

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

Yanqiu Li, Feifei Wang, Xin Li, Ling Wang, Zheng Yang, Zhongsheng You, Aimin Peng 

Department of Oral Biology, University of Nebraska Medical Center, Lincoln, Nebraska, USA • Department of Cell Biology and Physiology, School of Medicine, Washington University in St. Louis, St. Louis, Missouri, USA

 https://en.wikipedia.org/wiki/Open_access

 <https://creativecommons.org/licenses/by/4.0/>

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2

July 12, 2023 (this version)

Reviewed preprint version 1

April 25, 2023

Posted to bioRxiv

February 22, 2023

Sent for peer review

February 22, 2023

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 reduced DNA damage signaling, and 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 study reports the **important** finding that there appears to be a timer that monitors the repair of DNA after damage and regulates whether cells are subsequently able to enter mitosis. The authors identify proteins **important** for this decision and propose a mechanism supported by **solid** but not conclusive data. This study will be of interest to researchers in the fields of DNA damage repair and cell cycle control.

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 (Ciccia and Elledge, 2010 [DOI](#); Jackson and Bartek, 2009 [DOI](#); Lou and Chen, 2005 [DOI](#)). 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 (Alvarez-Fernandez et al., 2010 [↗](#); Macurek et al., 2008 [↗](#); Mamely et al., 2006 [↗](#); Peng, 2013 [↗](#); Peschiaroli et al., 2006 [↗](#); 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.

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 ionizing radiation (IR, **Fig. 1** Supplemental 1A&B), and HeLa cells treated with DOX or HU (**Fig. 1** Supplemental 1C-E). 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.

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, as shown by immunoblotting (**Fig. 2B**), and by direct fluorescence (**Fig. 2** Supplemental 1A&B). Like the endogenous MASTL, accumulation of exogenous MASTL occurred concurrently with activation of DNA damage signaling, measured by phosphorylation of ATM/ATR substrates (**Fig. 2** Supplemental 1A-C). 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 1D&E).

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 partner 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). Taken together, our data characterized the MASTL-E6AP association which was likely mediated via direct protein interaction, although the potential involvement of additional binding partners was not excluded.

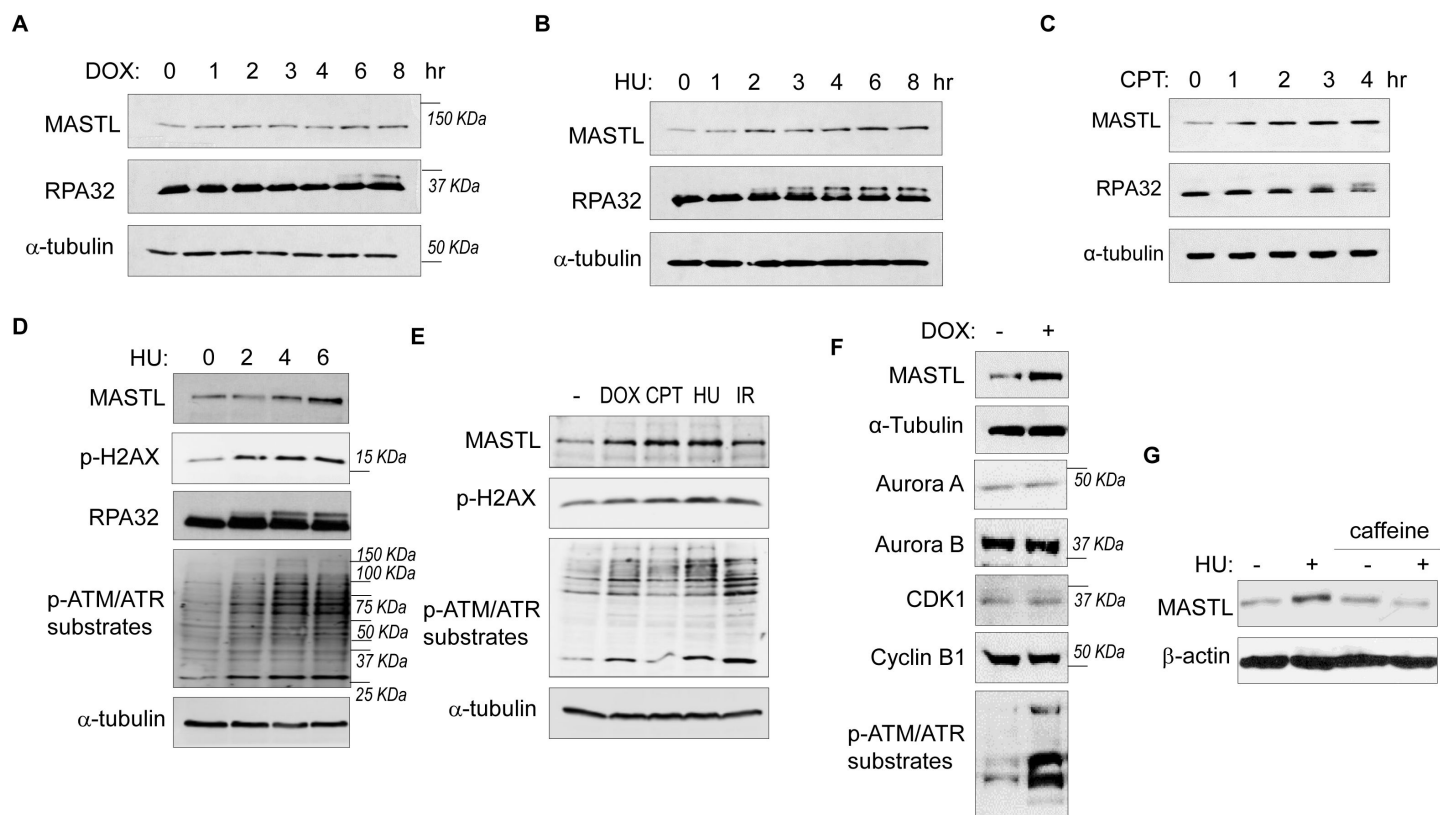


Figure 1.

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.

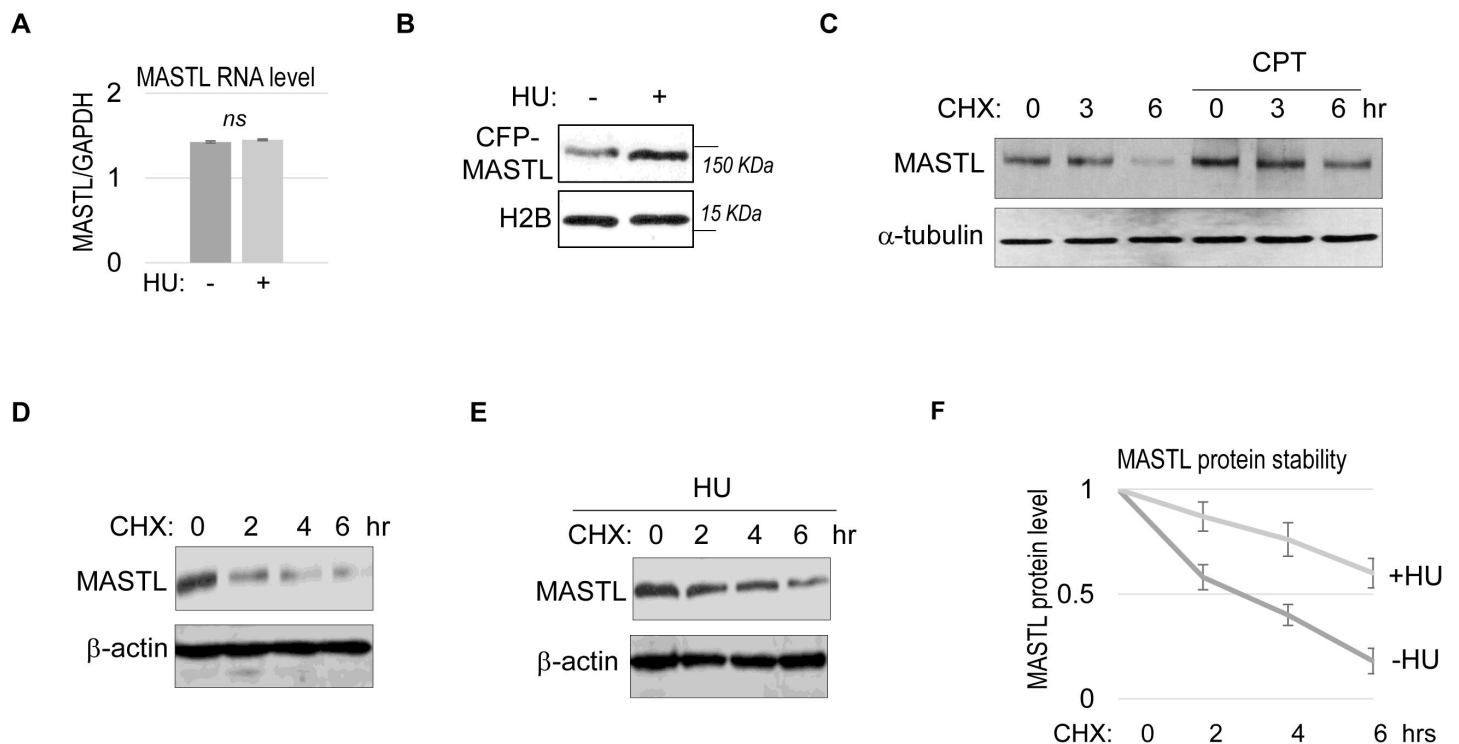


Figure 2.

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 μg/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 μg/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 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. 4B](#). 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. Consistently, E6AP depletion did not further increase MASTL level in cells treated with MG132 to suppress proteasome-mediated protein degradation ([Fig. 4C](#), Supplemental 1B). 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. 4C](#) Supplemental 1C); the MASTL mutant deleted of the E6AP-binding motif was not ubiquitinated ([Fig. 4C](#) Supplemental 1D). Together, these data established E6AP as a key modulator of MASTL stability.

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](#)). By comparison, MASTL knockdown did not significantly disrupt cell proliferation under the unperturbed condition ([Fig. 5C](#) Supplemental 1A).

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. 5C](#) Supplemental 1B). 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 CPF-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.

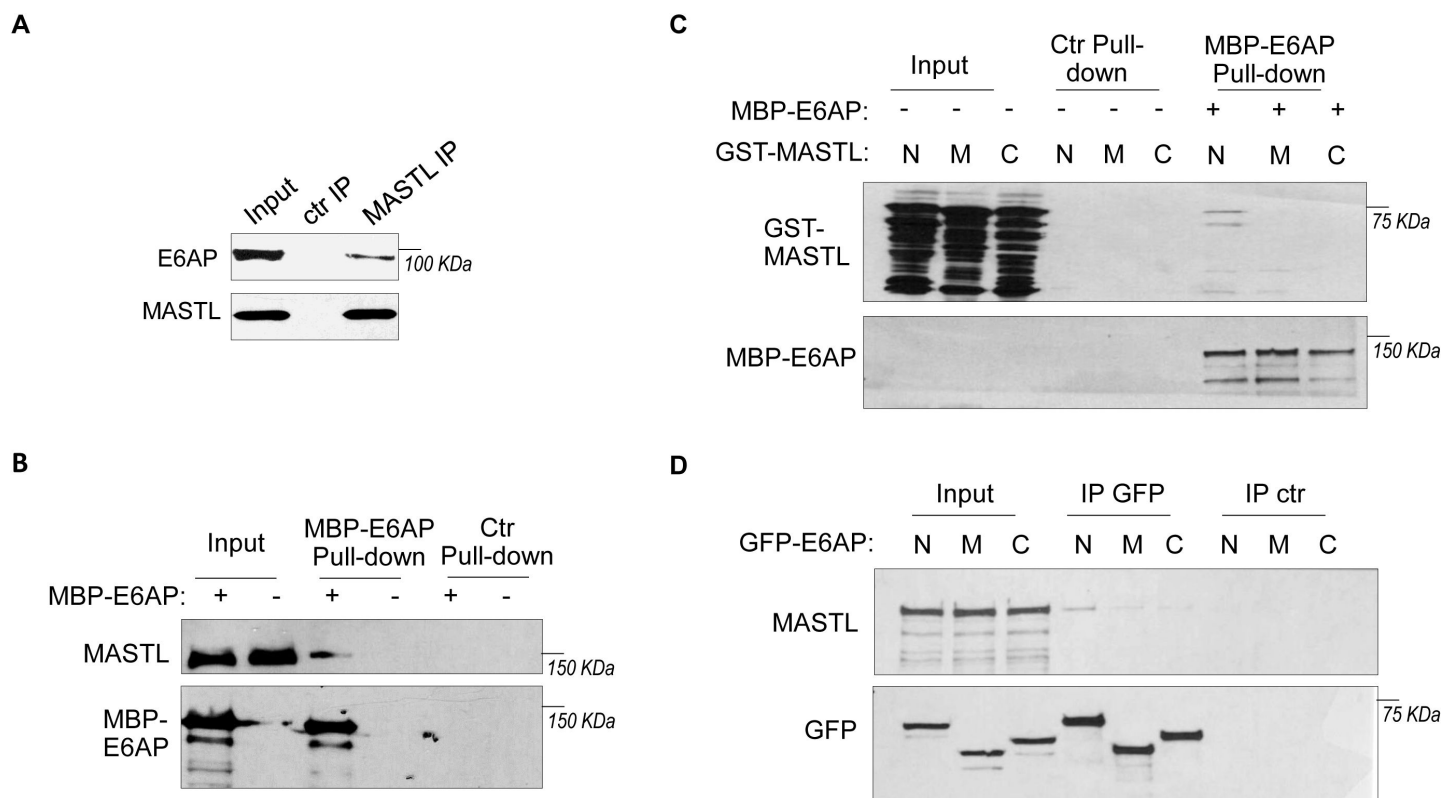


Figure 3.

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.

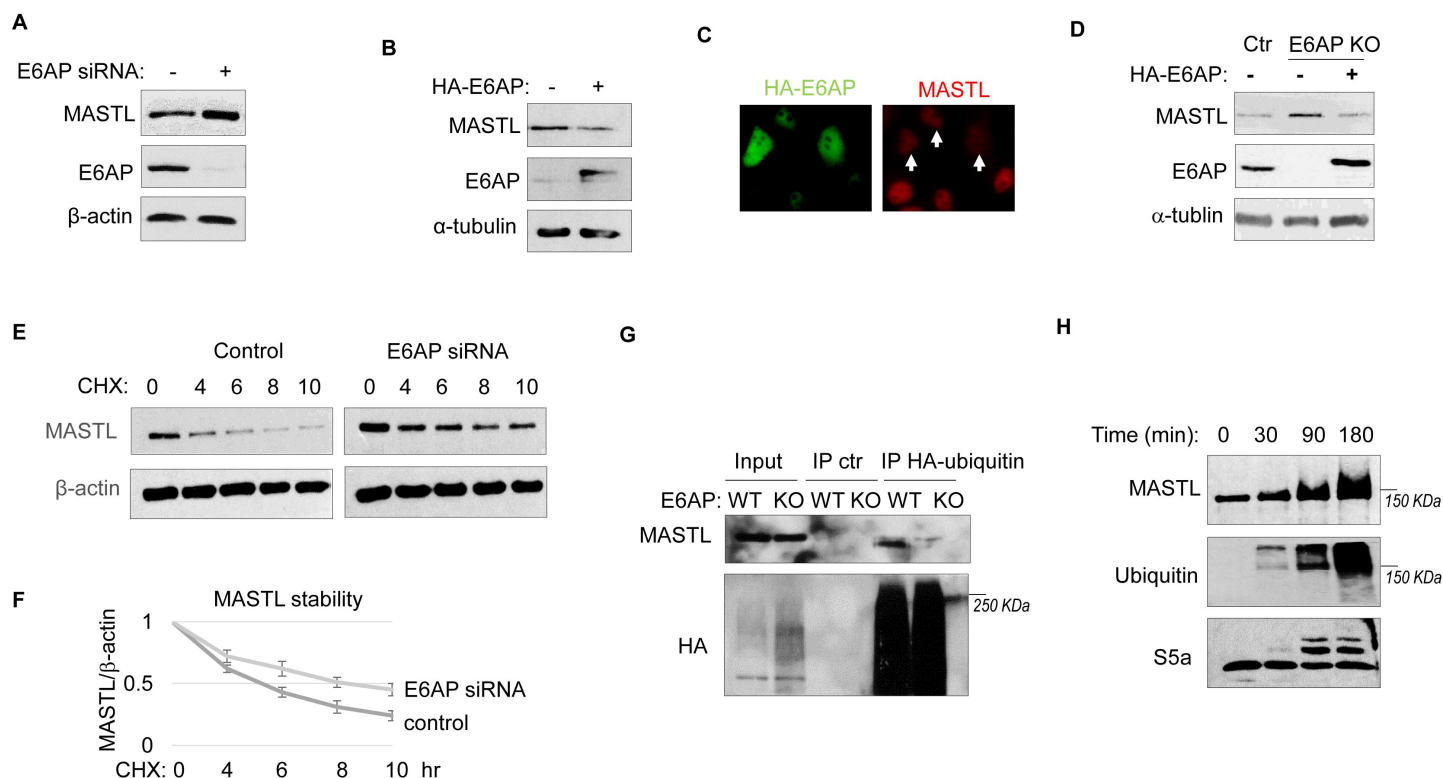


Figure 4.

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 harvest 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.

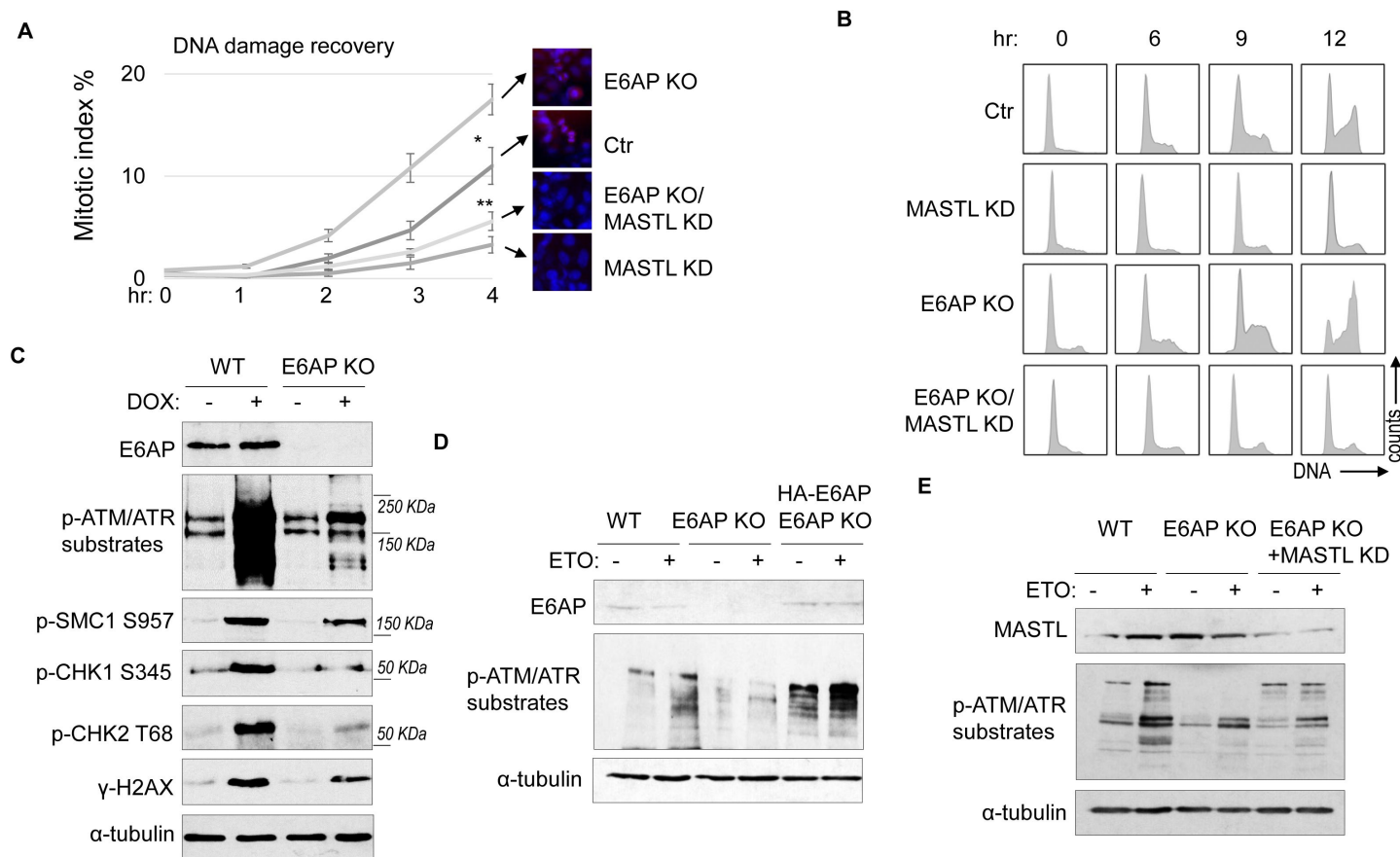


Figure 5.

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 manually 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 (* $p < 0.05$, ** $p < 0.01$, $n > 500$ cell number/measurement). 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.

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).

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**).

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

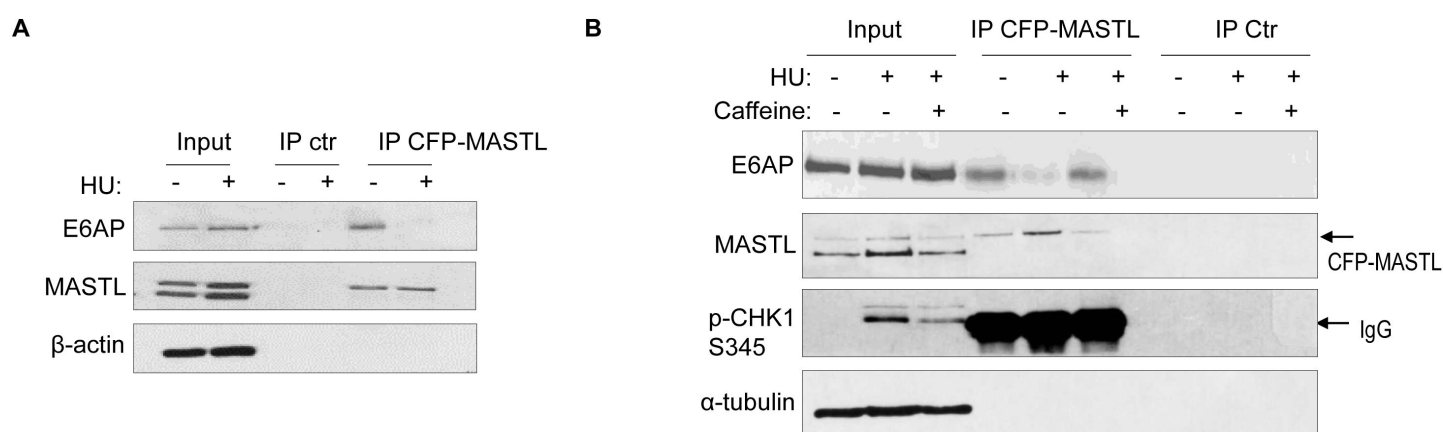


Figure 6.

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 for 2 hr. 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, for 2 hr. 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.

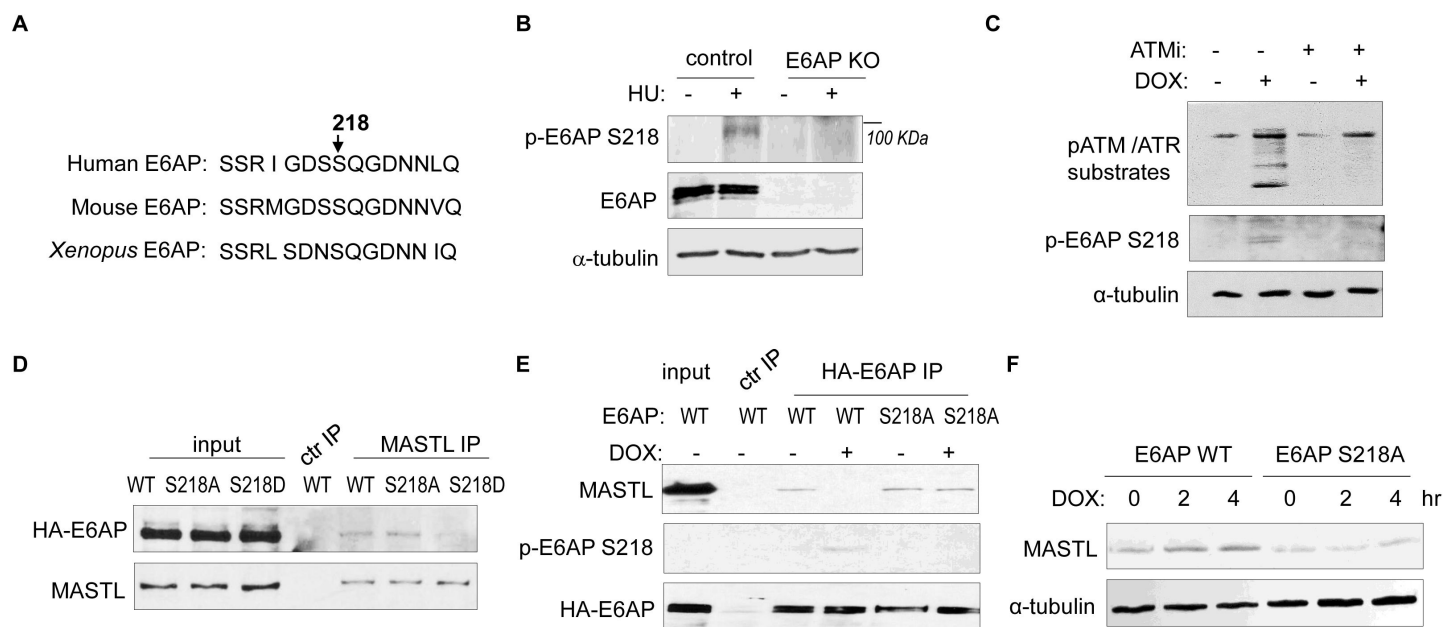


Figure 7.

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 for 4 hr, and analyzed by immunoblotting for phospho-E6AP Ser-218, E6AP, and α-tubulin. (C) HeLa cells were treated without or with 0.5 μM DOX and 5 μM KU55933 (ATMi), as indicated, for 1 hr, 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 μM 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 μM DOX, incubated as indicated, and harvested for immunoblotting for MASTL and α-tubulin.

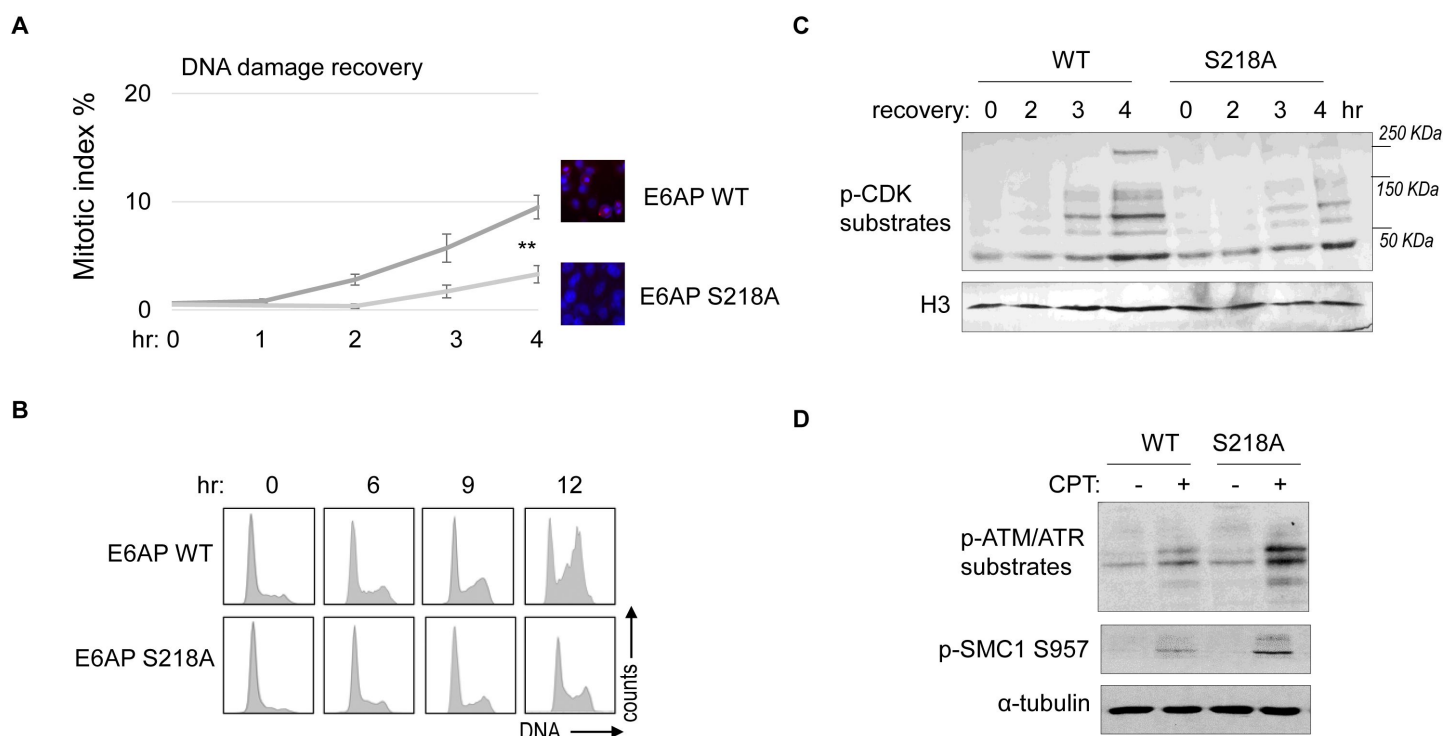


Figure 8.

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 μ M 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 manually 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 (** $p < 0.01$, $n > 500$ cell number/measurement). (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 μ M 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 μ M CPT for 90 minutes, and analyzed by immunoblotting for phospho-ATM/ATR substrates, phospho-SMC1 Ser-957, and α -tubulin.

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.

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; Wolynec 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; Wolynec 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 re-activation of CDK, PLK1, MASTL, and other cell cycle kinases promotes the de-activation of DNA damage checkpoint signaling and cell cycle resumption (Peng, 2013). For example, PLK1 and CDK can mediate the phosphorylation of DDR factors to suppress DNA damage checkpoint signaling (Alvarez-Fernandez et al., 2010; Macurek et al., 2008; Mamely et al., 2006; Peschiaroli et al., 2006; van Vugt et al., 2004). 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). In comparison to the recovery stage, the expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint, allowing efficient checkpoint activation. The subsequent impact of MASTL upregulation on promoting checkpoint recovery and cell cycle progression can be attributed to inhibition of PP2A/B55, although the potential involvement of additional mechanisms is not excluded. 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.

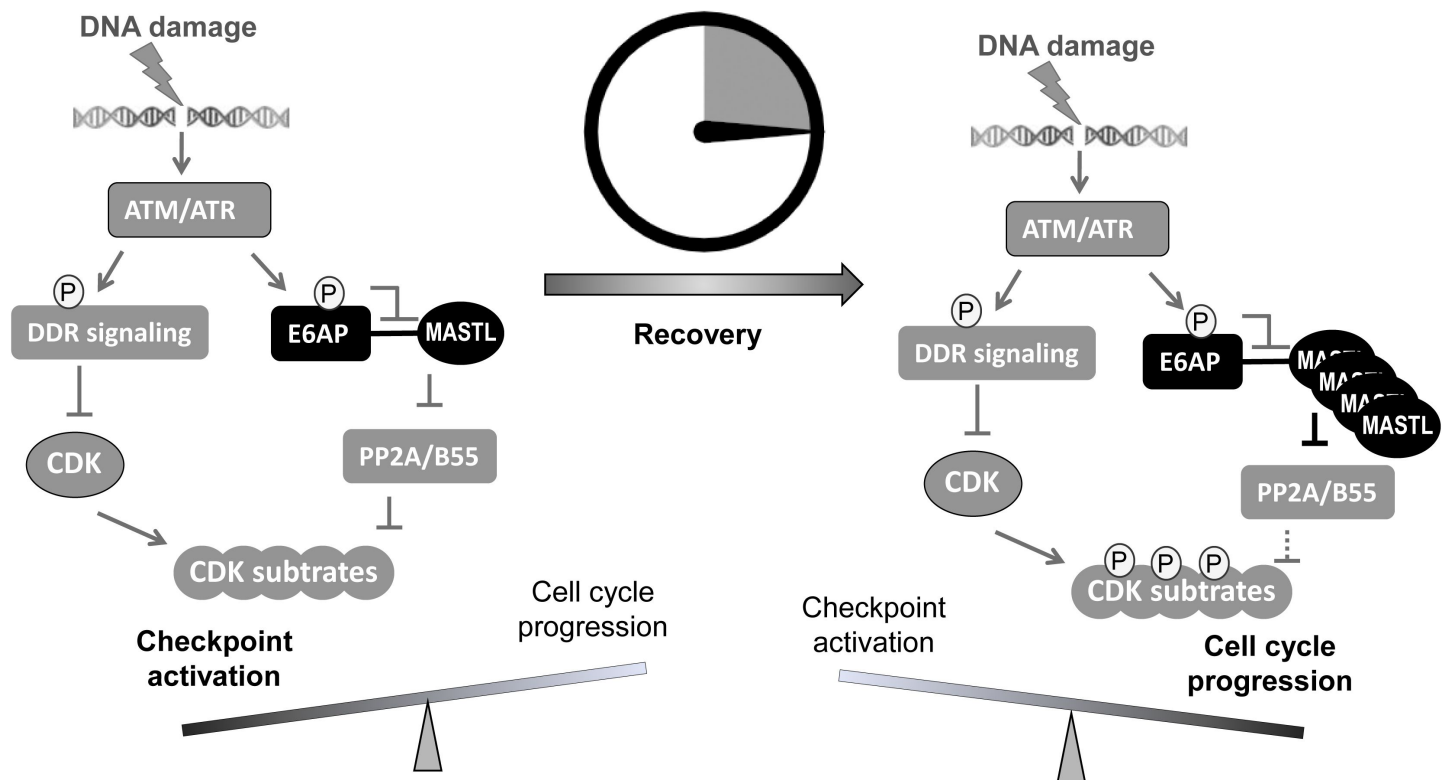


Figure 9.

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. At this stage, MASTL expression is normal (not upregulated). 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, at upregulated levels several hours after DNA damage, promotes de-activation of the DNA damage checkpoint and initiates cell cycle resumption by inhibiting dephosphorylation of CDK substrates.

Antibodies and chemicals

Mouse antibody to MASTL (clone 4F9, Millipore MABT372) was described previously (Wang et al., 2011 [\[4\]](#)). 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 [\[4\]](#)), was maintained in DMEM (HyClone) with 10% FBS(HyClone). As described previously (Li et al., 2021 [\[4\]](#)), 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'AGGAAUUGUCAUUCUUU; 5'-3' UCAGAAUAAAGAUUGACA. MASTL siRNA sequence:

5'-3'GUCUACUUGGUAUUGGAA; 5'-3' UAAGAUUUCCAUAUACCA. For fluorescence-activated cell sorting, cells were fixed in 70% cold ethanol, washed with cold PBS and stained with propidium iodide (20 μ g/ml propidium iodide and 200 μ g/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 μ g/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' CTACTACCACAGTAACTG 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 [DOI](#)). 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 [DOI](#)). 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 [DOI](#)). 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.

Acknowledgements

We thank Drs. Gregory G Oakley, Thomas M. Petro and Dr. Jixin Dong (University of Nebraska Medical Center, USA) for stimulating discussions. A.P. is supported by funding from the National Institutes of Health (CA233037; DE030427).

References

- Alvarez-Fernandez M., Halim V.A., Krenning L., Aprelia M., Mohammed S., Heck A.J., Medema R.H (2010) **Recovery from a DNA-damage-induced G2 arrest requires Cdk-dependent activation of FoxM1** *EMBO reports* **11**:452–458
- Archambault V., Zhao X., White-Cooper H., Carpenter A.T.C., Glover D.M (2007) **Mutations in drosophila Greatwall/Scant reveal its roles in mitosis and meiosis and interdependence with polo kinase** *PLoS genetics* **3**:2163–2179
- Bartek J., Lukas J (2007) **DNA damage checkpoints: from initiation to recovery or adaptation** *Current opinion in cell biology* **19**:238–245
- Beli P., Lukashchuk N., Wagner S.A., Weinert B.T., Olsen J.V., Baskcomb L., Mann M., Jackson S.P., Choudhary C (2012) **Proteomic investigations reveal a role for RNA processing factor THRAP3 in the DNA damage response** *Molecular cell* **46**:212–225
- Bernassola F., Karin M., Ciechanover A., Melino G (2008) **The HECT family of E3 ubiquitin ligases: multiple players in cancer development** *Cancer Cell* **14**:10–21
- Blake-Hodek K.A., Williams B.C., Zhao Y., Castilho P.V., Chen W., Mao Y., Yamamoto T.M., Goldberg M.L (2012) **Determinants for activation of the atypical AGC kinase Greatwall during M phase entry** *Mol Cell Biol* **32**:1337–1353
- Buiting K., Williams C., Horsthemke B (2016) **Angelman syndrome - insights into a rare neurogenetic disorder** *Nature reviews Neurology* **12**:584–593
- Castilho P.V., Williams B.C., Mochida S., Zhao Y., Goldberg M.L (2009) **The M Phase Kinase Greatwall (Gwl) Promotes Inactivation of PP2A/B55 delta, a Phosphatase Directed Against CDK Phosphosites** *Mol Biol Cell* **20**:4777–4789
- Castro A., Lorca T (2018) **Greatwall kinase at a glance** *Journal of cell science* **131**
- Ciccia A., Elledge S.J. (2010) **The DNA damage response: making it safe to play with knives** *Molecular cell* **40**:179–204
- Clemenson C., Marsolier-Kergoat M.C (2009) **DNA damage checkpoint inactivation: Adaptation and recovery** *DNA repair* **8**:1101–1109
- Fatima I., Singh A.B., Dhawan P (2020) **MASTL: A novel therapeutic target for Cancer Malignancy** *Cancer Med* **9**:6322–6329
- Flynn R.L., Zou L (2011) **ATR: a master conductor of cellular responses to DNA replication stress** *Trends in biochemical sciences* **36**:133–140
- Franz-Wachtel M., Eisler S.A., Krug K., Wahl S., Carpy A., Nordheim A., Pfizenmaier K., Hausser A., Macek B (2012) **Global detection of protein kinase D-dependent phosphorylation events in nocodazole-treated human cells** *Mol Cell Proteomics* **11**:160–170

Gharbi-Ayachi A., Labbe J.C., Burgess A., Vigneron S., Strub J.M., Brioudes E., Van-Dorselaer A., Castro A., Lorca T (2010) **The Substrate of Greatwall Kinase, Arpp19, Controls Mitosis by Inhibiting Protein Phosphatase 2A** *Science* **330**:1673–1677

Jackson S.P., Bartek J (2009) **The DNA-damage response in human biology and disease** *Nature* **461**:1071–1078

Kettenbach A.N., Schweppe D.K., Faherty B.K., Pechenick D., Pletnev A.A., Gerber S.A (2011) **Quantitative phosphoproteomics identifies substrates and functional modules of Aurora and Polo-like kinase activities in mitotic cells** *Sci Signal* **4**

Klammer M., Kaminski M., Zedler A., Oppermann F., Blencke S., Marx S., Muller S., Tebbe A., Godl K., Schaab C (2012) **Phosphosignature predicts dasatinib response in non-small cell lung cancer** *Mol Cell Proteomics* **11**:651–668

Levav-Cohen Y., Wolyniec K., Alsheich-Bartok O., Chan A.L., Woods S.J., Jiang Y.H., Haupt S., Haupt Y (2012) **E6AP is required for replicative and oncogene-induced senescence in mouse embryo fibroblasts** *Oncogene* **31**:2199–2209

Li Y., Kardell M.B., Wang F., Wang L., Zhu S., Bessho T., Peng A (2021) **The Sm core components of small nuclear ribonucleoproteins promote homologous recombination repair** *DNA repair* **108**

Lou Z., Chen J (2005) **Mammalian DNA damage response pathway** *Advances in experimental medicine and biology* **570**:425–455

Macurek L., Lindqvist A., Lim D., Lampson M.A., Klompaker R., Freire R., Clouin C., Taylor S.S., Yaffe M.B., Medema R.H (2008) **Polo-like kinase-1 is activated by aurora A to promote checkpoint recovery** *Nature* **455**:119–U188

Mamely I., van Vugt M.A.T.M., Smits V.A.J., Semple J.I., Lemmens B., Perrakis A., Medema R.H., Freire R. (2006) **Polo-like kinase-1 controls proteasome-dependent degradation of claspins during checkpoint recovery** *Current Biology* **16**:1950–1955

Marzec K., Burgess A (2018) **The Oncogenic Functions of MASTL Kinase** *Frontiers in cell and developmental biology* **6**

Matsuoka S., Ballif B.A., Smogorzewska A., McDonald E.R., Hurov K.E., Luo J., Bakalarski C.E., Zhao Z., Solimini N., Lerenthal Y., et al. (2007) **ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage** *Science* **316**:1160–1166

Medema R.H (2010) **Greatwall in control of recovery** *Cell Cycle* **9**:4264–4265

Mochida S., Ikeo S., Gannon J., Hunt T (2009) **Regulated activity of PP2A-B55 delta is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts** *Embo Journal* **28**:2777–2785

Mochida S., Maslen S.L., Skehel M., Hunt T (2010) **Greatwall Phosphorylates an Inhibitor of Protein Phosphatase 2A That Is Essential for Mitosis** *Science* **330**:1670–1673

Olsen J.V., Vermeulen M., Santamaria A., Kumar C., Miller M.L., Jensen L.J., Gnad F., Cox J., Jensen T.S., Nigg E.A., et al. (2010) **Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis** *Sci Signal* **3**

- Peng A (2013) **Working hard for recovery: mitotic kinases in the DNA damage checkpoint** *Cell & bioscience* **3**
- Peng A., Maller J.L (2010) **Serine/threonine phosphatases in the DNA damage response and cancer** *Oncogene* **29**:5977–5988
- Peng A., Wang L., Fisher L.A (2011) **Greatwall and Polo-like kinase 1 coordinate to promote checkpoint recovery** *J Biol Chem* **286**:28996–29004
- Peng A.M., Yamamoto T.M., Goldberg M.L., Maller J.L (2010) **A novel role for greatwall kinase in recovery from DNA damage** *Cell Cycle* **9**:4364–4369
- Peschiaroli A., Dorrello N.V., Guardavaccaro D., Venere M., Halazonetis T., Sherman N.E., Pagano M (2006) **SCF beta TrCP-mediated degradation of claspin regulates recovery from the DNA replication checkpoint response** *Molecular cell* **23**:319–329
- Ren D., Fisher L.A., Zhao J., Wang L., Williams B.C., Goldberg M.L., Peng A (2017) **Cell Cycle-dependent Regulation of Greatwall Kinase by Protein Phosphatase 1 and Regulatory Subunit 3B** *J Biol Chem*
- Scheffner M., Kumar S (2014) **Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects** *Biochimica et biophysica acta* **1843**:61–74
- Schweppe D.K., Rigas J.R., Gerber S.A (2013) **Quantitative phosphoproteomic profiling of human non-small cell lung cancer tumors** *Journal of proteomics* **91**:286–296
- Sell G.L., Margolis S.S. (2015) **From UBE3A to Angelman syndrome: a substrate perspective** *Frontiers in neuroscience* **9**
- Shiloh Y (2003) **ATM and related protein kinases: Safeguarding genome integrity** *Nat Rev Cancer* **3**:155–168
- Syljuasen R.G (2007) **Checkpoint adaptation in human cells** *Oncogene* **26**:5833–5839
- Toczyski D.P., Galgoczy D.J., Hartwell L.H (1997) **CDC5 and CKII control adaptation to the yeast DNA damage checkpoint** *Cell* **90**:1097–1106
- van Vugt M.A.T.M., Bras A., Medema R.H. (2004) **Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells** *Molecular cell* **15**:799–811
- van Vugt M.A.T.M., Medema R.H. (2004) **Checkpoint adaptation and recovery - Back with polo after the break** *Cell Cycle* **3**:1383–1386
- Vigneron S., Brioudes E., Burgess A., Labbe J.C., Lorca T., Castro A (2009) **Greatwall maintains mitosis through regulation of PP2A** *The EMBO journal* **28**:2786–2793
- Vigneron S., Gharbi-Ayachi A., Raymond A.A., Burgess A., Labbe J.C., Labesse G., Monsarrat B., Lorca T., Castro A (2011) **Characterization of the Mechanisms Controlling Greatwall Activity** *Mol Cell Biol*
- Voets E., Wolthuis RM (2010) **MASTL is the human orthologue of Greatwall kinase that facilitates mitotic entry, anaphase and cytokinesis** *Cell Cycle* **9**

- Wang F., Wang L., Fisher L.A., Li C., Wang W., Peng A (2019) **Phosphatase 1 Nuclear Targeting Subunit (PNUTS) Regulates Aurora Kinases and Mitotic Progression** *Molecular cancer research : MCR* **17**:10–19
- Wang L., Fisher L.A., Wahl J.K., Peng A. (2011) **Monoclonal antibodies against Xenopus greatwall kinase** *Hybridoma (Larchmt)* **30**:469–474
- Wang L., Luong V.Q., Giannini P.J., Peng A (2014) **Mastl kinase, a promising therapeutic target, promotes cancer recurrence** *Oncotarget* **5**:11479–11489
- Wang P., Larouche M., Normandin K., Kachaner D., Mehse H., Emery G., Archambault V (2016) **Spatial regulation of greatwall by Cdk1 and PP2A-Tws in the cell cycle** *Cell Cycle* **15**:528–539
- Weber C., Schreiber T.B., Daub H (2012) **Dual phosphoproteomics and chemical proteomics analysis of erlotinib and gefitinib interference in acute myeloid leukemia cells** *Journal of proteomics* **75**:1343–1356
- Wolyniec K., Levav-Cohen Y., Jiang Y.H., Haupt S., Haupt Y (2013) **The E6AP E3 ubiquitin ligase regulates the cellular response to oxidative stress** *Oncogene* **32**:3510–3519
- Wong P.Y., Ma H.T., Lee H.J., Poon R.Y (2016) **MASTL(Greatwall) regulates DNA damage responses by coordinating mitotic entry after checkpoint recovery and APC/C activation** *Scientific reports* **6**
- Yamamoto T.M., Wang L., Fisher L.A., Eckerdt F.D., Peng A (2014) **Regulation of Greatwall kinase by protein stabilization and nuclear localization** *Cell Cycle* **13**:3565–3575
- Yoo H.Y., Kumagai A., Shevchenko A., Shevchenko A., Dunphy W.G (2004) **Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase** *Cell* **117**:575–588
- Yu J., Qin B., Lou Z (2020) **Ubiquitin and ubiquitin-like molecules in DNA double strand break repair** *Cell & bioscience* **10**
- Yu J.T., Fleming S.L., Williams B., Williams E.V., Li Z.X., Somma P., Rieder C.L., Goldberg M.L (2004) **Greatwall kinase: a nuclear protein required for proper chromosome condensation and mitotic progression in Drosophila** *Journal of Cell Biology* **164**:487–492
- Yu J.T., Zhao Y., Li Z.X., Galas S., Goldberg M.L (2006) **Greatwall kinase participates in the Cdc2 autoregulatory loop in Xenopus egg extracts** *Molecular cell* **22**:83–91
- Zhou B.B.S., Elledge S.J (2000) **The DNA damage response: putting checkpoints in perspective** *Nature* **408**:433–439
- Zhu S., Peng A (2016) **Non-homologous end joining repair in Xenopus egg extract** *Scientific reports* **6**

Author information

Yanqiu Li

Department of Oral Biology, University of Nebraska Medical Center, Lincoln, Nebraska, USA

Feifei Wang

Department of Oral Biology, University of Nebraska Medical Center, Lincoln, Nebraska, USA

Xin Li

Department of Oral Biology, University of Nebraska Medical Center, Lincoln, Nebraska, USA

Ling Wang

Department of Oral Biology, University of Nebraska Medical Center, Lincoln, Nebraska, USA

Zheng Yang

Department of Cell Biology and Physiology, School of Medicine, Washington University in St. Louis, St. Louis, Missouri, USA

Zhongsheng You

Department of Cell Biology and Physiology, School of Medicine, Washington University in St. Louis, St. Louis, Missouri, USA

ORCID ID: [0000-0002-9719-8791](https://orcid.org/0000-0002-9719-8791)

Aimin Peng

Department of Oral Biology, University of Nebraska Medical Center, Lincoln, Nebraska, USA

For correspondence: aimin.peng@unmc.edu

ORCID ID: [0000-0002-2452-1949](https://orcid.org/0000-0002-2452-1949)

Editors

Reviewing Editor

Jon Pines

Institute of Cancer Research Research, United Kingdom

Senior Editor

Kevin Struhl

Harvard Medical School, United States of America

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.

and 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.

Revised manuscript:

I think the authors did a good job in revising the paper, and provide compelling support for a timer function in the checkpoint. I do think they still have missed one important point how MASTL could act as a timer to control recovery. The data clearly show that MASTL somehow controls ATM/ATR activity, whilst their final model (fig.9) places MASTL upstream of CDK activity, without mentioning its feedback on ATM/ATR. I think there are 2 possible explanations for the timer function of MASTL they have discovered here, both may be relevant. The first is enhanced CDK activation by direct control of CDK phosphorylation through MASTL/B55/PP2A. The second is through MASTL-mediated shut-down of ATM/ATR activation (mechanism to be determined) which is also reported here. Their final model and discussion do not display sufficient appreciation for this latter option, and I would argue that the HU-recovery experiment shown in Fig.5B is actually in strong support of the second explanation, rather than the first.

Reviewer #2 (Public Review):

This manuscript describes a role for the ATM-E6AP-MASTL pathway in DNA damage checkpoint recovery. However, the data in the first version of the manuscript strongly suggest that E6AP is involved in checkpoint activation, which raises doubts about the exact function of this pathway. Additional minor issues were raised regarding the quality of some of the data. Although some minor points were addressed in the revised manuscript, the major issue whether the E6AP-MASTL pathway mediates checkpoint activation or checkpoint recovery was not experimentally addressed. Instead, the authors state that "the expression level of MASTL is not upregulated during the activation stages of the DNA damage checkpoint". However, their data show otherwise: MASTL upregulation coinciding with RPA phosphorylation and p-ATM/ATR signal.

I am therefore not convinced the revised manuscript sufficiently addressed the comments to fully support the conclusions.

Author Response

The following is the authors' response to the original reviews.

We thank the reviewers for their insightful comments. The main issue raised by the reviewers was that because E6AP depletion reduced checkpoint signaling via MASTL upregulation, this pathway is likely to be involved also in DNA damage checkpoint activation, in addition to checkpoint recovery. Hence, the proposed "timer"-like model is not fully supported. However, it is important to note that, the expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint (unless E6AP is depleted). DNA damage signaling, via ATM-dependent E6AP phosphorylation, caused MASTL

accumulation over time. This ultimately shifts the balance toward checkpoint recovery and cell cycle re-entry. As such, the role of MASTL (and E6AP-depletion) in suppressing DNA damage checkpoint is in harmony with the proposed role of MASTL upregulation in promoting checkpoint recovery. We have made additional clarifications about this point in the revised manuscript.

We have also addressed other concerns raised by the reviewers, as explained in the point-to-point responses below. With the addition of new modifications and data, we believe the revised manuscript is complete and conclusive.

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.

We thank the reviewer for his/her insightful comments. The main issue raised by the reviewers was that because E6AP depletion reduced checkpoint signaling via MASTL upregulation, this pathway is likely to be involved also in DNA damage checkpoint activation, in addition to checkpoint recovery. Hence, the proposed “timer”-like model is not fully supported. However, it is important to note that, the expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint (unless E6AP is depleted). DNA damage signaling, via ATM-dependent E6AP phosphorylation, caused MASTL accumulation over time. This ultimately shifts the balance toward checkpoint recovery and cell cycle re-entry. As such, the role of MASTL (and E6AP-depletion) in suppressing DNA damage checkpoint is in harmony with the proposed role of MASTL upregulation in promoting checkpoint recovery. We have made additional clarifications about this point in the revised manuscript.

As suggested by the reviewer, we have rephrased the statement in abstract to “E6AP depletion reduced DNA damage signaling, and promoted cell cycle recovery from the DNA damage checkpoint, in a MASTLdependent manner”.

As a mitotic kinase, MASTL promotes mitotic entry and progression. This is well in line with our findings that DNA damage-induced MASTL upregulation promotes cell cycle re-entry into mitosis. MASTL upregulation could also inhibit DNA damage signaling. This manner of feedback, inhibitory, modulation of DNA damage signaling by mitotic kinases (e.g., PLK1, CDK) has been implicated in previous studies (reviewed in Cell & Bioscience volume 3, Article number: 20 (2013)). In the revised manuscript, we have included more discussions about this aspect of checkpoint regulation.

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.

Detailed time point information is now added in the figure legends for E6AP phosphorylation data. We were able to observe this event during early stages (e.g., 1 hr, or 2-4 hr) of the DNA damage response, prior to significant MASTL protein accumulation.

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.*
- 1. Greatwall directly interacts with E6-AP and this interaction is suppressed by ATM dependent phosphorylation of E6-AP on S218*
- 1. E6-AP mediates Greatwall stability directly via ubiquitylation*
- 1. E6-AP knock out cells show reduced ATM/ATR activation and quicker checkpoint recovery following ETO and HU treatment*
- 1. 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.*

We thank the reviewer for his/her insightful comments.

Charrasse et al. showed ENSA knockout prolonged, but not stopped the progression of S-phase. In our experiments, MASTL (partial) knockdown did not significantly impact HeLa cells proliferation in the absence of DNA damage (Fig. 5, supplemental 1A). The reported role of MASTL in checkpoint recovery was consistently seen in response to various drugs, including etoposide which typically induces G2 arrest. Thus, we do not believe a prolonged S-phase accounts for the checkpoint recovery phenotype.

1. 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.

DNA damage-induced MASTL upregulation was observed in various cell lines and after different treatments. To further strengthen this point, as suggested by the reviewer, we have included quantification of fluorescent measurements (Fig. 2, supplemental 1 A-C). Quantification of immunoblots for MASTL upregulation was also added in Fig. 1, supplemental 1E. The effects of E6AP depletion were consistently shown for both siRNA and stable KO.

1. 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.

The role of MASTL in promoting cell cycle progression via suppressing PP2A/B55 has been well established. As suggested by the reviewer, we have included discussions to acknowledge that “The role of MASTL upregulation in promoting checkpoint recovery and cell cycle progression can be attributed to inhibition of PP2A/B55, although the potential involvement of additional mechanisms is not excluded”.

1. 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).

We do not propose that PP2A/B55 directly dephosphorylates ATM/ATR-mediated phosphorylation. In fact, PP2A/B55 dephosphorylates and inactivates mitotic kinases and substrates which can feedback inhibit DNA damage checkpoint signaling (as previously

shown for PLK1 and CDK). We included in a discussion about this point in the revised manuscript.

The point regarding checkpoint activation vs recovery is addressed below (point 5).

1. 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.

It is important to note that, the expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint (unless E6AP is depleted). DNA damage signaling, via ATM-dependent E6AP phosphorylation, caused MASTL accumulation over time. This ultimately shifts the balance toward checkpoint recovery and cell cycle re-entry. As such, the role of MASTL (and E6APdepletion) in suppressing DNA damage checkpoint is in harmony with the proposed role of MASTL upregulation in promoting checkpoint recovery. We have made additional clarifications about this point in the revised manuscript.

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.

We thank the reviewer for his/her insightful comments. As suggested, the previously reported role of PLK1 and other cell cycle kinases in DNA damage checkpoint recovery is discussed in more details in the revised manuscript. As for PP2A/B55, we do not think it promotes checkpoint recovery, e.g., by dephosphorylating ATM/ATR or their substrates. Instead, this phosphatase dephosphorylates cell cycle kinases or their substrates, such as CDK1 or PLK1.

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.

We showed that E6AP depletion reduced checkpoint signaling via MASTL upregulation, so this pathway is likely to be involved also in DNA damage checkpoint activation, in addition to checkpoint recovery. However, it is important to note that, the expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint (unless E6AP is depleted). DNA damage signaling, via ATM-dependent E6AP phosphorylation, caused MASTL accumulation over time. This ultimately shifts the balance toward checkpoint recovery and cell cycle re-entry. As such, the role of MASTL (and E6AP depletion) in suppressing DNA damage checkpoint is in harmony with the proposed role of MASTL upregulation in promoting checkpoint recovery. We have made additional clarifications about this point in the revised manuscript.

Reviewer #1 (Recommendations For The Authors):

In principle a very interesting story, that attempts 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 it will maintain stability, if not then stability is compromised. However, the story in its present form suffers from a number of major gaps that will need to be addressed

Major point:

My primary concern regarding the main conclusion is that altered MASTL regulation seems to be doing much more than simply promoting more rapid recovery after DNA damage. This concern comes from the following gaps that I noted whilst reading the paper:

- *Knock out of E6AP, is leading to a dramatic inhibition of ATM/ATR activation after damage (Fig. 5C,D,E), this is (partially) rescued by co-depletion of MASTL (Fig 5E). The authors will have to show that the primary effect of altered MASTL regulation is improved recovery, rather than reduced checkpoint activation. In other words, is initial checkpoint activation in cells that have lost E6AP normal, or do these cells fail to mount a proper checkpoint response? If the latter is true, that could completely alter the take home-message of this paper, because it could mean that E6AP/MASTL do not act as a "timer", but as a "tuner" to set checkpoint strength at the start of the DNA damage response. The authors themselves 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?*

The expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint (unless E6AP is depleted). DNA damage signaling, via ATM-dependent E6AP phosphorylation, caused MASTL accumulation over time. This ultimately shifts the balance toward checkpoint recovery and cell cycle re-entry. As such, the role of MASTL (and E6AP-depletion) in suppressing DNA damage checkpoint is in harmony with the proposed role of MASTL upregulation in promoting checkpoint recovery. We have made additional clarifications about this point in the revised manuscript. We have also made clarification to the statement indicated by the reviewer.

- *MASTL KD has a rather unexpected effect on cell cycle progression after HU synchronization (Fig. 5B). It seems that the MASTL KD cells fail to exit from the HU-imposed G1/S arrest, an effect that is not rescued in the E6AP knock-outs. Inversely, E6AP knock-outs seem to more readily exit from the HU-imposed arrest, an effect that is completely lost after knock-down of MASTL. How do the authors interpret these results? Their conclusions are entirely based on a role for MASTL as a driver of mitotic entry, with E6AP in control of its levels, but this experiment suggests that MASTL and E6AP are controlling very different aspects of cell cycle control in their system.*

As the reviewer pointed out, our data in checkpoint signaling and cell cycle progression suggested that MASTL upregulation could also inhibit DNA damage signaling, in addition to promoting cell cycle progression. This manner of feedback, inhibitory, modulation of DNA damage signaling by mitotic kinases (e.g., PLK1, CDK) has been implicated in previous studies (reviewed in Cell & Bioscience volume 3, Article number: 20 (2013)). In the revised manuscript, we have included discussions about this aspect of checkpoint regulation.

- *It is not possible to evaluate the validity of the conclusions that are based on Figure 6. We need to know how long the cells were treated with HU to disrupt the interaction between E6AP and MASTL. Is the timing of this in the range of the timing of MASTL increase after damage? A time course experiment is required here.*
- *The data obtained on E6AP-S218 phosphorylation and with the S218A mutant during damage and recovery look very promising. But again, the release from HU is confusing me as to what to conclude from them. Also, the authors should show how S218A expression affects MASTL levels (before and after damage). Also, a time course of ATM/ATR activation is required to decide if initial or late ATM/ATR signaling is affected.*

Detailed time point information is now added in the figure legends for E6AP phosphorylation and E6AP-MASTL dissociation data. We were able to observe these events during early stages (e.g., 1 hr, or 2-4 hr) of the DNA damage response, prior to significant MASTL protein accumulation.

- *The conclusion that "and was not likely to be caused by the completion of DNA repair, as judged by the phosphorylation of replication protein A" (page 5) is based on western blots that represent the average across the entire population. It is possible that MASTL expression is still low in the cells that have not completed repair, while its increase on blots comes from a subset of cells where repair is complete. The authors should perform immunofluorescence so that expression levels of MASTL can be directly compared to levels of phospho-RPA in individual cells. In fact, the manuscript could benefit a lot from a more in-depth single-cell (microscopy)-based analysis of the relations over time between ATM/ATR activation, E6AP phosphorylation, MASTL stabilization versus the checkpoint arrest and subsequent recovery.*

Time point analyses were provided for DNA damage-induced RPA phosphorylation and ATM/ATR substrate phosphorylation (Fig. 1). These data showed MASTL accumulation in the presence of active DNA damage checkpoint signaling. To further strengthen this point, as suggested by the reviewer, we have included quantification of fluorescent measurements

(Fig. 2, supplemental 1 A-C). IF data showed MASTL upregulation in correlation with ATM/ATR activation.

Minor points:

It's not "ionized radiation", but "ionizing radiation" (page 5)

We have made the correction as pointed out by the reviewer.

Expression levels of MASTL should be quantified over time after DNA damage. In some of the experiments the increase seems to plateau relatively quick (HU treatment, fig 1B, 1-2 hours), while in others the levels continue to increase over longer periods (HU treatment, fig 1D, 6 hours). This is relevant to the timer function of MASTL that is proposed here.

The kinetics of MASTL upregulation is generally consistent among all cell lines. As suggested, quantification of immunoblots is provided (Fig. 1, supplemental 1E); additional quantification of IF signals is also included (Fig. 2, supplemental 1 A-C).

The experiment executed with caffeine (page 5) should be repeated with more selective/potent ATM/ATR inhibitors that are commercially available.

Specific ATM inhibitor was used to confirm the caffeine result in Fig. 7 supplemental 1B&C.

"a potential binding pattern" (page 6) should be "a potential binding partner"

We have made the correction as pointed out by the reviewer.

Reviewer #2 (Recommendations For The Authors):

1. All western blots require size markers. The FACS blots shown do not have any axis labels.

We have included size markers for blots, at the first appearance of each antibody. Labels are added for FACS blots.

1. The quantification of mitotic cells does not indicate how many cells were counted and if this was done by eye or using software.

The missing experimental information is included in the figure legends, as suggested.

1. The western blots demonstrating ubiquitylation of Greatwall (Figure 4D) are of very poor quality and impossible to interpret.

The ubiquitination of MASTL did not show clear ladders, possibly due to its relative protein size.

Reviewer #3 (Recommendations For The Authors):

Specific suggestions to improve the manuscript:

1. Include literature regarding known mediators of DNA damage checkpoint recovery, including MASTL/Greatwall and PP2A, in the manuscript and discuss the observations from this manuscript in relationship with the literature.

Related literatures are included in the discussion.

1. The increase in MASTL protein levels upon DNA damage are not always clear, for example Fig. 1A. The same for MASTL stability after DNA damage, such as in Fig. 2C. Quantification of the westerns would help demonstrating a significant effect.

As suggested by the reviewer, we have included quantification of fluorescent measurements (Fig. 2, supplemental 1 A-C). Quantification of immunoblots for MASTL upregulation was also added in Fig. 1, supplemental 1E.

1. The E6AP-MASTL in vitro interaction studies shown in Fig. 3 raise doubts. First, beads only are used as negative control, whereas MBP only-beads are a better control. The westerns in top panels of 3B (MASTL), 3C (GST-MASTL) and 3D (MASTL) should be improved. In addition, in Fig. 3C, different GSTMASTL fragments are used in an MBP-E6AP pull down, but the GST-MASTL input does not show any specific band to demonstrate that these fragments are correct. The same for the GFP-E6AP fragments in Fig. 3 Suppl. 1C The input does not show any proteins, there is no N fragment present in the IP and the size of the fragment N3 in the IP GFP does not seem correct.

Altogether, it makes me doubt that the interaction between E6AP and MASTL is direct. Better data with appropriate controls should show whether the interaction is direct or mediated via another protein.

Purified proteins used for the in vitro interaction had significant degradation, causing many bands in the input. We included a lighter exposure of the input here as Author response image 1. MBP alone did not bind MASTL, as both M and C segments of MASTL were MBP-tagged, and did not pull down MASTL. We agree with the reviewer that our direct interaction data showed rather weak MASTL/E6AP interaction, suggesting the interaction is dynamic or possibly mediated by additional binding proteins. We have included this statement in the revised manuscript “Taken together, our data characterized MASTL-E6AP association which was likely mediated via direct protein interaction, although the potential involvement of additional binding partners was not excluded”.

Author response image 1.



1. Fig. 4B. Overexpression of HA-E6AP results in a decrease in MASTL protein levels. Can this effect be rescued by treatment with proteasome inhibitor MG132?

As expected, MG132 stabilized MASTL, with or without E6AP overexpression. We have added this new data in Fig. 4, supplemental 1B.

1. Fig. 4G. MASTL interacts with HA-ubiquitin in WT, but not E6AP KO cells. These cells are treated with MG132, so if E6AP really ubiquitinates MASTL, I would expect MASTL to be polyubiquitinated. However, the "interaction signal" does not show polyubiquitination. In fact, this band actually runs lower than MASTL in input samples, which even could be an artifact. Please explain.

The ubiquitination of MASTL did not show clear ladders, possibly due to its relative protein size. As the reviewer noted, the band position in the HA-Ub IP lanes seemed slightly shifted, compared to the input. We have noticed in many experiments that bands in the IP lanes did not perfectly align with the input lanes.

1. *The DNA damage recovery experiments measuring mitotic index after washing off etoposide (Fig. 5A and Fig. 8A): What are the time points taken? And importantly, why are there no error bars on these intermediate time points, but only on the 4 hour time point?*

As suggested, time point information and additional error bars are included.

1. *Fig. 5E. According to the authors, depletion of MASTL rescues the effect of KO of E6AP. However, no increase in pATM/ATR substrate signal is seen upon etoposide treatment in these samples so I am not convinced this experiment demonstrates a rescue.*

The rescue was evident, especially for many high molecular weight bands which were more effectively detected by this phospho-specific antibody.

1. *Fig. 5C and 8D strongly suggest that E6AP is involved in checkpoint activation. How do these data relate to DNA damage recovery? Is the recovery in E6AP KO cells faster as a consequence of reduced checkpoint signaling or is the recovery effect really specific by stabilization of MASTL? These data should be explained, also taken the data from Wong et al. (Sci. Rep. 2016) into account, that demonstrate that G2 checkpoint activation is independent of MASTL.*

The expression level of MASTL is not upregulated during the activation stage of the DNA damage checkpoint (unless E6AP is depleted). DNA damage signaling, via ATM-dependent E6AP phosphorylation, caused MASTL accumulation over time. This ultimately shifts the balance toward checkpoint recovery and cell cycle re-entry. As such, the role of MASTL (and E6AP-depletion) in suppressing DNA damage checkpoint is in harmony with the proposed role of MASTL upregulation in promoting checkpoint recovery. We have made additional clarifications about this point in the revised manuscript.

1. *The model presented in Fig. 9 is puzzling because there does not seem to be a difference between phosphorylation of E6AP and the interaction with MASTL on early versus late times after DNA damage. And this exactly is what is missing in the manuscript: A more detailed evaluation of the timing of E6APSer218 phosphorylation and the E6AP-MASTL interaction in response to DNA damage.*

More clarification is given to explain this model in the figure legend of Fig. 9. Time point analyses were provided for DNA damage-induced RPA phosphorylation and ATM/ATR substrate phosphorylation (Fig. 1). These data showed MASTL accumulation in the presence of active DNA damage checkpoint signaling. To further strengthen this point, we have included quantification of fluorescent measurements (Fig. 2, supplemental 1 A-C). IF data showed MASTL upregulation in correlation with ATM/ATR activation. Time point information was also added for Ser-218 phosphorylation and MASTL-ENSA dissociation which were observed in early stages of the DNA damage response (1 hr, or 2-4 hr).