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# USP7 counteracts SCF$^{\beta TrCP}$- but not APC$^{Cdh1}$-mediated proteolysis of Claspin

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Claspin is an adaptor protein that facilitates the ataxia telangiectasia and Rad3-related (ATR)-mediated phosphorylation and activation of Chk1, a key effector kinase in the DNA damage response. Efficient termination of Chk1 signaling in mitosis and during checkpoint recovery requires SCF$^{\beta TrCP}$-dependent destruction of Claspin. Here, we identify the deubiquitylating enzyme ubiquitin-specific protease 7 (USP7) as a novel regulator of Claspin stability. Claspin and USP7 interact in vivo, and USP7 is required to maintain steady-state levels of Claspin. Furthermore, USP7-mediated deubiquitylation markedly prolongs the half-life of Claspin, which in turn increases the magnitude and duration of Chk1 phosphorylation in response to genotoxic stress. Finally, we find that in addition to the M phase–specific, SCF$^{\beta TrCP}$-mediated degradation, Claspin is destabilized by the anaphase-promoting complex (APC) and thus remains unstable in G1. Importantly, we demonstrate that USP7 specifically opposes the SCF$^{\beta TrCP}$- but not APC$^{Cdh1}$-mediated degradation of Claspin. Thus, Claspin turnover is controlled by multiple ubiquitylation and deubiquitylation activities, which together provide a flexible means to regulate the ATR–Chk1 pathway.

## Introduction

In response to genotoxic stress, eukaryotic cells elicit DNA damage checkpoint responses, which delay cell cycle progression and stimulate DNA repair to restore genomic integrity (Zhou and Elledge, 2000; Bartek and Lukas, 2007). DNA damage triggers rapid degradation of the Cdk-activating phosphatase, Cdc25A, by the SCF$^{\beta TrCP}$ ubiquitin ligase to arrest the cell cycle in a reversible fashion (Mailand et al., 2000; Busino et al., 2003). This process requires priming phosphorylation of Cdc25A by the checkpoint kinase Chk1, which is itself activated by phosphorylation by the upstream kinase ataxia telangiectasia and Rad3-related (ATR). Efficient ATR-mediated phosphorylation of Chk1 occurs only in the presence of the checkpoint mediator Claspin, a key determinant for Chk1 activation (Kumagai and Dunphy, 2000). Upon entry into mitosis and during recovery from DNA damage–induced cell cycle arrest, Claspin undergoes proteasomal degradation, and such control of Claspin levels plays a pivotal role in restraining Chk1 activity under these conditions (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Like in the case of Cdc25A, the destruction of Claspin is also mediated by SCF$^{\beta TrCP}$; hence, this complex plays a key role in initiating as well as terminating DNA damage checkpoints. These findings have helped to establish regulated ubiquitylation as a major signaling mechanism in the DNA damage response.

The removal of ubiquitin conjugates from target proteins by deubiquitylating enzymes (DUBs) has emerged as an important regulatory mechanism in a range of cellular processes. An estimated 79 functional DUBs are encoded by the human genome, but as yet, only few of these have been assigned functions or substrates (Nijman et al., 2005b). Available evidence suggests that the specificity and regulatory potential of DUBs may be comparable to that of E3 ubiquitin ligases, underscoring the dynamic and reversible nature of protein ubiquitylation. Several DUBs have been found to function in the DNA damage response, including ubiquitin-specific protease 1 (USP1), USP7, and USP28 (Nijman et al., 2005a; Huang et al., 2006; Zhang et al., 2006). For instance, USP7 (also known as HAUSP) is a DUB for Mdm2 (Li et al., 2004), a ubiquitin ligase for p53, and is thus an important factor in the control of p53 abundance during the cell cycle.

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Abbreviations used in this paper: APC, anaphase-promoting complex; ATR, ataxia telangiectasia and Rad3-related; CI, catalytically inactive; DOX, doxycycline; DUB, deubiquitylating enzyme; HU, hydroxyurea; IB, immunoblotting; IP, immunoprecipitation; USP, ubiquitin-specific protease; WT, wild type.

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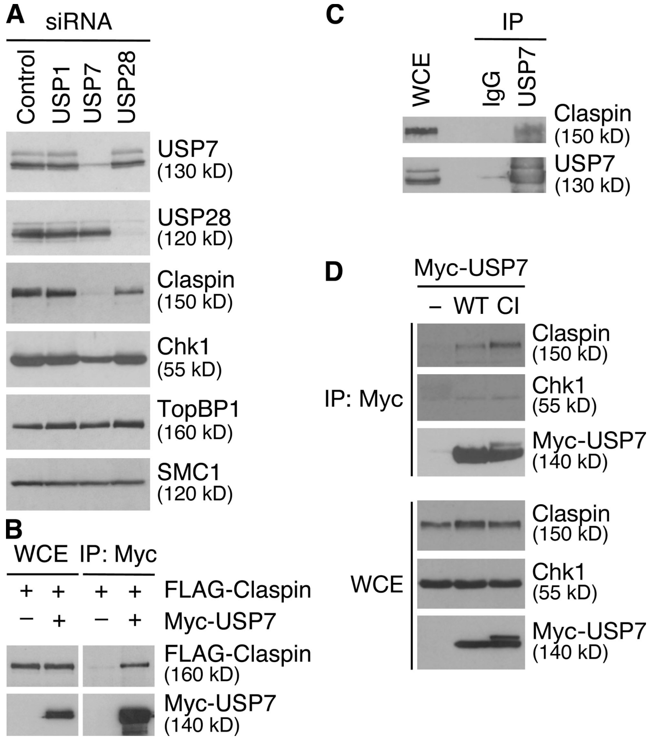

Figure 1. **Claspin interacts with USP7.** (A) U2OS cells were transfected with indicated siRNAs for 72 h and analyzed by IB with the indicated antibodies. The functionality of the USP1 siRNA has been demonstrated previously (Huang et al., 2006). (B) Lysates of 293T cells transfected with indicated plasmids for 24 h were subjected to Myc IP followed by IB. (C) Lysates of 293T cells were subjected to IP with USP7 antibody or preimmune rabbit serum (IgG) followed by IB. (D) 293T cells were transfected with WT or CI forms of Myc-USP7, and processed as in B. The extra band in the USP7 CI lane corresponds to ubiquitylated USP7 (unpublished data).

In this report, we show that the control of Claspin stability is more complex than previously anticipated, involving both ubiquitylation and deubiquitylation to allow cells to finely gauge the levels of Claspin. We identify USP7 as a major DUB for Claspin, with a consequent impact on Chk1 phosphorylation and checkpoint recovery. We also demonstrate that Claspin is degraded by the anaphase-promoting complex (APC) in the G1 phase, and that USP7 specifically counteracts SCF$^{\beta TRCP}$- but not APC$^{Cdh1}$-mediated ubiquitylation of Claspin.

## Results and discussion

### Claspin interacts with USP7

To explore the dynamic control of Claspin stability, we investigated whether Claspin is regulated by deubiquitylation activities. Focusing on a selected set of DUBs, which have previously been implicated in the DNA damage response, we monitored the response of Claspin levels to siRNA-mediated down-regulation of these DUBs. Consistent with published results (Zhang et al., 2006), we observed a partial reduction in Claspin level after knocking down USP28 (Fig. 1 A). Strikingly, however, we consistently detected a much more prominent decrease of Claspin expression after depletion of USP7, which suggests that USP7 might protect Claspin from ubiquitylation-dependent degradation (Fig. 1 A). However, the abundance of TopBP1, which is also required for Chk1 activation in response to genotoxic insults, was not affected by knockdown of USP7 or other DUBs (Fig. 1 A). A mixture of three independent oligonucleotides was used to efficiently knock down USP7 expression, and the effect of USP7 depletion on Claspin levels could be reproduced with individual siRNAs to USP7 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200807137/DC1). To clarify whether Claspin is a direct target of USP7, we tested whether the two proteins copurify in immunoprecipitation (IP) experiments. Indeed, USP7 and Claspin readily interacted under conditions where both proteins were overexpressed (Fig. 1 B), and we could also detect association between the endogenous proteins (Fig. 1 C). In contrast, we did not observe binding of Claspin to several other DUBs (unpublished data), underscoring the specificity of the Claspin–USP7 interaction. To further probe the relationship between USP7 and Claspin, we assessed the Claspin-binding capability of wild-type (WT) or catalytically inactive (CI) USP7. In such experiments, USP7 CI interacted more strongly with endogenous Claspin than did WT USP7 (Fig. 1 D), which is consistent with a substrate-trapping mechanism in which an inability of inactive USP7 to deubiquitylate Claspin would manifest as a prolonged binding and thus a tighter interaction. These observations suggest that Claspin is a novel substrate for USP7.

Interestingly, depletion of USP7 also led to a significant down-regulation of total Chk1 levels (Fig. 1 A), but we failed to produce credible evidence that Chk1 is a direct target for USP7. In particular, whereas Claspin avidly interacted with USP7, the amount of Chk1 coimmunoprecipitated with ectopic USP7 did not significantly exceed that observed in the control cells (Fig. 1 D). In addition, knockdown of Claspin or Chk1 negatively affected the expression level of the other protein (Fig. S1 C), which suggests that Claspin and Chk1 may promote the stability of each other, in agreement with previous findings (Yang et al., 2008). Thus, it seems likely that the effects on Chk1 levels observed in response to up- or down-regulation of USP7 may be indirectly mediated through its impact on Claspin.

### USP7 deubiquitylates and stabilizes Claspin

To corroborate the emerging link between USP7 and Claspin, we generated cell lines capable of conditionally expressing WT or CI mutant forms of Myc-tagged USP7. Induction of USP7 in these cell lines resulted in homogenous nuclear expression of the transgenes in virtually all cells, but had little impact on cell cycle distribution (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200807137/DC1; and not depicted). Using these cell lines, we asked whether overexpression of USP7 would have a stabilizing effect on endogenous Claspin. Expression of USP7 WT clearly resulted in an elevation of Claspin levels, whereas no such effect could be seen in cells induced to express USP7 CI (Fig. 2 A). Only a slight increase in Chk1 levels was evident under these conditions, which further supports the notion that Claspin rather than Chk1 is a target of USP7. As a control for the functionality of ectopic USP7 in the cell lines, we analyzed its impact on Mdm2, a known target for USP7. Indeed, Mdm2 abundance was strongly elevated in a manner dependent on the catalytic activity of USP7 but independent of cell cycle

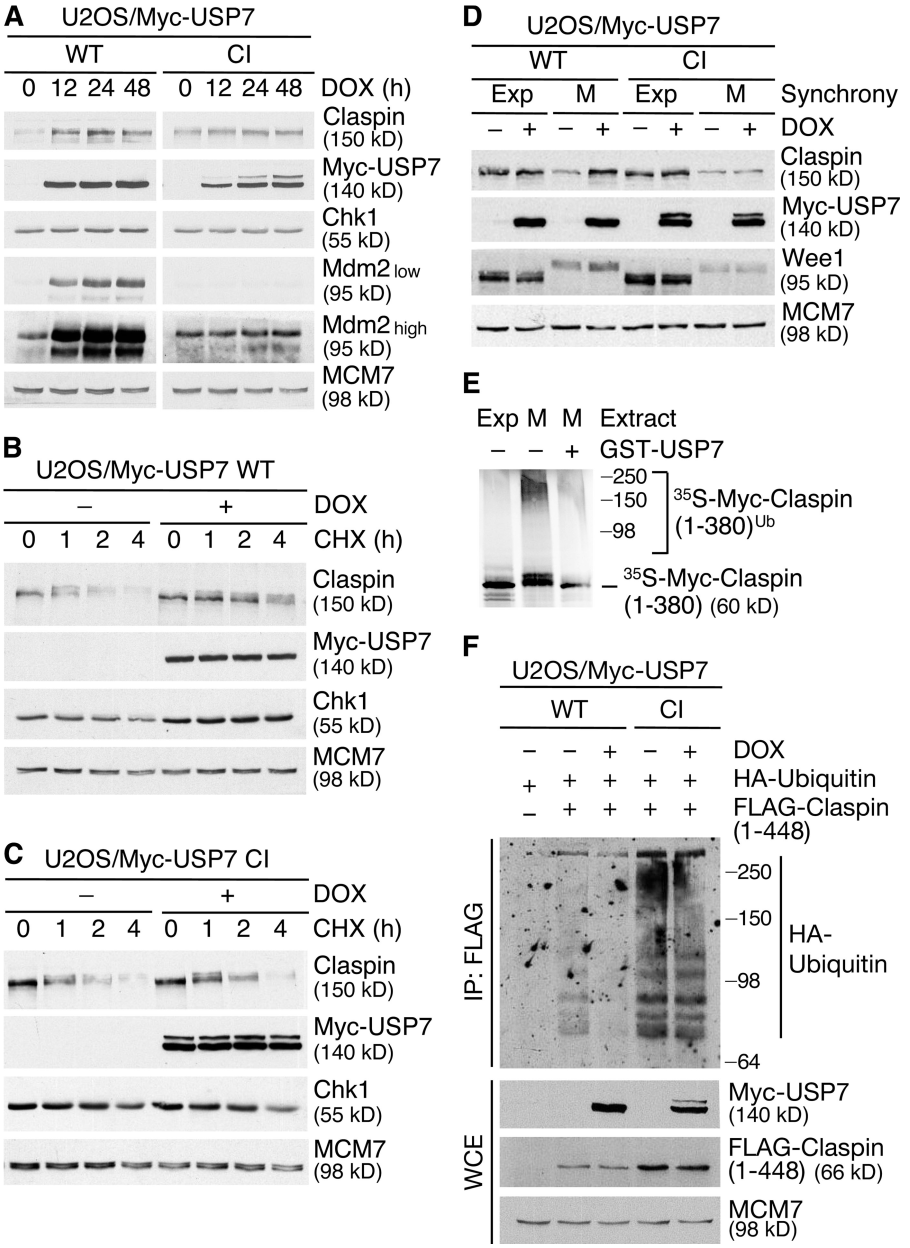

Figure 2. **USP7 deubiquitylates and stabilizes Claspin.** (A) U2OS/Myc-USP7 cell lines were induced with doxycycline (DOX), harvested at the indicated times, and processed for IB with the indicated antibodies. (B) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 30 h, after which cycloheximide (CHX) was added to the cultures for the indicated times. The half-life of Claspin was estimated by IB of total cell extracts. (C) U2OS/Myc-USP7 CI cells were treated as in B. (D) U2OS/Myc-USP7 WT or CI cells were induced or not induced for 18 h with DOX, and left untreated (Exp) or incubated with nocodazole for an additional 12 h to synchronize cells in mitosis (M). The cell extracts were then analyzed by IB. Mobility-shifted Wee1 served as a marker for mitotically synchronized cells. (E) [$^{35}$S]-labeled Claspin (amino acids 1–380) was incubated in ubiquitylation reaction mix, then supplemented with extracts of exponentially growing or mitotic U2OS cells and in vitro–translated βTrCP1. Where indicated, bacterially purified GST-USP7 WT was added to the reaction. Claspin ubiquitylation was visualized by autoradiography. Numbers to the right of the gel blots indicate molecular mass standards in kD. (F) U2OS/Myc-USP7 cell lines were transfected with indicated constructs for 24 h, and lysates were processed for IP with FLAG antibody and IB.

stage (Fig. 2 A and Fig. S2 C). To clarify further whether Claspin is stabilized by USP7-dependent deubiquitylation, we assessed the half-life of Claspin in cells expressing ectopic USP7 by adding cycloheximide to block protein synthesis. Overexpression of USP7 WT brought about a robust, three- to fourfold increase in the stability of Claspin as compared with uninduced cells, in which the half-life of Claspin was very short (~1 h, Fig. 2 B). In contrast, little if any effect on Claspin stability was evident in cells expressing USP7 CI (Fig. 2 C), which supports the idea that Claspin is deubiquitylated by USP7.

### USP7 reverses SCF$^{\beta TrCP}$-dependent ubiquitylation of Claspin

We have previously shown that the levels of Claspin oscillate in a cell cycle–dependent manner, being high in S and G2 phases and declining sharply upon entry into mitosis and throughout G1 (Mailand et al., 2006). Degradation of Claspin at the onset of mitosis is driven by its ubiquitylation by SCF$^{\beta TrCP}$, and we therefore asked whether overexpression of USP7 could counteract this process. To this end, we assessed the impact of ectopic USP7 on the levels of Claspin in mitotic cells. In uninduced cells, Claspin was indeed expressed at much lower levels in mitotic cells than in exponentially growing cells (Fig. 2 D). However, as would be expected if USP7 was able to reverse SCF$^{\beta TrCP}$-mediated ubiquitylation of Claspin, induction of USP7 WT fully restored Claspin levels to those seen in asynchronous cells (Fig. 2 D). Again, this propensity of USP7 was dependent on the catalytic activity of USP7, as the abundance of Claspin remained low in mitotic cells expressing USP7 CI (Fig. 2 D). Because extracts from mitotic cells readily support ubiquitylation of Claspin in a βTrCP-dependent fashion (Mailand et al., 2006), we used such an approach to test whether USP7 directly deubiquitylates Claspin in vitro. As shown in Fig. 2 E, the polyubiquitylation of Claspin observed in mitotic cells was inhibited when purified GST-tagged USP7 WT was added to the reaction. In addition, induction of USP7 WT but not USP CI quantitatively suppressed the appearance of polyubiquitylated Claspin species in cells (Fig. 2 F), which indicates that USP7 is a major Claspin-directed DUB. Together, these results demonstrate that USP7 stabilizes Claspin by directly opposing its βTrCP-mediated ubiquitylation. Hence, the balance of ubiquitylation and deubiquitylation activities allow for subtle control of steady-state levels of Claspin.

### Claspin is degraded by the APC in G1

Although elevated levels of USP7 WT were able to maintain Claspin expression during mitosis, we noted that it was still degraded upon reentry into G1 phase (Fig. 3 A). This suggested that once in G1, Claspin becomes susceptible to degradation by a βTrCP-independent mechanism refractory to USP7 activity. We reasoned that under these conditions, the destruction of Claspin might instead be driven by the APC, which promotes the degradation of numerous regulatory proteins in late mitosis and G1 (Peters, 2006). To test this possibility, we coexpressed Claspin with Cdh1, the substrate-specific activator of the APC in G1, using the finding that elevated levels of Cdh1 are sufficient to trigger APC activation irrespective of cell cycle stage (Sorensen et al., 2000). In the presence of high levels of Cdh1, the expression of Claspin was strongly suppressed (Fig. 3 B), which suggests that Claspin is indeed a target for APC-mediated degradation. To further probe this proteolytic mechanism, we used an immunofluorescence-based approach to monitor Claspin abundance in G1 cells. Although Claspin was detectable only at background levels in G1 phase (Cyclin B1–negative) nuclei, treatment with a proteasome inhibitor (MG132) was sufficient to restore Claspin expression to levels comparable with S and G2 phase cells (Fig. 3 C), demonstrating that Claspin is actively degraded by the proteasome in G1. Next, we subjected cells to siRNA-mediated depletion of βTrCP or Cdh1 to test whether the degradation of Claspin in G1 cells was predominantly mediated by $\mathrm{SCF}^{\beta\mathrm{TrCP}}$ or $\mathrm{APC}^{\mathrm{Cdh1}}$. Although Claspin did not accumulate in G1 cells in response to knockdown of βTrCP, its expression in G1 was efficiently restored upon depletion of Cdh1 (Fig. 3 C), indicating that the degradation of Claspin in G1 phase is mediated by $\mathrm{APC}^{\mathrm{Cdh1}}$ but not $\mathrm{SCF}^{\beta\mathrm{TrCP}}$. In contrast, we had previously shown that the APC was unable to promote the degradation of Claspin in early mitosis (Mailand et al., 2006). Thus, two distinct pathways operate to limit Claspin abundance during the cell cycle, in a manner much like Cdc25A, whose destruction is also controlled by both $\mathrm{SCF}^{\beta\mathrm{TrCP}}$ and $\mathrm{APC}^{\mathrm{Cdh1}}$ (Donzelli et al., 2002). We speculate that the APC-mediated degradation of Claspin in G1 is an important means of suppressing inappropriate Chk1 activation during this window of the cell cycle. Indeed, cells expressing elevated levels of Cdh1 were markedly impaired in their ability to activate Chk1 in response to UV, whereas the phosphorylation of other ATR targets remained virtually normal, as exemplified by p53 (Fig. 3 D). The choice of APC as the machinery for Claspin ubiquitylation in G1 may reflect the fact that priming phosphorylation of Claspin by the Plk1 kinase is required for its $\mathrm{SCF}^{\beta\mathrm{TrCP}}$-mediated destruction (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006); the absence of Plk1 in G1, because of its destruction by $\mathrm{APC}^{\mathrm{Cdh1}}$ (Peters, 2006), thus necessitates a Plk1-independent mechanism for the G1-specific degradation of Claspin.

As mentioned previously, the $\mathrm{APC}^{\mathrm{Cdh1}}$-mediated degradation of Claspin appeared insensitive to USP7 activity (Fig. 3 A), and to confirm this, we tested the ability of $\mathrm{APC}^{\mathrm{Cdh1}}$ to degrade Claspin in the presence of elevated levels of USP7. Unlike inactivation of $\mathrm{APC}^{\mathrm{Cdh1}}$, expression of ectopic USP7 WT did not restore Claspin expression in G1 (Fig. 3 E), which indicates that the stabilization of Claspin observed in these cells (Fig. 2, A and B) happens outside G1 phase. In addition, overexpressed USP7 failed to counteract Claspin degradation mediated by activated $\mathrm{APC}^{\mathrm{Cdh1}}$ (Fig. 3 F). We conclude from these experiments that USP7 selectively opposes the $\mathrm{SCF}^{\beta\mathrm{TrCP}}$- but not $\mathrm{APC}^{\mathrm{Cdh1}}$-dependent degradation of Claspin, which suggests that the molecular nature of ubiquitin chains attached to Claspin by $\mathrm{APC}^{\mathrm{Cdh1}}$ and $\mathrm{SCF}^{\beta\mathrm{TrCP}}$ differ in terms of their susceptibility or accessibility to USP7-mediated deubiquitylation.

### USP7 controls the timing of checkpoint-induced Chk1 phosphorylation through regulation of Claspin stability

The role of USP7 in maintaining steady-state levels of Claspin suggested that modulation of USP7 activity might affect Chk1 regulation during checkpoint responses. To test this, we first assessed the ability of USP7-depleted cells to activate Chk1. Consistent with the quantitative loss of Claspin in such cells, knockdown of USP7 strongly impaired UV-dependent activation of Chk1, as judged from its phosphorylation on Ser317 (Fig. 4 A). As with Claspin levels, this effect was specific to USP7, as neither depletion of USP1 or USP28 significantly compromised UV-induced Chk1 phosphorylation (Fig. 4 A). Hence, these data suggest that USP7 is required for Chk1 activation in response to DNA damage.

Destruction of Claspin by $\mathrm{SCF}^{\beta\mathrm{TrCP}}$ promotes the timely inactivation of Chk1 during recovery from DNA damage–induced cell cycle arrest (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006), and because USP7 protects Claspin from βTrCP-dependent degradation, we reasoned that the activity of USP7 might be a contributing factor in timing the duration of Chk1 phosphorylation during checkpoint responses. To this end, we analyzed the kinetics of Chk1 dephosphorylation, and thus checkpoint termination, in USP7 cell lines released from a replication block induced by hydroxyurea (HU). We consistently observed a strong delay in the ability of USP7 WT-induced cells to degrade Claspin and inactivate Chk1 after release from the HU-induced arrest, relative to uninduced cells (Fig. 4 B). This was accompanied by a slower rate of cell cycle progression upon HU removal, which might at least partially reflect the delayed kinetics of Claspin degradation in these cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200807137/DC1). The overall levels of Chk1 remained somewhat higher in cells induced to express USP7 WT, which is in agreement with the previously observed correlation

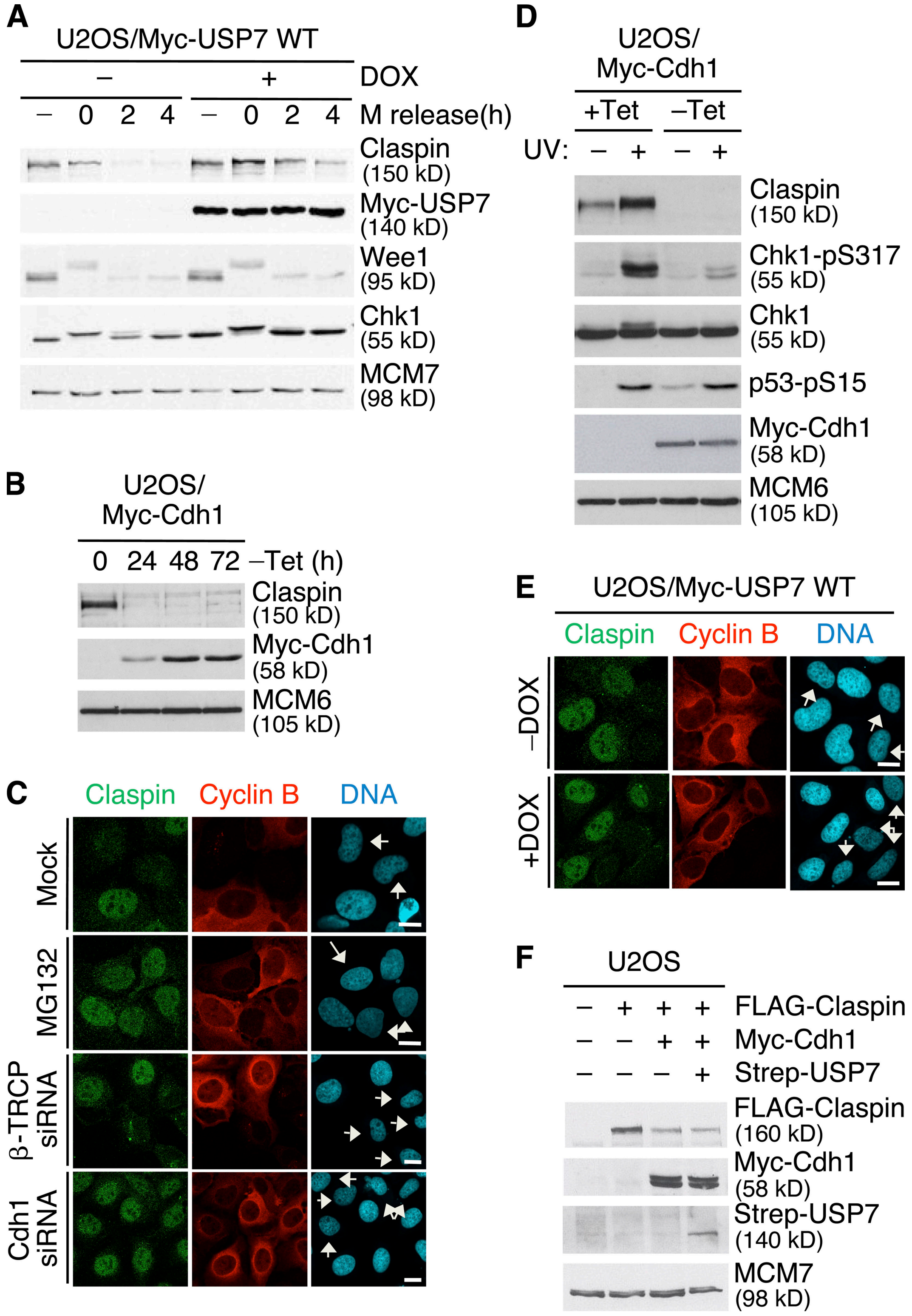

Figure 3. **Degradation of Claspin by APC$^{Cdh1}$ in G1 is not opposed by USP7.** (A) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 18 h, and left untreated or synchronized in mitosis by nocodazole treatment for an additional 12 h. Mitotic cells were washed, plated in fresh medium, and collected at the indicated times after release. Lysates of these and asynchronous control cells (–) were analyzed by IB. (B) U2OS/Myc-Cdh1 cells were induced by removal of tetracycline (Tet) for the indicated times and processed for IB. (C) U2OS cells were treated with MG132 for 4 h or transfected with βTrCP or Cdh1 siRNAs for 48 h. The cells were then fixed and immunostained with indicated antibodies. DNA was visualized by counterstaining with ToPro3. Arrows indicate G1 cells. Bars, 10 µm. (D) U2OS/Myc-Cdh1 cells were induced or not induced by tetracycline withdrawal for 48 h, exposed to UV (20 J/m$^2$), and harvested 1 h later. Total cell extracts were processed for IB. (E) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 48 h, and processed for immunofluorescence as in C. Arrows indicate G1 cells. Bars, 10 µm. (F) U2OS cells were transfected with the indicated plasmids for 24 h and processed for IB.

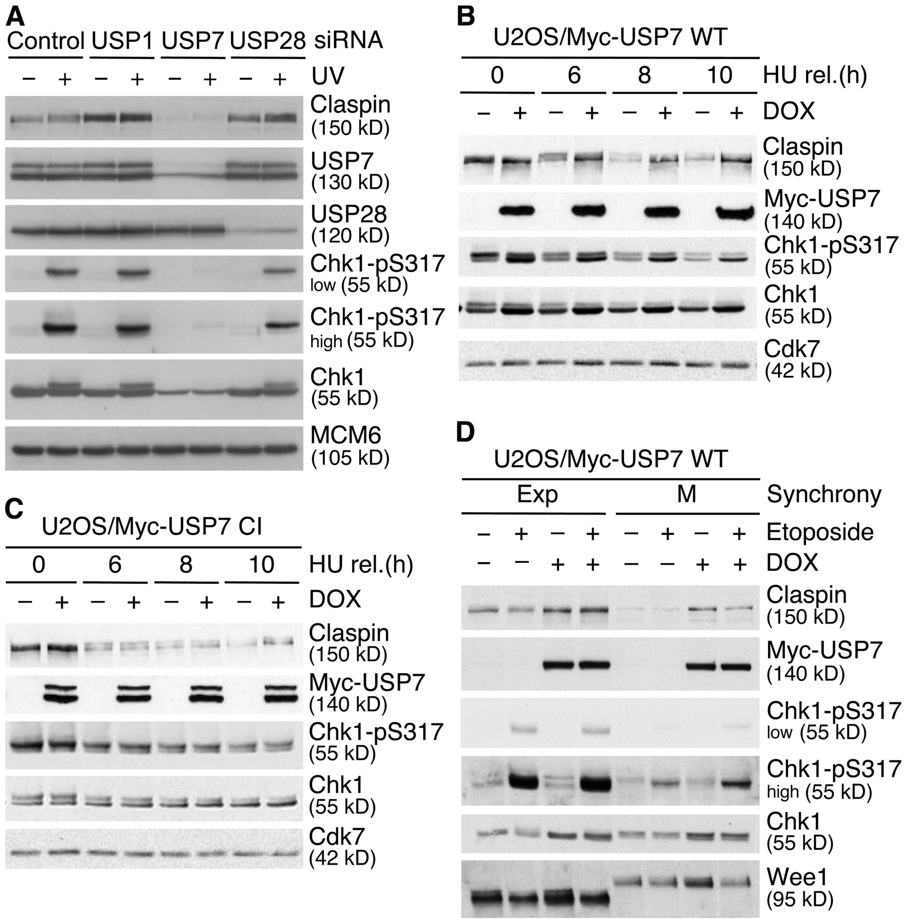

Figure 4. **USP7 controls the timing of Chk1 phosphorylation through regulation of Claspin stability.** (A) U2OS cells were transfected with indicated siRNAs for 72 h, then subsequently treated with UV (20 J/m$^2$) and harvested 1 h later. Cells were then processed for IB. (B) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 12 h, and treated with HU for an additional 24 h. Cells were then released from the HU block, harvested at the indicated times, and processed for IB. (C) U2OS/Myc-USP7 CI cells were treated as in B. (D) U2OS/Myc-USP7 WT cells were induced or not induced with DOX for 24 h, and left untreated or synchronized in mitosis with nocodazole for an additional 12 h. Cells were then treated with etoposide for 2 h where indicated, and processed for IB.

between Chk1 and Claspin levels. Consistent with the inability of USP7 CI to stabilize Claspin, cells expressing this mutant did not display any checkpoint recovery delay (Fig. 4 C). Hence, increased activity of USP7 interferes with the cellular ability to timely degrade Claspin and inactivate Chk1 upon checkpoint termination.

In addition to its role during checkpoint recovery, βTrCP-dependent degradation of Claspin helps prevent inappropriate activation of Chk1 during mitosis (Mailand et al., 2006; Mamely et al., 2006). Consequently, mitotic cells depleted for βTrCP display partial Chk1 phosphorylation after exposure to genotoxic agents. Because USP7 stabilized Claspin in mitosis, we tested if mitotic cells overexpressing USP7 WT would allow Chk1 activation. Indeed, like βTrCP depletion, elevated levels of USP7 WT partially enabled phosphorylation of Chk1 upon exposure of mitotic cells to the DNA-damaging agent etoposide (Fig. 4 D). These observations suggest that the ubiquitylation and deubiquitylation activities toward Claspin must be tightly coordinated to enforce Claspin degradation and thus Chk1 inactivation in mitosis.

Collectively, our results uncover a dynamic and complex mode of controlling Claspin stability during the cell cycle and upon checkpoint-inducing stimuli involving both ubiquitylation and deubiquitylation activities. These findings highlight the tuning of Claspin availability as a key regulatory event in pathways that govern Chk1 activation, and likely reflect the fact that Claspin is specifically required for ATR-mediated phosphorylation of Chk1 but not other targets (Liu et al., 2006). Most importantly, we identified USP7 as a DUB opposing βTrCP-mediated ubiquitylation of Claspin, thus broadening the scope of USP7 functions in the maintenance of genomic integrity. By protecting Claspin from degradation, USP7 may directly impact the initiation as well as termination of Chk1-mediated signaling responses, and hence it will be important to address if and how the Claspin-directed activity of USP7 is itself regulated during checkpoint responses. A recent study also identified Claspin as a novel APC target, and the DUB USP28 was found to oppose its APC-mediated ubiquitylation (Bassermann et al., 2008). Hence, two distinct DUBs may be used to counteract Claspin ubiquitylation mediated by SCF$^{\beta TrCP}$ or APC$^{Cdh1}$, further highlighting the importance of tightly controlling its steady-state expression levels during the cell cycle.

## Materials and methods

### Plasmids and RNA interference

Plasmids expressing WT and CI (C223S) Myc-tagged USP7 (pcDNA3-Myc-USP7) were gifts from R. Everett (MRC Virology Unit, University of Glasgow, Scotland, UK). The inserts were inserted into pcDNA4/TO (Invitrogen), allowing for doxycycline-inducible expression of Myc-USP7 WT and CI, and into pGEX-20T (GE Healthcare) for bacterial expression of GST-USP7 fusion proteins. Other plasmids used in this study included pIRES-FLAG-Claspin, pCMV2-FLAG-Claspin (amino acids 1–448), pX-Myc-Claspin (amino acids 1–380), pX-Myc-Cdh1, and pX-Myc-βTrCP1, all of which have been described previously (Mailand et al., 2006; Sorensen et al., 2000). Plasmid transfections were performed using FuGene6 (Roche).

A mixture of three different siRNAs were used to efficiently knock down USP7, as described previously (Canning et al., 2004). Other siRNAs used in this study included USP1 (5′-GGCAAUACUUGCUAUCUUA-3′; Nijman et al., 2005a) and USP28 (5′-CUGCAUUCACCUUAUCAUU-3′). siRNA to Cdh1 and βTrCP have been described previously (Mailand and Diffley, 2005; Mailand et al., 2006). All siRNA duplexes (purchased from Thermo Fischer Scientific) were transfected at a final concentration of 100 nM using Lipofectamine RNAiMAX (Invitrogen).

### Cell culture

Human U2OS osteosarcoma cells and HEK293T embryonic kidney cells were cultured in DME containing 10% fetal bovine serum. U2OS derivative cell lines expressing Myc-tagged USP7 WT or CI in a doxycycline-inducible fashion from pcDNA4/TO-Myc-USP7 vectors were generated and maintained as described previously (Mailand et al., 2007). The U2OS/Myc-Cdh1 cell line has been described previously (Sorensen et al., 2000). Cells were synchronized in mitosis by shaking off rounded cells after treatment with 40 ng/ml nocodazole (Sigma-Aldrich) for 12 h. Other drugs used in this study included: 1 μg/ml doxycycline (EMD), 2 μg/ml tetracycline (EMD), 2 mM HU (Sigma-Aldrich), 25 μg/ml cycloheximide (Sigma-Aldrich), and etoposide (50 μM for exponential and 100 μM for mitotic cells; EMD).

### Immunochemical methods and microscopy

Immunoblotting (IB), IP, and immunofluorescence were performed as described previously (Mailand et al., 2006). Antibodies used in this study included mouse monoclonals to Strep-tag (IBA BioTAGnology) and Mdm2 (sc-965; Santa Cruz Biotechnology, Inc.); rabbit polyclonals to USP7 (BL851; Bethyl Laboratories, Inc.), Claspin (BL-73 [Bethyl Laboratories, Inc.] and Ab3720 [Abcam], used for IB and immunofluorescence, respectively), Wee1 (sc-325; Santa Cruz Biotechnology, Inc.), and USP28 (a gift from S.J. Elledge, Harvard Medical School, Boston, MA). Antibodies to FLAG, Myc, HA, cyclin B1, Chk1, Cdk7, Chk1 S317, SMC1, TopBP1, and MCM6 have been described previously (Mailand et al., 2006). Acquisition of confocal images was done essentially as described previously (Bekker-Jensen et al., 2006), using a confocal microscope (LSM510; Carl Zeiss, Inc.) fitted with a Plan-Neofluar 40× NA 1.3 oil immersion objective lens (Carl Zeiss, Inc.). For dual-color imaging, secondary antibodies coupled to Alexa Fluor dyes with excitation wavelengths of 488 and 568 nm were used. Image acquisition and basic image processing were performed with LSM software (Carl Zeiss, Inc.).

### In vitro deubiquitylation assay

Claspin was ubiquitylated in vitro essentially as described previously (Mailand et al., 2006). In brief, in vitro–translated $[^{35}S]$Claspin (amino acids 1–380) was incubated with ubiquitylation reaction mix (40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 mg/ml ubiquitin, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, 0.5 mM ATP, 5 μM MG132 [all from Sigma-Aldrich], 5 μM human E1, and 2 μM hUbcH5C [both from Boston Biochem]), then supplemented with 30 μg of extract from exponentially growing or mitotic U2OS cells (Mailand et al., 2006) and unlabeled in vitro–translated βTrCP1. To assess the deubiquitylation activity of USP7 toward Claspin, 1 μg of GST-USP7 WT purified from *Escherichia coli* was included in the reaction. Reactions were incubated at 30°C for 1 h, stopped by addition of Laemmli sample buffer, resolved by SDS-PAGE, and visualized by autoradiography.

### Online supplemental material

Fig. S1 shows specificity and effects of USP7, Claspin, and Chk1 siRNAs. Fig. S2 shows characterization of the U2OS/Myc-USP7 inducible cell lines. Fig. S3 shows cell cycle profiles of cells in the experiment in Fig. 4 B at representative time points, determined by flow cytometric analysis. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200807137/DC1.

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