse et al. suggested). A simple control would be to monitor the accumulation of mitotic cells by microscopy or FACS following Greatwall depletion without any further checkpoint activation.
2. The changes in protein levels of Greatwall and the effects of E6-AP on Greatwall stability are rather subtle and depend mostly on a qualitative assessment of western blots. Where quantifications have been made (Figures 2D and 4F) the loading control and the starting conditions for Greatwall (0 timepoints in the right panel) appear saturated making precise quantification impossible. I would argue that the authors should at least quantify the immuno-blots that led them to conclude on changes in Greatwall levels and make sure that the exposure times used are in the dynamic range of the camera (or film). A more precise experiment would be to use the exogenously expressed CFP-Greatwall that is described in Figure 6 and measure the acute changes in protein levels using quantitative fluorescence microscopy in live cells. This is, in my opinion, a lot more trustworthy than quantitative immuno-blots. I also note here that most experiments linking Greatwall levels to E6-AP were done using siRNA, while the E6-AP ko cells would be a more reliable background for these experiments, especially with reconstituted controls.
3. This study has no data linking the effects of Greatwall to its canonical target PP2A:B55. The model shown in Figure 9 is therefore highly speculative. The possibility that Greatwall could act independently of PP2A:B55 should at least be considered in the discussion given the lack of experimental evidence.
4. The major effect of E6-AP depletion on the checkpoint appears to be a striking reduction in ATM/ATR activation, suggesting that this ubiquitin ligase is involved in checkpoint activation rather than recovery. It is not clear if this phenotype is dependent on Greatwall. If so it would be hard to reconcile with the default model that E6-AP acts via the destabilisation of Greatwall. In the permanent absence of E6-AP, increased Greatwall levels should inactivate B55:PP2A. How would this lead to a decrease in ATM/ATR activation? This is unlikely, and indeed Figure 5E shows that the reduction of MASTL in parallel to E6-AP does not result in elevated levels of ATR/ATM activation. Conversely, the S215A E6-AP mutant does have a strong rescue impact on ATR/ATM (Figure 8D).
5. In summary, I do not think that the presented experiments clearly dissect the involvement of E6-AP and Greatwall in checkpoint activation and recovery. E6-AP depletion has a strong effect on checkpoint activation while Greatwall depletion is likely to have various checkpoint-independent effects on cell cycle progression.

Reviewer #3 (Public Review):

In this manuscript, Li et al. describe the contribution of the ATM-E6AP-MASTL pathway in recovery from DNA damage. Different types of DNA damage trigger an increase in protein levels of mitotic kinase MASTL, also called Greatwall, caused by increased protein stability. The authors identify E3 ligase E6AP to regulate MASTL protein levels. Depletion or knockout of E6AP increases MASTL protein levels, whereas overexpression of E6AP leads to lower

MASTL levels. E6AP and MASTL were suggested to interact in conditions without damage and this interaction is abrogated after DNA damage. E6AP was shown to be phosphorylated upon DNA damage on Ser218 and a phosphomimicking mutant does not interact with MASTL. Stabilization of MASTL was hypothesized to be important for recovery of the cell cycle/mitosis after DNA damage.

The identification of this novel pathway involving ATM and E6AP in MASTL regulation in the DNA damage response is interesting. However, is surprising that authors state that not a lot is known about DNA damage recovery while Greatwall and MASTL have been described to be involved in DNA damage (checkpoint) recovery. In addition, PP2A, a phosphatase downstream of MASTL is a known mediator of checkpoint recovery, in addition to other proteins like Plk1 and Claspin. Although some of the publications regarding these known mediators of DNA damage recovery are mentioned, the discussion regarding the relationship to the data in this manuscript are very limited.

The regulation of MASTL stability by E6AP is novel, although the data regarding this regulation and the interaction are not entirely convincing. In addition, several experiments presented in this paper suggest that E6AP is (additionally) involved in checkpoint signalling/activation, whereas the activation of the G2 DNA damage checkpoint was described to be independent of MASTL. Has E6AP multiple functions in the DNA damage response or is ATM-E6AP-MASTL regulation not as straightforward as presented here?

Altogether, in my opinion, not all conclusions of the manuscript are fully supported by the data.