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$SCF^{\beta TrCP}$-Mediated Degradation of Claspin Regulates Recovery from the DNA Replication Checkpoint Response

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Summary

During replicative stress, Claspin mediates the phosphorylation and consequent activation of Chk1 by ATR. We found that during recovery from the DNA replication checkpoint response, Claspin is degraded in a βTrCP-dependent manner. In vivo, Claspin is phosphorylated in a canonical DSGxxS degron sequence, which is typical of βTrCP substrates. Phosphorylation of Claspin is mediated by Plk1 and is essential for binding to βTrCP. In vitro ubiquitylation of Claspin requires βTrCP, Plk1, and an intact DSGxxS degron. Significantly, expression of a stable Claspin mutant unable to bind βTrCP prolongs the activation of Chk1, thereby attenuating the recovery from the DNA replication stress response and significantly delaying entry into mitosis. Thus, the $SCF^{\beta TrCP}$-dependent degradation of Claspin is necessary for the efficient and timely termination of the DNA replication checkpoint. Importantly, in response to DNA damage in G2, Claspin proteolysis is inhibited to allow the prompt reestablishment of the checkpoint.

Introduction

Cyclin-dependent kinases (CDKs) are crucial regulators of the cell division cycle in eukaryotes (Murray, 2004; Pagano and Jackson, 2004). Both S phase and mitosis require CDK activity; however, whereas during the G1/S transition Cdk1, Cdk2, Cdk3, Cdk4, and Cdk6 have apparently redundant functions, at G2/M, Cdk1 is nonredundant (Bashir and Pagano, 2005). Despite the number of CDKs that are active at G1/S, the amount of total CDK activity required for DNA replication to occur is significantly less than the sole Cdk1 activity necessary for entry into mitosis. During S and G2 phases, Cdk1 activity is kept at a low level by phosphorylation on Thr14 and Tyr15, which are mediated by the Myt1 and Wee1 kinases, respectively. To prevent dephosphorylation of Cdk1, the double-specificity phosphatases Cdc25A, Cdc25B, and Cdc25C are kept in an inactive state, sequestered in the cytoplasm, or degraded. Cdc25A is likely to be the major activator of Cdk1 (Mailand et al., 2002), and in analogy to *Cdk1*, which is the only essential CDK gene in mice, of the three CDC25 paralogs, only *Cdc25A* is an essential gene in the mouse (Chen et al. [2001], Lincoln et al. [2002] and H. Kiyokawa, personal communication).

Rapid, specific, and timely proteolysis of cell cycle regulators by the ubiquitin-proteasome system represents an important mechanism that ensures proper progression through the cell division cycle in a unidirectional and irreversible manner (Guardavaccaro and Pagano, 2006). The proteolysis of many core components of the cell cycle machinery is controlled by Skp1-Cul1-F box protein (SCF) ubiquitin ligases. In humans, there are 68 SCF ligases (Cenciarelli et al., 1999; Jin et al., 2004; Winston et al., 1999), each characterized by a different F box protein subunit that provides specificity by directly recruiting the substrate to the rest of the ligase and, ultimately, to the ubiquitin-conjugating enzyme. Most SCF substrates are recognized and bound by the F box protein subunit only when phosphorylated on specific sites. Notably, only three out of 68 human SCF ubiquitin ligases (SCF^{Skp2}, $SCF^{\beta\text{-}TrCP}$, and SCF^{Fbw7}) have well-established substrates, many of which are involved in cell cycle control (e.g., Cdc25A, cyclin E, Emi1, p21, p27, and Wee1) (Ang and Harper, 2005; Cardozo and Pagano, 2004; Petroski and Deshaies, 2005). Mammals express two distinct paralogous βTrCP proteins (βTrCP1, also known as Fbw1, and βTrCP2, also called Fbw11), yet their biochemical properties are undistinguishable. For simplicity, we will therefore use the term βTrCP to refer to both, unless specified.

During S phase, the monitoring of incompletely replicated DNA and other replicative stresses by various checkpoint mechanisms ensures genomic integrity (Bartek et al., 2004). These DNA replication checkpoints ultimately impinge on Cdk1 by activating Wee1 and by keeping Cdc25 phosphatases at bay (van Vugt et al., 2005). Thus, this pathway ensures that mitosis is not executed until DNA replication has been properly completed and checked for fidelity. The DNA replication checkpoint response includes sensors of incompletely replicated or damaged DNA (e.g., Rad17), transducers (e.g., ATR), mediators (e.g., Claspin), effectors (e.g., Chk1), and targets (e.g., Cdc25A and Cdk1) (Melo and Toczyski, 2002). *ATR* and *Chk1* are essential genes required for cell cycle progression and the checkpoint response (Bartek and Lukas, 2003). ATR-mediated phosphorylation of Chk1 induces its activation; in turn, Chk1 phosphorylates substrates that regulate cell cycle progression, DNA repair, and apoptosis (Gottifredi and Prives, 2005).

A major target of Chk1 is Cdc25A that is phosphorylated on Ser76 and thereby primed for subsequent phosphorylation by an as yet unknown kinase. The latter phosphorylates Ser79 and Ser82, thus generating a phosphodegron to bind βTrCP (Busino et al., 2003; Jin et al., 2003). $SCF^{\beta TrCP}$ directs the ubiquitylation and

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degradation of Cdc25A, hence keeping Cdk1 activity low during S and G2 (apparently low levels of Cdc25A in S and G2 are able to sustain full Cdk2 activity). At G2/M, when the DNA replication checkpoint is turned off, Cdc25A accumulates and contributes to Cdk1 activation. Cdc25A accumulation is allowed at this time as for some unknown reason it is not recognized by $SCF^{\beta TrCP}$ any longer. It is likely that Chk1 stops phosphorylating Cdc25A, but how this process is terminated is currently not clear. Once Cdk1 is activated, it initiates two autoamplification loops: one involving the phosphorylation of Cdc25A (that is further stabilized by a not-yet understood mechanism [Mailand et al., 2002]) and the other involving the phosphorylation of Wee1. The latter constitutes a priming event for Plk1-mediated phosphorylation of Wee1 and its subsequent recognition by $SCF^{\beta TrCP}$, which results in the degradation of Wee1 (Watanabe et al., 2004). The result of these autoamplification loops is the full activation of Cdk1 that in turn executes crucial events of early mitosis.

Claspin is a ring-shaped protein that is loaded onto DNA just after initial unwinding of the two DNA strands for replication (Kumagai and Dunphy, 2000; Lee et al., 2003; Sar et al., 2004). Studies in *Xenopus* and mammals have shown that Claspin is a major mediator of Chk1 activation in response to DNA replication and DNA damage checkpoints (Chini and Chen, 2004; Melo and Toczyski, 2002; van Vugt and Medema, 2004). During replicative stresses that occur during S phase, Claspin promotes ATR-mediated phosphorylation and activation of Chk1 (Chini and Chen, 2003; Kumagai and Dunphy, 2000, 2003; Lee et al., 2003). *Xenopus* egg extracts depleted of Claspin are unable to arrest the cell cycle in response to DNA replication blocks (Kumagai and Dunphy, 2000). Similarly, silencing of Claspin in human cells decreases Chk1 activation in response to hydroxyurea (HU), resulting in the stabilization of Cdc25A (Chini and Chen, 2003; Sorensen et al., 2004).

We have identified Claspin as an interactor of βTrCP by using an immunopurification procedure followed by mass spectrometry analysis. In agreement with its role in the DNA replication stress response, we found that the protein levels of Claspin are cell cycle regulated, peaking in S phase and decreasing in G2. We hypothesized that the decrease in the levels of Claspin is mediated by βTrCP to terminate the Claspin-mediated activation of Chk1 and, consequently, to turn off the DNA replication checkpoint. In turn, this mechanism would allow the accumulation of Cdc25A and the subsequent activation of Cdk1. This hypothesis was investigated in the study presented herein.

Results

Claspin Is an Interactor of βTrCP

To identify new substrates of the $SCF^{\beta TrCP}$ ubiquitin ligase, we have optimized an immunopurification strategy that enriches for ubiquitylated substrates. Briefly, βTrCP2 was expressed in HEK293T cells, immunopurified according to our protocol (see Supplemental Experimental Procedures available in the Supplemental Data with this article online), and the final eluate was directly analyzed by liquid chromatography-tandem mass spectrometry. The examination of copurified endogenous proteins revealed the presence of unique peptides (larger than seven amino acids) derived from SCF subunits, including Cul1 (29 peptides) and Skp1 (nine peptides), as well as peptides derived from known $SCF^{\beta TrCP}$ substrates, including β-catenin (13 peptides), Cdc25A (one peptide), Wee1 (one peptide), and a pseudosubstrate, hnRNP-u (51 peptides). Finally, among a number of peptides from putative novel substrates, we recovered six unique peptides from Claspin (GSEQTTGAENE VETNALPVVSK, DLVKNEELEIQEK, ADVVPVTLAPK, QLGVDVSIKPR, AVGFLSVPK, and MGYFPTEEK).

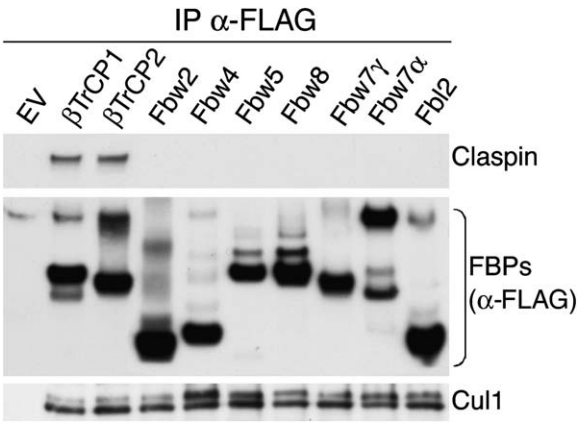

Figure 1. βTrCP1 and βTrCP2 Interact with Claspin In Vivo

HEK293T cells were transfected with the indicated FLAG-tagged F box protein constructs (FBPs). During the last 6 hr before harvesting, cells were treated with the proteasome inhibitor MG132. Exogenous proteins were immunoprecipitated (IP) from cell extracts with anti-FLAG resin, and immunocomplexes were probed with antibodies to the indicated proteins. (EV, empty vector.)

To confirm the specific binding between Claspin and βTrCP, we screened 19 human F box proteins. FLAG-tagged versions of these proteins were transfected into HEK293T cells (adding the proteasome inhibitor MG132 for 6 hr before harvesting the cells) and then immunoprecipitated to evaluate their interaction with endogenous Claspin. We found that the only F box proteins able to coimmunoprecipitate with Claspin were βTrCP1 and its paralog βTrCP2 (Figure 1). In contrast, related F box proteins such as Fbw2, Fbw4, Fbw5, Fbw7α, Fbw7γ, or Fbw8, which all contain WD-40 repeats like βTrCP, did not bind endogenous Claspin. Similarly, various additional F box proteins (Fbl2, Fbl16, Fbx1, Fbx2, Fbx9, Fbx11, Fbx28, Fbx31, Fbx46, Nipa, and Skp2) did not coimmunoprecipitate with Claspin either (Figure 1 and data not shown).

Claspin Is Degraded in G2 during Recovery from the DNA Replication Checkpoint, but It Rapidly Reaccumulates in Response to DNA Damage

Before investigating a possible role of βTrCP in the degradation of Claspin, we sought to find the physiological conditions required to degrade Claspin in the cellular context. Claspin is an E2F target gene (Balciunaite et al., 2005; Iwanaga et al., 2005), and accordingly, its expression has been reported to increase during S phase (Chini and Chen, 2003). To study the expression of Claspin during the cell cycle, we conducted synchronization experiments in human cells. U2OS cells arrested in prometaphase were collected by mitotic shake off and

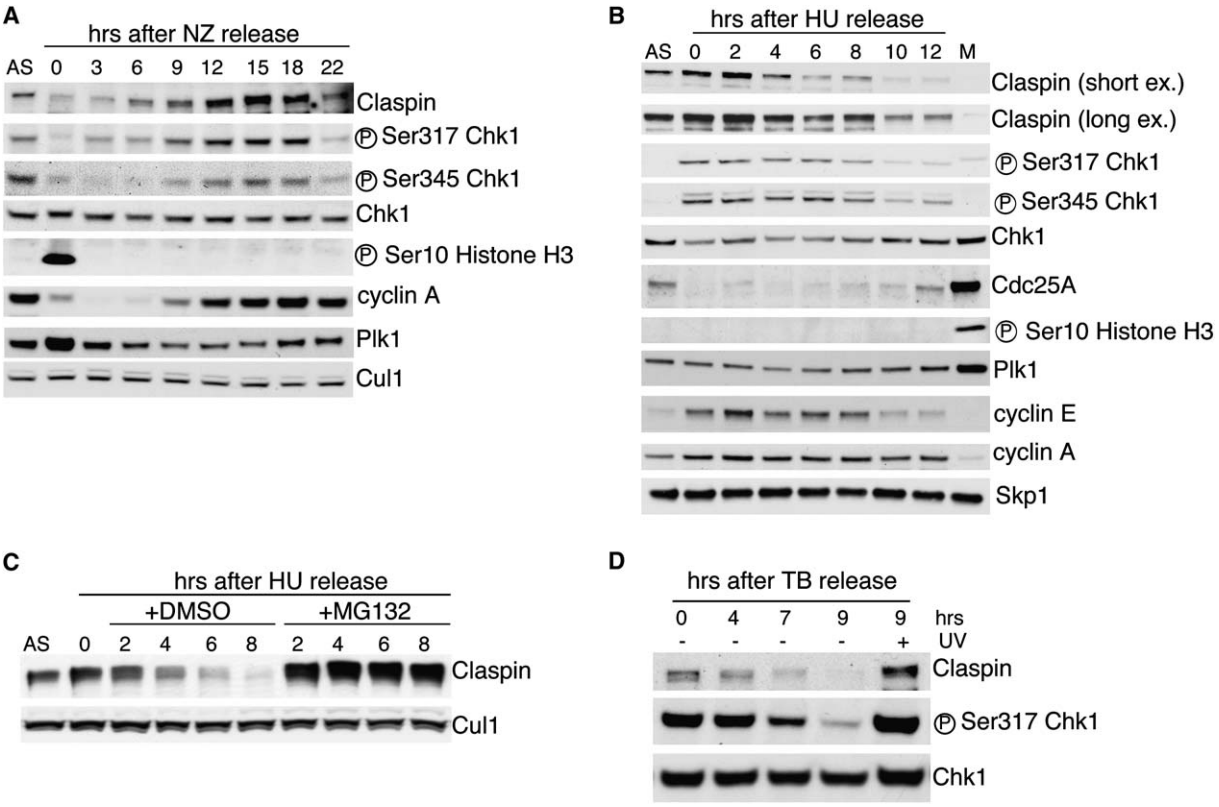

Figure 2. Claspin Is Degraded in G2, during Recovery from the DNA Replication Stress Response, and It Is Rapidly Stabilized in G2 by DNA Damage

(A) U20S cells were released from nocodazole (NZ)-induced prometaphase arrest (indicated as time 0) and collected at the indicated times. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. (AS, asynchronous cells.)

(B) U20S cells were synchronized at G1/S with hydroxyurea (HU) for 20 hr (indicated as time 0). Cells were subsequently washed and allowed to progress through the cell cycle for the indicated times. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. The last lane represents protein extract from NZ-induced prometaphase cells (indicated as M). (AS, asynchronous cells.) Note that due to the higher degree of DNA replication stress induced by HU, Chk1 was strongly phosphorylated and, therefore, blots were exposed less time than those in (A).

(C) The experiment was performed as in (B) except that after release from HU, either DMSO or MG132 was added, as indicated.

(D) U20S cells were synchronized at G1/S by using a thymidine block (TB). Cells were subsequently washed and allowed to progress through the cell cycle for the indicated times. The last lane represents protein extract from cells released for 7.5 hr from the block, treated with UV (50 J/m^2), and then left in culture for an additional 90 min. Protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins.

then allowed to progress through mitosis and into the next cell cycle. The levels of Claspin were found to be low in mitosis and G1, accumulated during S phase (marked by high expression of cyclin A), and decreased again between 18 and 22 hr after release from prometaphase (Figure 2A). At this time, the levels of cyclin A were still elevated and histone H3 was not phosphorylated on Ser10, indicating that the cells had not yet approached mitosis. Claspin is a mediator of ATR-mediated phosphorylation (and consequent activation) of Chk1. As expected, the activation of Chk1, examined with two antibodies that specifically recognize Chk1 phosphorylated on Ser317 and Ser345, respectively, paralleled the levels of Claspin.

To pinpoint the time when Claspin levels decrease during the cell cycle, U2OS cells were synchronized at G1/S with HU and then released into drug-free medium to allow synchronous progression toward mitosis. The levels of Claspin were high in S and started to steadily decrease 4 hr after release from the HU block, paralleling the decrease in Chk1 phosphorylation and inversely correlating with the accumulation of Cdc25A (Figure 2B). Treatment of the cells with the proteasome inhibitor MG132 prevented the disappearance of Claspin observed after the release from the HU block (Figure 2C).

A similar pattern of Claspin expression was observed by using different G1/S synchronization methods (i.e., aphidicolin or thymidine block) (Figure 2D and data not shown) and in different cell types, including HeLa, T98G, IMR-90 (nontransformed, nonimmortalized diploid fibroblasts), and SV40 Large T-immortalized IMR-90 cells (Figure S1). In addition, the half-life of Claspin was found to be longer in S compared to G2 phase (see below, Figure 3C).

Thus, although E2F-dependent transcription plays a major role in the accumulation of Claspin at G1/S, degradation controls the decrease in Claspin levels during G2. Strikingly, Claspin rapidly reaccumulates in G2 cells subjected to DNA damage with UV (Figure 2D), ionizing radiation, or doxorubicin (data not shown).

We concluded that Claspin is degraded by the proteasome in G2 when cells recover from DNA replication stress. However, the inhibition of this mechanism by DNA-damaging agents induces the rapid accumulation

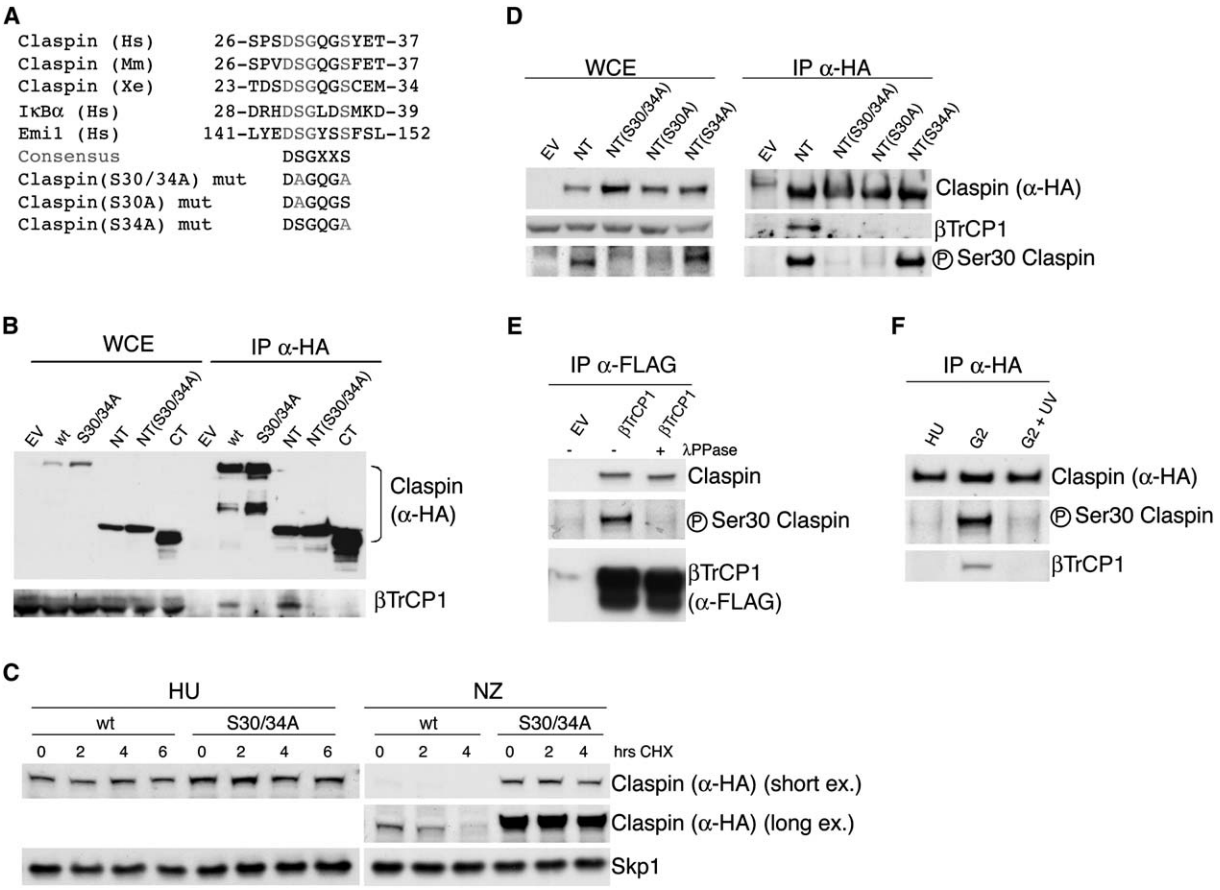

Figure 3. The DSGxxS Phosphodegron of Claspin Is Required for Binding to βTrCP and Is Phosphorylated In Vivo

(A) Alignment of the amino acid regions corresponding to the putative βTrCP binding motif in Claspin orthologs and DSGxxS motifs in previously reported βTrCP substrates.

(B) βTrCP binding to Claspin depends on the DSGxxS motif present in Claspin. HEK293T cells were transfected, as indicated, with an empty vector (EV), HA-tagged wild-type Claspin (wt), HA-tagged Claspin(S30/34A) mutant, HA-tagged N-terminal deletion mutant comprising amino acids 1–680 (NT), HA-tagged NT(S30/34A) mutant, or HA-tagged C-terminal deletion mutant encompassing amino acids 681–1332 (CT). Whole-cell extracts (WCEs) were either subjected directly to immunoblotting to analyze the expression levels of wt and mutant Claspin proteins or to immunoprecipitation (IP) with an anti-HA resin followed by immunoblotting with antibodies to the indicated proteins.

(C) Claspin(S30/34A) is stable in mitosis. U2OS cells were infected with a retrovirus expressing either HA-tagged wt Claspin or HA-tagged Claspin(S30/34A). Left panels, cells were synchronized at G1/S wit HU for 20 hr and then incubated with cycloheximide (CHX) for the indicated times. Right panels, after treatment with nocodazole (NZ) for 18 hr, prometaphase cells were collected by mitotic shake off and incubated with CHX for the indicated times in the presence of NZ. Round cells were then collected and analyzed by immunoblotting.

(D) Claspin is phosphorylated on Ser30 in vivo. The experiment was performed as in (B) except that single-point mutants (Claspin NT[S30A] and NT[S34A]) were also expressed and an anti phospho-Ser30-specific antibody to Claspin was used in the immunoblots.

(E) Claspin bound to βTrCP1 is phosphorylated on Ser30. The experiment was performed as in Figure 1 except that the sample in lane 3 was treated with λ-phosphatase (λPPase) prior to SDS-PAGE.

(F) Stabilized Claspin is not phosphorylated on Ser30 and does not associate with βTrCP1. U2OS cells were retrovirally infected with HA-tagged wt Claspin and then treated with HU for 18 hr. The protein extracts shown in the last two lanes were from cells released from the HU block for a 10 hr period. The protein extract shown in the last lane was from cells furthermore treated with UV (50 J/m^2) 90 min prior to harvesting. In all cases, cells were treated with the proteasome inhibitor MG132 for 3 hr prior to harvesting and lysis. Cell extracts were then subjected to immunoprecipitation with an anti-HA resin followed by immunoblotting with antibodies to the indicated proteins.

of Claspin and possibly provides a means to promptly reestablish the checkpoint in G2.

The Binding of Claspin to βTrCP Requires Phosphorylation in the DSGxxS Degron

Claspin contains a conserved canonical DSGxxS degron (Figure 3A), typical of βTrCP substrates (Cardozo and Pagano, 2004). All substrates investigated so far require the phosphorylation of the two serine residues present in this motif to allow recognition by βTrCP. We generated a number of Claspin deletion mutants as well as mutants in which Ser30 and/or Ser34 was mutated to alanine. After expression of these proteins (all HA tagged) in HEK293T cells, we immunoprecipitated them with an anti-HA resin. Whereas wild-type Claspin and an N-terminal deletion mutant encompassing the first 680 amino acids (NT) coimmunoprecipitated with endogenous βTrCP1, a C-terminal deletion mutant encompassing amino acids 681–1332 of Claspin (CT), Claspin(S30/34A), NT-Claspin(S30A), NT-Claspin(S34A), and NT-Claspin(S30/34A) were unable to do so (Figures 3B and 3D). This result shows that the presence of both serines in the degron of Claspin is necessary for stable interaction with βTrCP. Significantly, all mutants unable

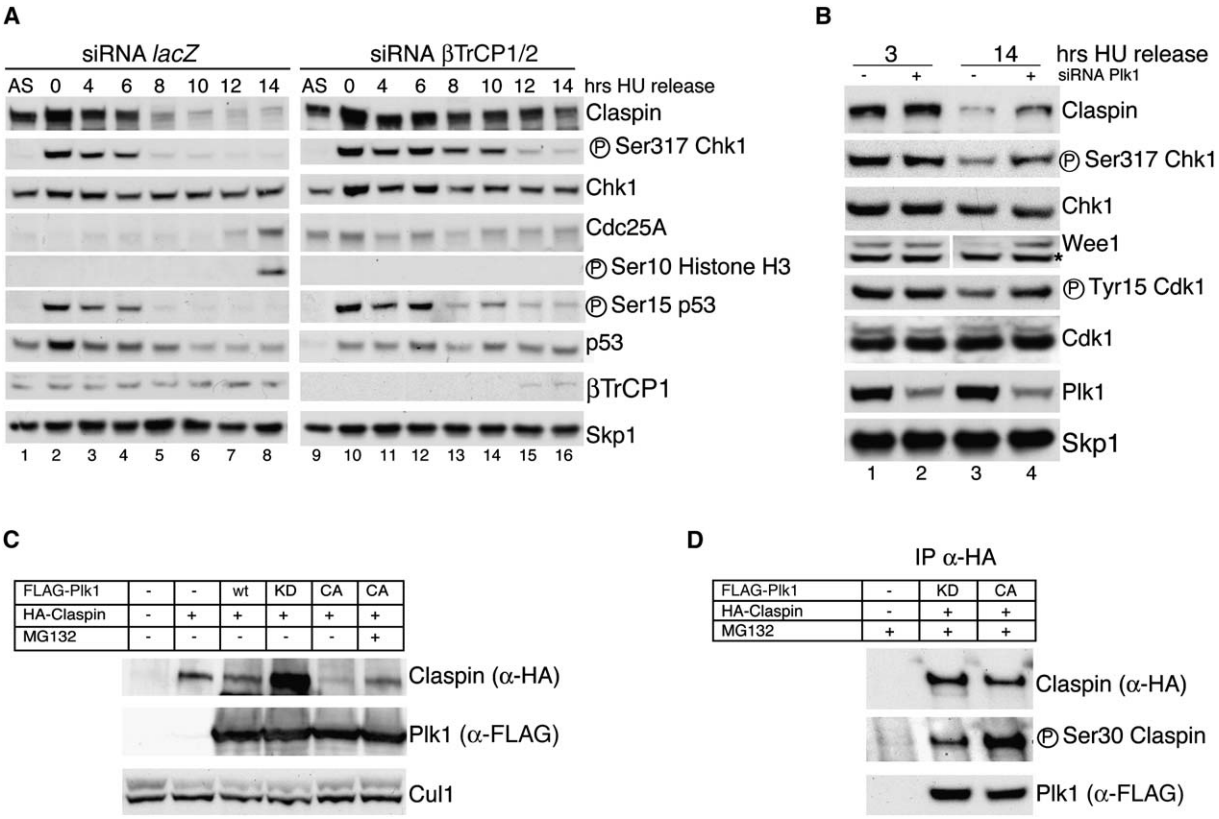

Figure 4. βTrCP and Plk1 Control the Degradation of Claspin In Vivo

(A) U2OS cells were transfected twice with siRNA molecules to a nonrelevant mRNA (*lacZ*) or to both βTrCP1 and βTrCP2 mRNAs. Cells were then treated with HU for 20 hr (lanes 2–8 and 10–16). After this period, HU was removed for the indicated hours. Lanes 1 and 8 show extracts from asynchronously growing cells (AS). Protein extracts were probed with antibodies to the indicated proteins.

(B) The experiment was performed as in (A) except that a dsRNA oligo to Plk1 (lanes 2 and 4) was compared to an oligo targeting a nonrelevant mRNA (lanes 1 and 3). The asterisk indicates a nonspecific band recognized by the anti-Wee1 antibody.

(C) HEK293T cells were cotransfected with HA-tagged Claspin and either wild-type (wt), kinase-defective K82R (KD), or constitutively active T210D (CA) FLAG-tagged Plk1. Where indicated, cells were treated with the proteasome inhibitor MG132 for 2 hr prior to harvesting and lysis. Cell extracts were then subjected to immunoblotting with antibodies to the indicated proteins.

(D) The experiment was performed as in (C) except that MG132 was added in all cases. Cell extracts were subjected to immunoprecipitation (IP) with an anti-HA resin followed by immunoblotting with antibodies to the indicated proteins.

to bind βTrCP were consistently expressed at higher levels than wild-type Claspin or NT-Claspin (see whole-cell extracts [WCE] in Figures 3B and 3D), indicating that mutations in the degron stabilize Claspin. Indeed, during mitosis the half-life of Claspin(S30/34A) was found to be significantly longer than that of wild-type Claspin (Figure 3C). In contrast at G1/S, when Claspin needs to accumulate, both wild-type Claspin and Claspin(S30/34A) were similarly stable.

To investigate whether Ser30 is phosphorylated in vivo, we generated a phospho-specific antibody against the ^{25}DSPSDS*GQGSYET^{37} peptide with a phosphoserine at position 30 (S*). This antibody recognized endogenous Claspin (Figure 3E) as well as exogenous NT-Claspin, but not NT-Claspin(S30A) and NT-Claspin(S30/34A) (Figure 3D). Treatment of endogenous Claspin coimmunoprecipitated with βTrCP with λ-phosphatase accelerated Claspin's migration in SDS-PAGE and abolished its recognition by the phospho-Ser30 specific antibody (Figure 3E). Significantly, stabilized Claspin (in cells treated with HU or UV) was not phosphorylated on Ser30 and did not associate with βTrCP1 (Figure 3F). These results, together with the observation that the NT-Claspin(S30A) mutant does not associate with βTrCP (Figures 3B and 3D), show that Ser30 is phosphorylated in vivo and that this event is necessary for the binding of Claspin to βTrCP. Importantly, phosphorylation of Claspin is inhibited during an HU block or in response to DNA damage, when Claspin needs to accumulate.

βTrCP and Plk1 Control Claspin Stability In Vivo

The results presented above prompted us to test whether βTrCP is involved in regulating the stability of Claspin. To this end, we employed the small interfering RNA (siRNA) technique to reduce the expression of βTrCP in U2OS cells by using a previously validated double-stranded RNA (dsRNA) oligo that efficiently targets both βTrCP1 and βTrCP2 (Fong and Sun, 2002; Guardavaccaro et al., 2003; Jin et al., 2003; Margottin-Goguet et al., 2003). We synchronized U2OS cells at G1/S and induced chronic DNA replication stress by using HU treatment for 20 hr. As previously shown, levels of Claspin increased and Chk1 was activated (as shown by its phosphorylation on Ser317) under these conditions (Figure 4A). After removal of HU, levels of Claspin steadily

decreased in parallel with the inactivation of Chk1. Downregulation of βTrCP strongly inhibited the degradation of Claspin after release from the HU block (Figure 4A, compare lanes 4–8 and 12–16) and concomitantly induced a prolonged activation of Chk1 (as shown by extended phosphorylation of both Chk1 and its target, p53). The fact that the depletion of βTrCP does not stabilize Claspin in untreated cells or cells arrested at G1/S (Figure 4A, lanes 1 and 2 versus 9 and 10) demonstrates that βTrCP promotes the degradation of Claspin only in cells recovering from the DNA replication checkpoint response.

The serine residues in the DSGxxS motif of Emi1 and the DSAxxE motif of Wee1 are targeted by Plk1 (to create a βTrCP binding site) (Hansen et al., 2004; Moshe et al., 2004). We hypothesized that a similar scenario may hold true for Claspin. To test this prediction, we investigated the effect of silencing Plk1 in cultured cells. Two different dsRNA oligos validated in previous publications (Eldridge et al., 2006; Moshe et al., 2004) inhibited the degradation of Claspin (Figure 4B and data not shown). The effect was only observed in G2, but not in S phase, highlighting the timing of Plk1 action on Claspin.

We also examined the effects of ectopic Plk1 expression on Claspin stability in HEK293 cells (Figure 4C). Compared to the control, wild-type Plk1 slightly reduced Claspin levels, an effect that was enhanced by using a constitutively active (CA) Plk1 mutant (T210D). This reduction was prevented by the addition of the proteasome inhibitor MG132 (for 2 hr prior to collecting the cells), demonstrating that Plk1(T210D) indeed induces the degradation of Claspin. Finally, a kinase-dead (KD) Plk1 mutant (K82R) robustly increased Claspin levels. Significantly, the expression of Plk1 KD decreased the phosphorylation of Claspin on Ser30 (Figure 4D). The residual phosphorylation of Claspin in cells cotransfected with Plk1 KD could be due to the failure to coexpress Claspin and Plk1 KD in a subpopulation of transfected cells.

Taken together, these results strongly indicate that Plk1-mediated phosphorylation generates the phosphodegron in Claspin necessary for the recruitment of βTrCP.

Ubiquitylation of Claspin Requires βTrCP, Plk1, and an Intact DSGxxS Degron

To test whether Claspin is ubiquitylated via the $SCF^{\beta TrCP}$ ubiquitin ligase, we reconstituted the ubiquitylation of Claspin in vitro. In vitro-transcribed/translated wild-type Claspin, but not the Claspin(S30/34A) mutant, was efficiently ubiquitylated and degraded only when both βTrCP1 and Plk1 were present in the reaction mix (Figure 5A). A different F box protein, Fbw5, or a different kinase, Cdk1, was unable to trigger the ubiquitylation and degradation of Claspin. A short exposure of the autoradiogram showed that the addition of Plk1 to the reaction mix induced an upshift and the appearance of a double band, probably as a result of Claspin phosphorylation by Plk1. This doublet was less evident in Claspin(S30/34A), likely due to the absence of Ser30 and Ser34. In addition, the effect of Plk1 on Claspin ubiquitylation was found to be dose and time dependent (Figures 5B and 5C). Addition of Cdk1-cyclin B or Cdk2-cyclin A had no effect on the Plk1-dependent ubiquitylation of Claspin at any of the Plk1 concentrations tested in Figure 5B (data not shown). When methylated ubiquitin (which is chemically modified to block all of its free amino groups) was added to the in vitro reactions, the highest molecular weight forms of Claspin almost completely disappeared, while faster migrating bands appeared (Figure 5C). The fact that methylated ubiquitin changes the pattern of bands confirms that the high molecular weight forms of Claspin are indeed Claspin polyubiquitylated species.

Thus, in agreement with our findings in vivo, the in vitro results show that βTrCP and Plk1 promote the ubiquitylation and consequent degradation of Claspin in a DSGxxS-dependent manner.

Claspin Degradation Is Necessary for Efficient Recovery from the DNA Replication Checkpoint Response and Entry into Mitosis

The experiments shown in Figure 4 strongly support a model in which βTrCP and Plk1 direct the proteolysis of Claspin in G2, at a time when cells recover from the DNA replication stress response. To study the biological significance of Claspin proteolysis, we retrovirally infected U2OS cells with HA-tagged wild-type Claspin and HA-tagged Claspin(S30/34A). Cells were then blocked with HU at G1/S, released, and collected at the indicated times. Significantly, expression of the stable Claspin mutant unable to bind βTrCP induced slower kinetics of (1) Chk1 inactivation, (2) Cdc25A accumulation, (3) Wee1 degradation, (4) dephosphorylation of phospho-Tyr15 in Cdk1, (5) dephosphorylation of phospho-Ser15 in p53, (6) p53 degradation, and (7) phosphorylation of histone H3 on Ser10 (Figure 6A). All these results show that Claspin(S30/34A) attenuates the recovery from the DNA replication stress response and strongly delays entry into mitosis. The mitotic phenotype was also quantified counting the percentage of U2OS cells positive for histone H3 phosphorylated on Ser10 (Figure 6B).

These experiments demonstrate that the degradation of Claspin in G2 allows the timely and efficient termination of the DNA replication checkpoint, which is necessary for progressing into mitosis.

Discussion

The levels of Claspin increase in response to replicative stress and DNA damage that often arise during DNA replication. This accumulation is a prerequisite for Claspin to mediate the phosphorylation and consequent activation of Chk1 by ATR (Chini and Chen, 2003; Kumagai and Dunphy, 2000). Depletion of Claspin in mammalian cells decreases Chk1 activation in response to HU and consequently induces the stabilization of Cdc25A (Chini and Chen, 2003; Sorensen et al., 2004). In the present report, we show that during recovery from the DNA replication checkpoint response Claspin is phosphorylated by Plk1, which generates a phosphodegron for the recruitment of the $SCF^{\beta TrCP}$ ubiquitin ligase. This event triggers the ubiquitylation and degradation of Claspin. Our findings demonstrate that βTrCP- and Plk1-mediated degradation of Claspin allows the efficient and timely termination of the DNA replication checkpoint.

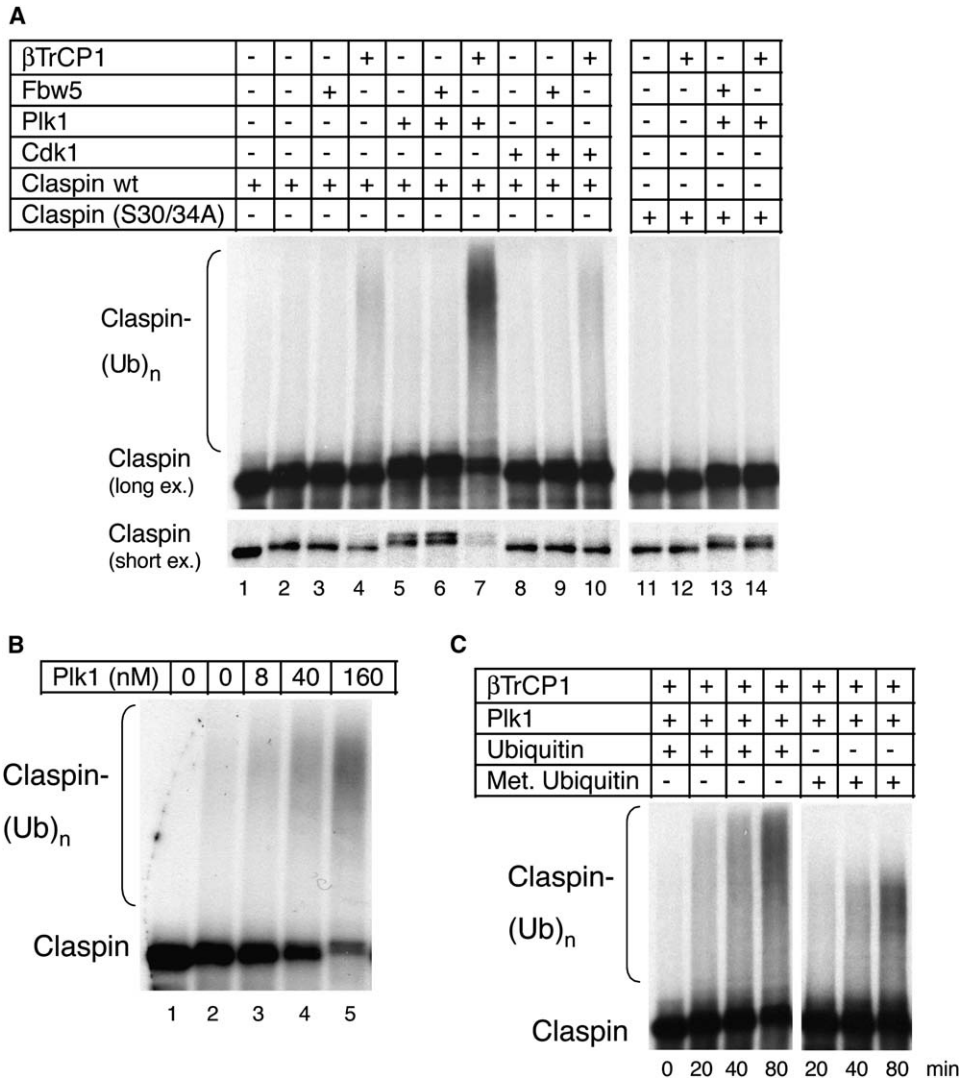

Figure 5. βTrCP, Plk1, and the DSGxxS Phosphodegron Are Required for the In Vitro Ubiquitylation of Claspin

(A) In vitro ubiquitin ligation assay of ^{35}S labeled in vitro-transcribed/translated Claspin (NT or [NTS30/34A]) was conducted in the presence or absence of the following proteins (used in different combinations as indicated): cold in vitro-transcribed/translated βTrCP, cold in vitro-transcribed/translated Fbw5, purified recombinant Plk1, or purified recombinant Cdk1-cyclin B complex. Samples were incubated at 30°C for 90 min except that in lane 1 that was immediately added to sample buffer. The lower autoradiography images represent a short exposure time, and the upper ones represent a long exposure time. The bracket on the left side of the top panels marks a ladder of bands corresponding to polyubiquitylated Claspin.

(B) The experiment was performed as in (A) except that increasing amounts of Plk1 were added to the reaction mix, as indicated. Samples were incubated at 30°C for 90 min except the one in lane 1, which was immediately added to sample buffer.

(C) The experiment was performed as in (A) except that, where indicated, methylated ubiquitin, rather than ubiquitin, was added to the reaction mix. Samples were incubated at 30°C for the indicated times.

A fascinating aspect of the model arising from recent work is the central role of βTrCP in modulating Cdk1 activity during the cell cycle (Figures 7A and 7B). During S phase, βTrCP directs the degradation of Cdc25A, preventing premature hyperactivation of Cdk1 and entry into mitosis prior to the completion of DNA synthesis (Busino et al., 2003; Jin et al., 2003). During recovery from the DNA replication checkpoint response in G2, βTrCP targets Claspin for degradation, allowing the effective inactivation of Chk1 and the consequent accumulation of Cdc25A (shown in this report). The latter event triggers the activation of Cdk1, which induces the βTrCP-mediated elimination of the antagonist of Cdc25A, Wee1 (Watanabe et al., 2004). This autoamplification loop ends with the full activation of Cdk1, which is now able to execute mitotic events. At the beginning of mitosis, βTrCP contributes to the activation of APC/C by directing the degradation of Emi1 (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). Significantly, this leads to the APC/C-mediated degradation of mitotic cyclins and, thus, the inactivation of Cdk1, resetting the cell cycle clock to low CDK activity for the next G1 phase (Guardavaccaro and Pagano, 2006).

It is evident that Plk1 is another key player in this pathway. The data presented herein suggest that during S phase low levels and low activity of Plk1 contribute to

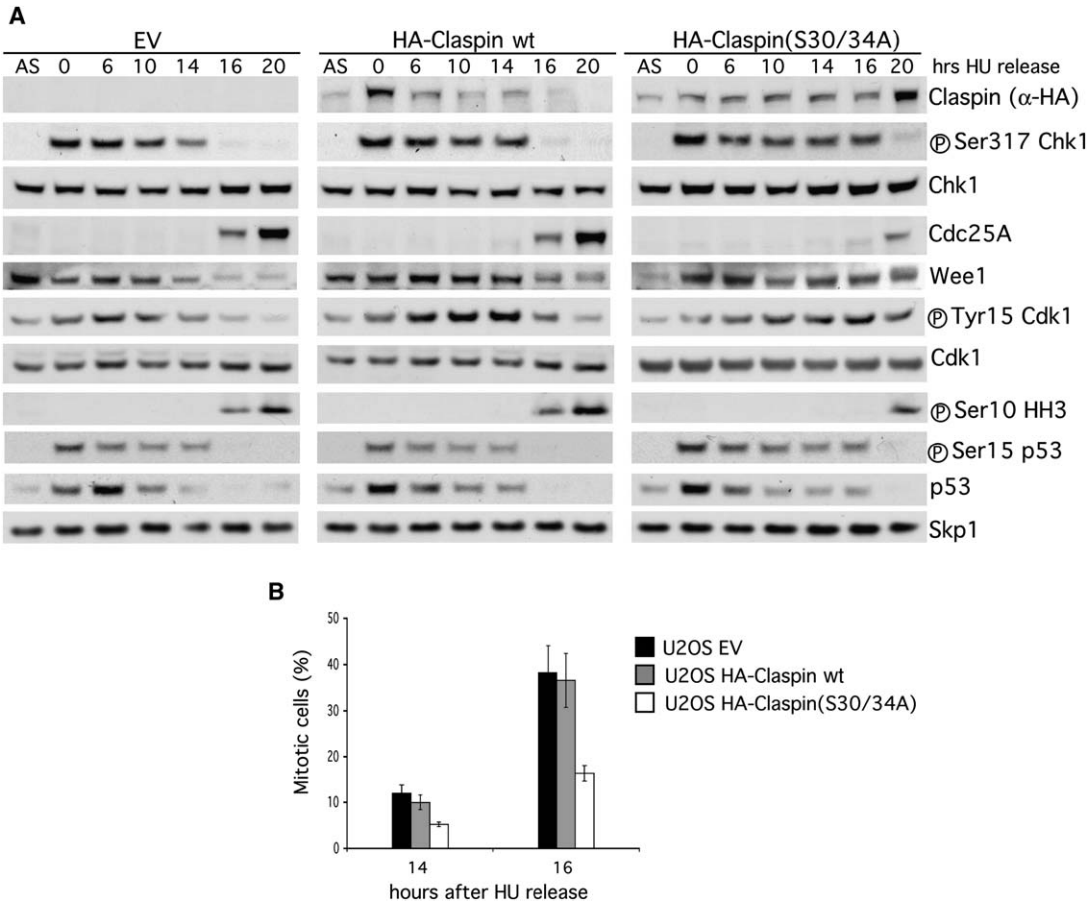

Figure 6. Claspin Degradation Is Necessary for Efficient Inactivation of Chk1 and Recovery from the DNA Replication Checkpoint

(A) U2OS cells were either infected with an empty vector (EV) or infected with a retrovirus expressing HA-tagged wild-type Claspin or HA-tagged Claspin(S30/34A). After treatment with HU for 20 hr, G1/S cells were replated in drug-free medium for the indicated times. Cells were harvested and analyzed by immunoblotting. (HH3, histone H3.)

(B) The percentage of phospho-Ser10 histone H3-positive mitotic cells was determined by immunofluorescence analysis in three different retroviral infections each performed as in (A). Error bars represent the standard deviations of three experiments.

the accumulation of Claspin, which mediates the activation of Chk1 by ATR. In turn, Chk1 sustains the stability of Claspin (Chini et al., 2006), possibly by maintaining the checkpoint. Moreover, active Chk1 inhibits Cdk1 by inducing the βTrCP-mediated degradation of Cdc25A (Busino et al., 2003; Jin et al., 2003) and the activation of Wee1 (Lee et al., 2001) (Figure 7A). In G2, during the recovery from the checkpoint, Plk1 is activated and promotes the βTrCP-mediated degradation of Claspin, resulting in the cessation of the checkpoint, the accumulation of Cdc25A, and the activation of Cdk1 (Figure 7B). Successively, Cdk1 further stabilizes Cdc25A by a mechanism not yet understood (Mailand et al., 2002) and cooperates with Plk1 in directing the βTrCP-mediated degradation of Wee1 (Watanabe et al., 2004).

The Plk1-dependent degradation of Wee1 at G/M has been implicated as an important event for entry into mitosis (van Vugt et al., 2004). Our study shows that the degradation of Claspin is also induced by Plk1, but this process occurs during recovery, upstream of Wee1 degradation. The expression of a Claspin mutant unable to be degraded during G2 induces a block of Wee1 degradation (Figure 6A), strongly supporting our model.

Contrary to the view that Plk1 is exclusively a mitotic kinase, our findings are in agreement with several studies showing that Plk1 is already active during G2 (Lowery et al., 2005; van Vugt and Medema, 2005). This would explain the need for Evi5 to protect Emi1 from βTrCP- and Plk1-mediated degradation during this cell cycle phase (Eldridge et al., 2006).

During G2, Plk1 activity is inhibited in response to different DNA stresses, resulting in a G2 arrest (Smits et al., 2000; van Vugt et al., 2001). We propose that inhibition of the Plk1-mediated degradation of Claspin in G2 by DNA-damaging agents induces a transcription-independent, rapid reaccumulation of Claspin (Figure 2D and data not shown), providing a mechanism to promptly reestablish the checkpoint in G2. Having repaired the DNA damage, Claspin is degraded again via βTrCP to allow progression of cells into mitosis (our unpublished data and accompanying manuscript by J. Lukas and colleagues [Mailand et al., 2006]).

We show that Ser30, present in the DSG motif of Claspin, is phosphorylated in vivo and necessary for its

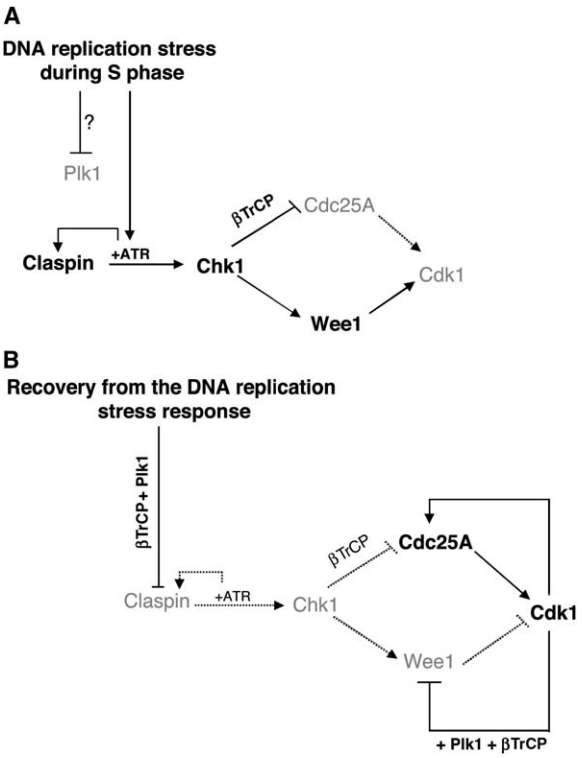

Figure 7. Model of How βTrCP and Plk1 Control the Efficient Attenuation of the DNA Replication Checkpoint Response by Promoting the Degradation of Claspin

Black color signifies activated forms of the respective proteins, and gray indicates inactive forms or degraded proteins. See text for details.

binding to and ubiquitylation via βTrCP (Figures 3–5). These two functions also require the presence of Ser34 (Figure 3D and data not shown) and, although a phosphospecific antibody to phospho-Ser34 has not been generated, it is very likely that Ser34 is also phosphorylated in vivo. This hypothesis is supported by the extensive literature on βTrCP substrates (Cardozo and Pagano, 2004) and by the structure of the Skp1-βTrCP1-β-catenin complex, demonstrating that βTrCP recognizes the doubly phosphorylated DpSGXXpS degron (Wu et al., 2003). Our results suggest that the two serine residues in the degron of Claspin are phosphorylated by Plk1.

Phosphorylation of substrates by Plk1 requires a priming kinase that phosphorylates the polo-box domain binding site (PBD-BS) in the substrate to allow docking and stable interaction with Plk1. The minimal PBD-BS contains a SerpSer/pThr sequence with a Pro in position 3 in most cases (Lowery et al., 2005). In several substrates of Plk1 (e.g., Wee1 and Emi1), the priming kinase is Cdk1. In the case of Claspin, neither Cdk1-cyclin B1 nor Cdk2-cyclin A2 (although active in promoting Emi1 ubiquitylation in the presence of Plk1) produced any effect on the Plk1-dependent in vitro ubiquitylation of Claspin (data not shown). Thus, the priming kinase of the PBD-BS in human Claspin awaits to be identified.

In addition to a role in checkpoint control (mediated by its binding to Chk1), Claspin has a second function in promoting efficient DNA replication (likely mediated by its interaction with Polε, Cdc45, RPA, etc.). Overexpression of Claspin in human cells enhances the rate of cell proliferation (Lin et al., 2004). Similarly, depletion of Claspin from *Xenopus* extracts has been shown to slow DNA replication down (Lee et al., 2003). Furthermore, the putative yeast ortholog of Claspin, Mrc1, has a well-known role in DNA replication (Alcasabas et al., 2001; Osborn and Elledge, 2003; Xu et al., 2004). The mechanism by which Claspin promotes DNA replication is, however, still subject to speculation.

Claspin has been shown to bind stably to the chromatin, and a recent study has demonstrated that this stable interaction may be related to Claspin's function in DNA synthesis (Lee et al., 2005). In contrast, stable association to the chromatin is not necessary for the activation of Chk1 by Claspin. Accordingly, Chk1 binds only transiently to chromatin. Interestingly, in *Xenopus*, the dissociation of Claspin from chromatin requires the Plk1-mediated phosphorylation on Ser934 (Yoo et al., 2004). It is possible that in *Xenopus* Plk1 induces the dissociation of Claspin from chromatin (to restrain its positive effects on DNA replication via phosphorylation on Ser934), whereas in humans Plk1 promotes the degradation of Claspin (to turn the DNA replication checkpoint off via phosphorylation on Ser30 and Ser34). The latter event is likely to be present in *Xenopus* as Ser30 and Ser34 are conserved throughout evolution (Figure 3A). In *Xenopus* egg extracts, phosphorylation of Ser934 is primed by the ATR-mediated phosphorylation of Thr906 (Yoo et al., 2004). Ser934 is conserved in humans (Ser983), whereas the residue corresponding to frog Thr906 (Thr954) has lost the phosphorylation consensus site for ATR, indicating that a different kinase(s) and/or a different mechanism controls the dissociation of Claspin from chromatin.

Often tumor cell lines display high levels of Claspin (Lin et al., 2004), probably due to the deregulation of the E2F-pRB axis (Balciunaite et al., 2005; Iwanaga et al., 2005). Accordingly, in human fibroblasts retrovirally infected to express SV40 Large T antigen, the expression of Claspin is dramatically higher than in control fibroblasts (Figure S1). Yet, when cells synchronously progress through G2, Claspin is still efficiently degraded. Our results indicate that if this was not the case, mitotic entry would be delayed. Thus, tumor cells may gain an advantage from high expression of Claspin, allowing it to execute DNA replication efficiently but maintain a very effective Claspin-degradation machinery to be able to terminate the checkpoint response. It is tempting to speculate that certain tumor cells may acquire gain of function that deregulates the DNA replication checkpoint by decreasing its effectiveness (e.g., through mutations that decrease the affinity of Claspin for Chk1 and/or ATR) or promoting the premature degradation of Claspin (e.g., through elevation of Plk1 levels, which is observed in many human neoplasms [Nakayama and Nakayama, 2006; van Vugt and Medema, 2005]).

A plethora of information is available about how the DNA replication checkpoint is activated in mammalian cells. However, apart from very few reports (Lu et al., 2005; Zhang et al., 2005), the regulation of checkpoint termination has remained largely unknown. Our findings implicate Plk1 in the generation of the phosphodegron

for the recognition of Claspin by the SCF$^{\beta TrCP}$ complex and underscore the importance of the ubiquitin system in the recovery from the DNA replication checkpoint response.

Experimental Procedures

Cell Culture, Synchronization, and Drug Treatment

U20S, HEK293T, IMR90, and SV40 Large T-expressing IMR90 cells were maintained in DME medium containing 10% FBS. Cells were synchronized at G1/S with HU (1.5 mM for 20 hr), aphidicolin (2 μg/ml for 24 hr), or thymidine (2.5 mM for 24 hr). Synchronization in prometaphase with nocodazole and mitotic shake off were performed as described (Guardavaccaro et al., 2003). Where indicated, 10 μM MG132 was added for 3–6 hr prior to harvesting the cells. To test the interaction between HA-Claspin and endogenous βTrCP (Figures 3B and 3D), G1-depleted HEK293T cells, prepared as described (Montagnoli et al., 1999), were used. To measure protein half-lives, mitotic cells were incubated in the presence of 100 μg/ml cycloheximide (Sigma) diluted in 100% ethanol.

Biochemical Methods

Extract preparation, immunoprecipitation, and immunoblotting were previously described (Bashir et al., 2004; Guardavaccaro et al., 2003). Antibodies are described in the Supplemental Experimental Procedures. Ubiquitylation and degradation assays are described in the Supplemental Experimental Procedures.

Transient Transfections and Retrovirally Mediated Gene Transfer

Plasmids are described in the Supplemental Experimental Procedures. HEK293T cells were transfected by using the calcium phosphate method as described (Bashir et al., 2004). Retroviral-mediated gene transfer was previously described (Carrano and Pagano, 2001).

Gene Silencing by Small Interfering RNA

siRNA was previously described (Bashir et al., 2004; Guardavaccaro et al., 2003). The siRNA oligonucleotide sequence for βTrCP was 5′-GUGGAAUUUGUGGAACAUCdTdT-3′ (corresponding to both nt 515–535 of human βTrCP1 and 262–282 of human βTrCP2). The siRNA oligonucleotide sequence for Plk1 was 5′-AGAUUGUGCC UAAGUCUCU-3′, corresponding to nt 245–263 of human Plk1 cDNA. A 21 nt siRNA duplex corresponding to a nonrelevant gene (*lacZ*) was used as control.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/23/3/319/DC1/.

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References

Alcasabas, A.A., Osborn, A.J., Bachant, J., Hu, F., Werler, P.J., Bousset, K., Furuya, K., Diffley, J.F., Carr, A.M., and Elledge, S.J. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat. Cell Biol. *3*, 958–965.

Ang, X., and Harper, J.W. (2005). SCF-mediated protein degradation and cell cycle control. Oncogene *24*, 2860–2870.

Balciunaite, E., Spektor, A., Lents, N.H., Cam, H., Te Riele, H., Scime, A., Rudnicki, M.A., Young, R., and Dynlacht, B.D. (2005). Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol. Cell. Biol. *25*, 8166–8178.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell *3*, 421–429.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat. Rev. Mol. Cell Biol. *5*, 792–804.

Bashir, T., and Pagano, M. (2005). Cdk1: the dominant sibling of Cdk2. Nat. Cell Biol. *7*, 779–781.

Bashir, T., Dorrello, N.V., Amador, V., Guardavaccaro, D., and Pagano, M. (2004). Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature *428*, 190–193.

Busino, L., Donzelli, M., Chiesa, M., Guardavaccaro, D., Ganoth, D., Dorrello, N.V., Hershko, A., Pagano, M., and Draetta, G.F. (2003). Degradation of Cdc25A by βTrCP during S phase and in response to DNA damage. Nature *426*, 87–91.

Cardozo, T., and Pagano, M. (2004). The SCF ubiquitin ligase: insights into a molecular machine. Nat. Rev. Mol. Cell Biol. *5*, 739–751.

Carrano, A.C., and Pagano, M. (2001). Role of the F-box protein Skp2 in adhesion-dependent cell cycle progression. J. Cell Biol. *153*, 1381–1389.

Cenciarelli, C., Chiaur, D.S., Guardavaccaro, D., Parks, W., Vidal, M., and Pagano, M. (1999). Identification of a human family of F-box proteins. Curr. Biol. *9*, 1177–1179.

Chen, M.S., Hurov, J., White, L.S., Woodford-Thomas, T., and Piwnica-Worms, H. (2001). Absence of apparent phenotype in mice lacking Cdc25C protein phosphatase. Mol. Cell. Biol. *21*, 3853–3861.

Chini, C.C., and Chen, J. (2003). Human claspin is required for replication checkpoint control. J. Biol. Chem. *278*, 30057–30062.

Chini, C.C., and Chen, J. (2004). Claspin, a regulator of Chk1 in DNA replication stress pathway. DNA Repair (Amst.) *3*, 1033–1037.

Chini, C.C., Wood, J., and Chen, J. (2006). Chk1 is required to maintain Claspin stability. Oncogene. Published online February 27, 2006. 10.1038/sj.onc.1209447.

Eldridge, A.G., Loktev, A.V., Hansen, D.V., Verschuren, E.W., Reimann, J.D., and Jackson, P.K. (2006). The Evi5 oncogene regulates cyclin accumulation by stabilizing the anaphase-promoting complex inhibitor Emi1. Cell *124*, 367–380.

Fong, A., and Sun, S.C. (2002). Genetic evidence for the essential role of beta-transducin repeat-containing protein in the inducible processing of NF-kappa B2/p100. J. Biol. Chem. *277*, 22111–22114.

Gottifredi, V., and Prives, C. (2005). The S phase checkpoint: when the crowd meets at the fork. Semin. Cell Dev. Biol. *16*, 355–368.

Guardavaccaro, D., and Pagano, M. (2006). Stabilizers and destabilizers controlling cell cycle oscillators. Mol. Cell *22*, 1–4.

Guardavaccaro, D., Kudo, Y., Boulaire, J., Barchi, M., Busino, L., Donzelli, M., Margottin-Goguet, F., Jackson, P.K., Yamasaki, L., and Pagano, M. (2003). Control of meiotic and mitotic progression by the F box protein βTrcp1 in vivo. Dev. Cell *4*, 799–812.

Hansen, D.V., Loktev, A.V., Ban, K.H., and Jackson, P.K. (2004). Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCF$^{\beta TrCP}$-dependent destruction of the APC Inhibitor Emi1. Mol. Biol. Cell *15*, 5623–5634.

Iwanaga, R., Komori, H., Ishida, S., Okamura, N., Nakayama, K., Nakayama, K.I., and Ohtani, K. (2005). Identification of novel E2F1 target genes regulated in cell cycle-dependent and independent manners. Oncogene *25*, 1786–1798.

Jin, J., Shirogane, T., Xu, L., Nalepa, G., Qin, J., Elledge, S.J., and Harper, J.W. (2003). SCFβ-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. Genes Dev. *17*, 3062–3074.

Jin, J., Cardozo, T., Lovering, R.C., Elledge, S.J., Pagano, M., and Harper, J.W. (2004). Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev. *18*, 2573–2580.

Kumagai, A., and Dunphy, W.G. (2000). Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. Mol. Cell *6*, 839–849.

Kumagai, A., and Dunphy, W.G. (2003). Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1. Nat. Cell Biol. *5*, 161–165.

Lee, J., Kumagai, A., and Dunphy, W.G. (2001). Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. Mol. Biol. Cell *12*, 551–563.

Lee, J., Kumagai, A., and Dunphy, W.G. (2003). Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. Mol. Cell *11*, 329–340.

Lee, J., Gold, D.A., Shevchenko, A., and Dunphy, W.G. (2005). Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response. Mol. Biol. Cell *16*, 5269–5282.

Lin, S.Y., Li, K., Stewart, G.S., and Elledge, S.J. (2004). Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation. Proc. Natl. Acad. Sci. USA *101*, 6484–6489.

Lincoln, A.J., Wickramasinghe, D., Stein, P., Schultz, R.M., Palko, M.E., De Miguel, M.P., Tessarollo, L., and Donovan, P.J. (2002). Cdc25B phosphatase is required for resumption of meiosis during oocyte maturation. Nat. Genet. *30*, 446–449.

Lowery, D.M., Lim, D., and Yaffe, M.B. (2005). Structure and function of Polo-like kinases. Oncogene *24*, 248–259.

Lu, X., Nannenga, B., and Donehower, L.A. (2005). PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. Genes Dev. *19*, 1162–1174.

Mailand, N., Podtelejnikov, A.V., Groth, A., Mann, M., Bartek, J., and Lukas, J. (2002). Regulation of G2/M events by Cdc25A through phosphorylation-dependent modulation of its stability. EMBO J. *21*, 5911–5920.

Mailand, N., Bekker-Jensen, S., Bartek, J., and Lukas, J. (2006). Destruction of Claspin by $SCF^{\beta\text{-}TRCP}$ restrains Chk1 activation and facilitates recovery from genotoxic stress. Mol. Cell *23*, this issue, 307–318.

Margottin-Goguet, F., Hsu, J.Y., Loktev, A., Hsieh, H.M., Reimann, J.D., and Jackson, P.K. (2003). Prophase destruction of Emi1 by the $SCF^{\beta TrCP/Slimb}$ ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev. Cell *4*, 813–826.

Melo, J., and Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. Curr. Opin. Cell Biol. *14*, 237–245.

Montagnoli, A., Fiore, F., Eytan, E., Carrano, A.C., Draetta, G., Hershko, A., and Pagano, M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. *13*, 1181–1189.

Moshe, Y., Boulaire, J., Pagano, M., and Hershko, A. (2004). Role of Polo-like kinase in the degradation of early mitotic inhibitor *1*, a regulator of the anaphase promoting complex/cyclosome. Proc. Natl. Acad. Sci. USA *101*, 7937–7942.

Murray, A.W. (2004). Recycling the cell cycle: cyclins revisited. Cell *116*, 221–234.

Nakayama, K.I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. Nat. Rev. Cancer *6*, 369–381.

Osborn, A.J., and Elledge, S.J. (2003). Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. Genes Dev. *17*, 1755–1767.

Pagano, M., and Jackson, P.K. (2004). Wagging the dogma; tissue-specific cell cycle control in the mouse embryo. Cell *118*, 535–538.

Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat. Rev. Mol. Cell Biol. *6*, 9–20.

Sar, F., Lindsey-Boltz, L.A., Subramanian, D., Croteau, D.L., Hutsell, S.Q., Griffith, J.D., and Sancar, A. (2004). Human claspin is a ring-shaped DNA-binding protein with high affinity to branched DNA structures. J. Biol. Chem. *279*, 39289–39295.

Smits, V.A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E.A., and Medema, R.H. (2000). Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat. Cell Biol. *2*, 672–676.

Sorensen, C.S., Syljuasen, R.G., Lukas, J., and Bartek, J. (2004). ATR, Claspin and the Rad9-Rad1-Hus1 complex regulate Chk1 and Cdc25A in the absence of DNA damage. Cell Cycle *3*, 941–945.

van Vugt, M.A., and Medema, R.H. (2004). Checkpoint adaptation and recovery: back with Polo after the break. Cell Cycle *3*, 1383–1386.

van Vugt, M.A., and Medema, R.H. (2005). Getting in and out of mitosis with Polo-like kinase-1. Oncogene *24*, 2844–2859.

van Vugt, M.A., Smits, V.A., Klompmaker, R., and Medema, R.H. (2001). Inhibition of Polo-like kinase-1 by DNA damage occurs in an ATM- or ATR-dependent fashion. J. Biol. Chem. *276*, 41656–41660.

van Vugt, M.A., Bras, A., and Medema, R.H. (2004). Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol. Cell *15*, 799–811.

van Vugt, M.A., Bras, A., and Medema, R.H. (2005). Restarting the cell cycle when the checkpoint comes to a halt. Cancer Res. *65*, 7037–7040.

Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Hunter, T., and Osada, H. (2004). M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by $SCF^{\beta TrCP}$. Proc. Natl. Acad. Sci. USA *101*, 4419–4424.

Winston, J.T., Koepp, D.M., Zhu, C., Elledge, S.J., and Harper, J.W. (1999). A Family of mammalian F-box proteins. Curr. Biol. *9*, 1180–1182.

Wu, G., Xu, G., Schulman, B.A., Jeffrey, P.D., Harper, J.W., and Pavletich, N.P. (2003). Structure of a βTrCP-Skp1-β-catenin complex: destruction motif binding and lysine specificity of the $SCF^{\beta TrCP}$ ubiquitin ligase. Mol. Cell *11*, 1445–1456.

Xu, H., Boone, C., and Klein, H.L. (2004). Mrc1 is required for sister chromatid cohesion to aid in recombination repair of spontaneous damage. Mol. Cell. Biol. *24*, 7082–7090.

Yoo, H.Y., Kumagai, A., Shevchenko, A., and 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.

Zhang, Y.W., Otterness, D.M., Chiang, G.G., Xie, W., Liu, Y.C., Mercurio, F., and Abraham, R.T. (2005). Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway. Mol. Cell *19*, 607–618.