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Report

Polo-like Kinase-1 Controls Proteasome-Dependent Degradation of Claspin during Checkpoint Recovery

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Summary

DNA-damage checkpoints maintain genomic integrity by mediating a cell-cycle delay in response to genotoxic stress or stalled replication forks. In response to damage, the checkpoint kinase ATR phosphorylates and activates its effector kinase Chk1 in a process that critically depends on Claspin [1]. However, it is not known how exactly this kinase cascade is silenced. Here we demonstrate that the abundance of Claspin is regulated through proteasomal degradation. In response to DNA damage, Claspin is transiently stabilized, and its expression depends on Chk1 kinase activity. In addition, we show that Claspin is degraded upon mitotic entry, a process that depends on the β-TrCP-SCF ubiquitin ligase and Polo-like kinase-1 (Plk1). We demonstrate that Claspin interacts with both β-TrCP and Plk1 and that inactivation of these components or the β-TrCP recognition motif in Claspin prevents its mitotic degradation. Interestingly, expression of a nondegradable Claspin mutant inhibits recovery from a DNA-damage-induced checkpoint arrest. Thus, we conclude that Claspin levels are tightly regulated, both during unperturbed cell cycles and after DNA damage. Moreover, our data demonstrate that the degradation of Claspin at the onset of mitosis is an essential step for the recovery of a cell from a DNA-damage-induced cell-cycle arrest.

Results and Discussion

Claspin Turnover Is Regulated during the Cell Cycle and in Response to DNA Damage

Activation of Chk1 by the DNA-damage-responsive checkpoint kinase ATR critically depends on the presence of Claspin, which acts as an adaptor protein to link Chk1 and ATR [1, 2]. Interestingly, Claspin levels were shown to oscillate during the cell cycle, suggesting that the checkpoint response is cell-cycle dependent [2]. To investigate this dependence, we analyzed Claspin levels at different stages during the cell cycle (Figure 1A). Claspin levels are high during S and early G2 phase, whereas Claspin levels sharply decrease as cells accumulate in the G2 and M phases (Figure 1A). Indeed, we could show by immunofluorescence that Claspin is virtually absent in mitotic cells (Figure 1B). We further confirmed downregulation of Claspin in mitotic cells by using the microtubule-destabilizing drug nocodazole, which blocks cell-cycle progression in prometaphase of mitosis (Figure 1C). Moreover, Claspin levels rapidly increased upon release from the nocodazole block, at time points when cells progressively entered G1 and S phase (Figure S1A in the Supplemental Data available online). Finally, cells arrested in G2 after treatment with the DNA-damaging agent camptothecin displayed high levels of Claspin (Figure S1B). These results indicate that Claspin is stabilized in cells that are prevented from entering mitosis and suggest that Claspin is degraded at mitotic onset, possibly precluding further activation of Chk1 by ATR.

We also analyzed the levels of Claspin after different genotoxic insults. We observed an increase in Claspin levels shortly after UV treatment and a decrease at later time points (Figure 1D). These fluctuations in Claspin levels were also observed with other types of DNA-damaging agents, including hydroxyurea (causing stalled replication forks), etoposide, and camptothecin (the last two causing both single- and double-strand DNA breaks) (Figure 1D and data not shown). Thus, in addition to the observed decrease of Claspin at mitotic entry, we also found upregulation of Claspin levels in response to genotoxic stress.

The rapid increase in Claspin levels after DNA damage suggested that this could not be due to transcriptional regulation, and we tested whether altered proteasomal degradation is involved. Indeed, treating U2OS cells with the proteasome inhibitor MG132 resulted in an increase of Claspin levels, up to the maximal level of expression that is seen in response to DNA damage (Figures 2A and 2B). Interestingly, addition of MG132 to damaged cells could not elicit a further increase in Claspin expression (Figure 2B) but did prevent the

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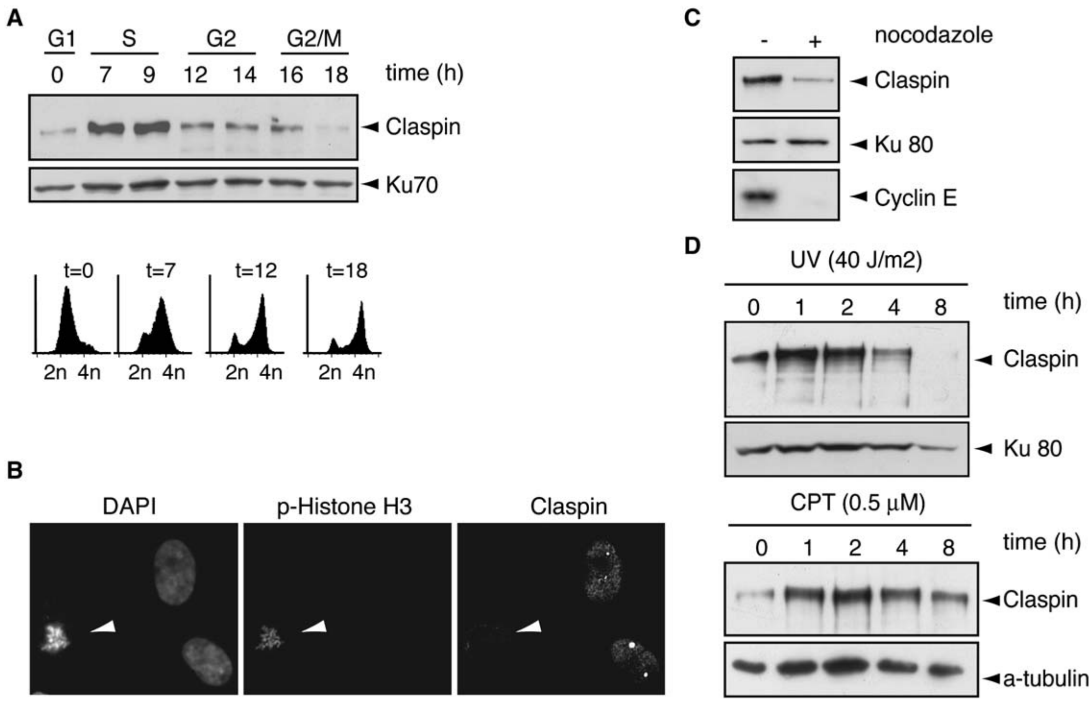

Figure 1. Claspin Regulation during the Cell Cycle and after DNA Damage

(A) U2OS cells were synchronized at the G1/S transition by double thymidine block, and cells were collected at different times after release for flow cytometry and western blot analysis.

(B) U2OS cells were analyzed by immunofluorescence using DAPI, anti-Claspin and anti-phospho-HistoneH3 as a mitotic marker. Arrowhead points at a mitotic cell.

(C) U2OS cells were left untreated or arrested with nocodazole for 18h and whole cell extracts (WCE) were prepared for analysis of Claspin expression. Cyclin E was used as an S-phase marker.

(D) U2OS cells were treated with UV light (40 J/m^2) or with 0.5 μM camptothecin (CPT) for 1 hr. Cells were collected at the indicated time points for analysis of Claspin expression.

reduction normally seen in Claspin levels at the later stages of the damage response (data not shown). Moreover, we found that the reduction seen for Claspin expression in mitotic cells was blocked by MG132, indicating that this reduction is due to enhanced processing of Claspin by the proteasome (Figure 2C). Altogether, these data demonstrate that Claspin is continuously turned over by the proteasome in undamaged cells, that it becomes transiently stabilized in response to DNA damage, and that its turnover is even further enhanced at mitotic entry. Consistent with this, we could detect a smear of higher molecular weight than Claspin in cells cotransfected with HA-tagged Claspin and His-tagged ubiquitin, indicating that Claspin is indeed poly-ubiquitinated (Figure 2D). Furthermore, we could demonstrate poly-ubiquitination of endogenous Claspin in cells treated with proteasome inhibitors (Figure 2E). Notably, Claspin ubiquitination was significantly enhanced in mitotic cells (Figure S2), whereas overall levels of HA-Claspin were considerably lower in mitotic cells than in the asynchronous cultures, consistent with the notion that Claspin turnover is increased upon entry into mitosis.

Chk1 and Polo-like Kinase-1 Exert antagonistic effects on Claspin turnover

Several examples of regulated proteasomal degradation by means of substrate phosphorylation exist in the literature, and because both Chk1 and Plx1 are known to interact with Claspin [1, 3], we tested whether Chk1 and Polo-like kinase-1 (Plk1) can affect Claspin turnover. Indeed, whereas expression of wild-type Chk1 did not destabilize Claspin, overexpression of a kinase-dead version of Chk1 resulted in destabilization of Claspin protein levels (Figure 3A). Consistent with these observations, downregulation of Chk1 by siRNA also resulted in a decrease in Claspin levels (Figure 3B). These data confirm recent findings by Chini et al. [4] but furthermore show that this Chk1-mediated stabilization of Claspin requires Chk1 kinase activity.

We next set out to determine whether Plk1, the human Plx1 homolog, was also able to modulate Claspin levels. Plk1 is expressed in G2 and mitosis but only becomes activated as cells enter mitosis, which is also when Claspin levels drop. Indeed, we could show that overexpression of Plk1 resulted in a downregulation of Claspin (Figure 3C). This effect depends on the catalytic activity of Plk1; expression of a kinase-defective mutant of Plk1 could not cause downregulation of Claspin (Figure 3C). In addition, by using GST fusions comprising different regions of Claspin [2], we were able to show that Plk1 can interact with the C-terminal half of Claspin (Figure S3A), consistent with findings of Yoo et al. in *Xenopus* [3]. We were able to confirm this interaction further by coimmunoprecipitation of Myc-tagged kinase-dead Plk1 with endogenous Claspin in vivo and vice

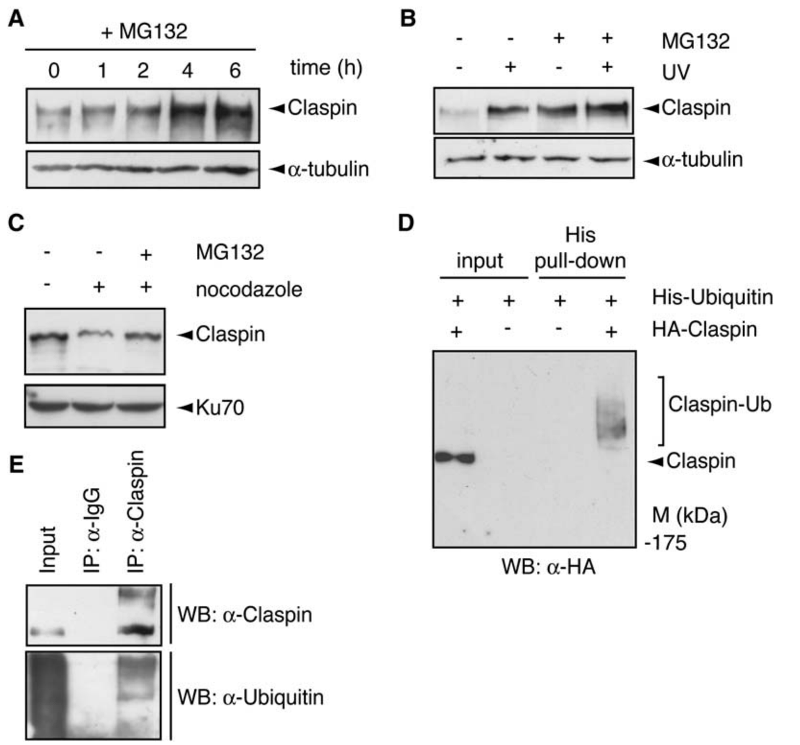

Figure 2. Claspin Protein Levels Are Regulated via Proteasome-Dependent Degradation

(A) U2OS cells were treated with MG132 for the indicated times before Western-blot analysis with the indicated antibodies.

(B) U2OS cells were incubated with MG132 for 3 hr before treatment with UV light (40 J/m^2). Cells were collected 1 hr after UV treatment for analysis of Claspin expression.

(C) Claspin expression in U2OS cells treated with nocodazole for 8 hr in the presence or absence of MG132. Untreated cells were taken along as a control.

(D) HEK 293T cells were transfected with indicated plasmids. After 36 hr, cells were lysed and extracts were incubated with Ni-NTA beads. After washing, the resin was analyzed for Claspin ubiquitination.

(E) U2OS cells were incubated for 3 hr in the presence of MG132 before lysis. Extracts were used for immunoprecipitations with Claspin or control IgG antibodies. Analysis was done by Western blotting with antibodies against Claspin and ubiquitin.

versa (Figure 3D and data not shown). This interaction of Plk1 with Claspin is mediated through the conserved Polo-box domain (PBD), as demonstrated by pull-down experiments with a GST fusion of the PBD of Plk1 (Figure 3E).

Using RNA-interference to deplete Plk1, we assessed whether Plk1 is required for the degradation of Claspin at mitotic onset. Indeed, depletion of Plk1 in HeLa and U2OS cells was able to prevent the degradation of Claspin in mitosis (Figure 3F), indicating that Plk1 is essential for mitotic Claspin degradation. Interestingly, whereas control-transfected mitotic cells show loss of Claspin during mitosis, no clear reduction in Claspin levels was observed in Plk1-depleted mitotic cells (Figure S3B).

β-TrCP Targets Claspin for Degradation

The SCF (Skp1-Cullin-F-box-protein) ubiquitin ligase complex is responsible for the degradation of multiple proteins during mitotic onset. In particular, key mitotic-entry regulators, such as Wee1 and Emi1 [5–7], are degraded by the β-TrCP-SCF complex in a Plk1-dependent fashion. To test whether Claspin might also be degraded by the β-TrCP-SCF complex, we downregulated β-TrCP1/2 by shRNA. Importantly, downregulation of β-TrCP1/2 resulted in elevation of Claspin protein levels during mitosis (Figure 4A), and we could demonstrate an interaction between Claspin, β -TrCP1/2 and Skp1 (Figure S4A and data not shown). Interestingly, a DSGxxS motif that is present in most SCF substrates [8] can be recognized in a conserved region in the N terminus of Claspin (Figure S4B). To investigate whether this DSGxxS motif is required for Claspin targeting for proteasomal degradation, we generated point mutants in which the two essential serines were replaced by alanines (Claspin S30/34A). Strikingly, the S30/34A mutant displayed a reduced affinity for β-TrCP1/2 as compared to wt-Claspin (Figure 4B), and disruption of the recognition motif abolished Claspin degradation in mitotic cells (Figure 4C).

Together, these results show the significant role for the β-TrCP1/2-SCF complex in Claspin degradation at mitotic onset. Also, they suggest that Plk1 might act at multiple points to promote checkpoint recovery. We have previously demonstrated that Plk1 promotes proteasomal degradation of the Cdk-inhibitory kinase Wee1 [7], which acts at the bottom of the checkpoint signaling cascade. Here we show that Plk1 is also involved in promoting the degradation of Claspin. Similar to Wee1, Plk1 can promote the proteasomal degradation of Claspin in a β-TrCP1/2-dependent fashion. This degradation requires the presence of a phosphodegron motif (DSGxxS), which is also found in other β-TrCP1/2 substrates, such as Emi1 [5, 6]. Presently, we do not know whether Plk1 directly phosphorylates the serines in the phosphodegron motif or whether Plk1-dependent degradation of Claspin by the β-TrCP1/2-SCF complex occurs in a more complex fashion, as was shown for Wee1 [9].

Claspin Degradation Is Required for Checkpoint Recovery

The downregulation of Claspin levels at mitotic entry might act to prevent Chk1 activation during mitosis. Indeed, we found that Chk1 can be activated in mitotic cells expressing the nondegradable S30/34A mutant of Claspin (Figure 4D), indicating that the degradation of Claspin is required to inactivate the checkpoint in mitosis. Similarly, degradation of Claspin might be a way to decrease Chk1 activity during recovery from a checkpoint arrest. To test this latter hypothesis, we arrested U2OS cells in G2 with a DNA-damaging agent and then treated them with caffeine to induce checkpoint

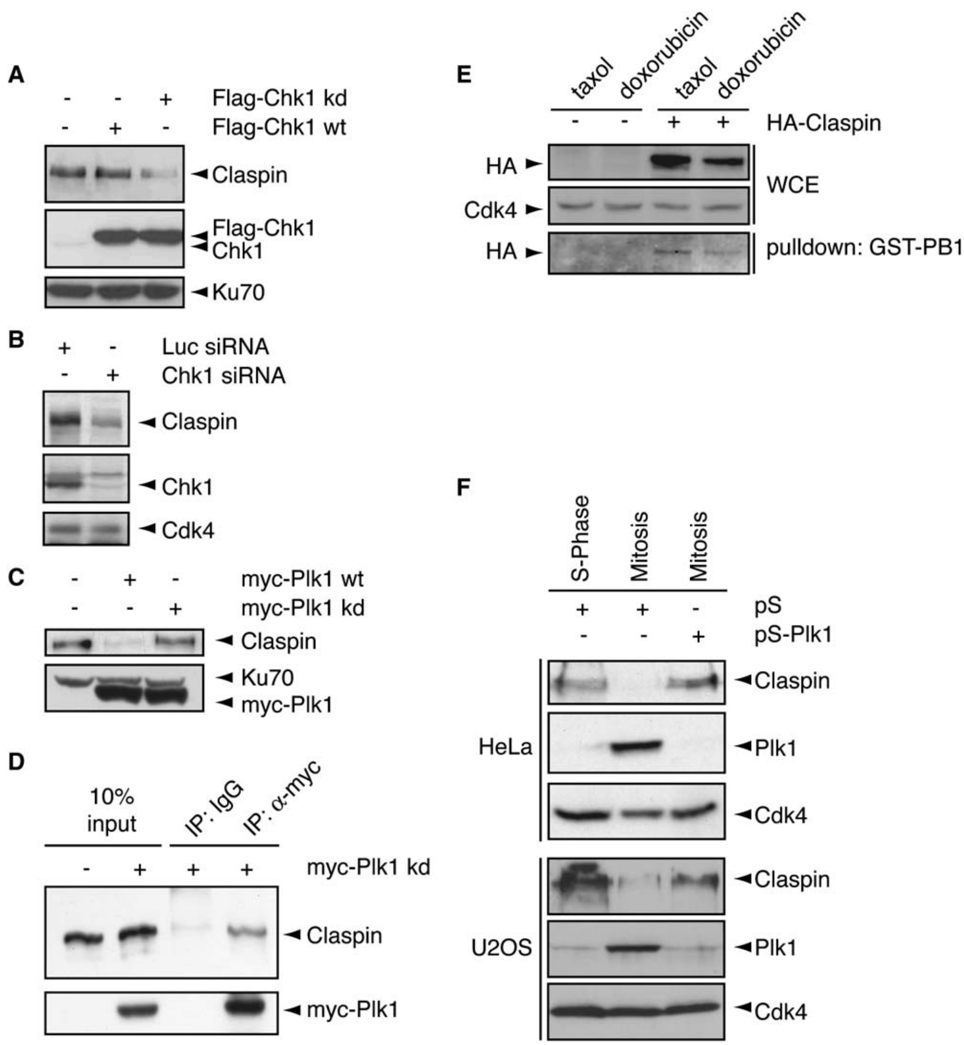

Figure 3. Chk1 and Plk1 Both Regulate Claspin Levels

(A) HEK 293T cells were transfected with wild-type or kinase-dead versions of Flag-Chk1. WCE were analyzed by Western blot for Claspin expression.

(B) U2OS cells were transfected with control (Luciferase) or Chk1 siRNA for 72 hr. WCE were analyzed by Western blotting.

(C) 293T cells were transfected with Myc-tagged versions of wild-type or kinase-dead Plk1. After 36 hr, WCE were made and analyzed.

(D) Immunocomplexes isolated with control or Myc antibodies from U2OS cells expressing Myc-kd-Plk1 were analyzed by Western blotting for associated Claspin.

(E) U2OS cells were transfected with HA-Claspin and treated for 16 hr with taxol. Alternatively, cells were pretreated with doxorubicin (0.5 μM) for 1 hr. WCE were subsequently incubated with recombinant GST-PBD. WCE and pull-down fractions were analyzed by Western blot.

(F) U2OS or HeLa cells were transfected with the pSuper or pSuper-Plk1 plasmids and subsequently arrested with thymidine for 24 hr. S-phase (2 hr after release) and mitotic shake-off fractions (18 hr after release, in the presence of nocodazole) were collected. WCE were analyzed by Western blot.

recovery. Caffeine inhibits the checkpoint kinases ATM and ATR and silences the checkpoint, allowing cells to recover from the arrest [10]. Indeed, whereas Chk1 phosphorylation is lost upon caffeine treatment in control cells, Plk1 depletion resulted in less-efficient Chk1 dephosphorylation after checkpoint silencing (Figure 4E). As expected, the status of Chk1 phosphorylation completely matched the presence or absence of Claspin (Figure 4E). To confirm that Claspin degradation is a key event in checkpoint recovery, we next assessed the effects of expressing the nondegradable Claspin mutant S30/34A. Expression of this mutant significantly lowered the extent of checkpoint recovery in response to different types of DNA damage. When caffeine was used to silence the G2 DNA-damage checkpoint induced by doxorubicin or camptothecin, a clear decrease in checkpoint recovery was visible in cells expressing nondegradable Claspin (Figure 4F). These results indicate that degradation of Claspin indeed is an important requirement during checkpoint recovery induced by caffeine treatment. We next investigated spontaneous checkpoint recovery by using hydroxyurea. Eighteen hours after a 1 hr pulse with 10 mM hydroxyurea, most cells had recovered, as evidenced by a high mitotic index (Figure 4F). However, cells expressing the nondegradable Claspin displayed a significant decrease in checkpoint recovery (Figure 4F). Together, these results demonstrate that the Plk1-dependent process of Claspin

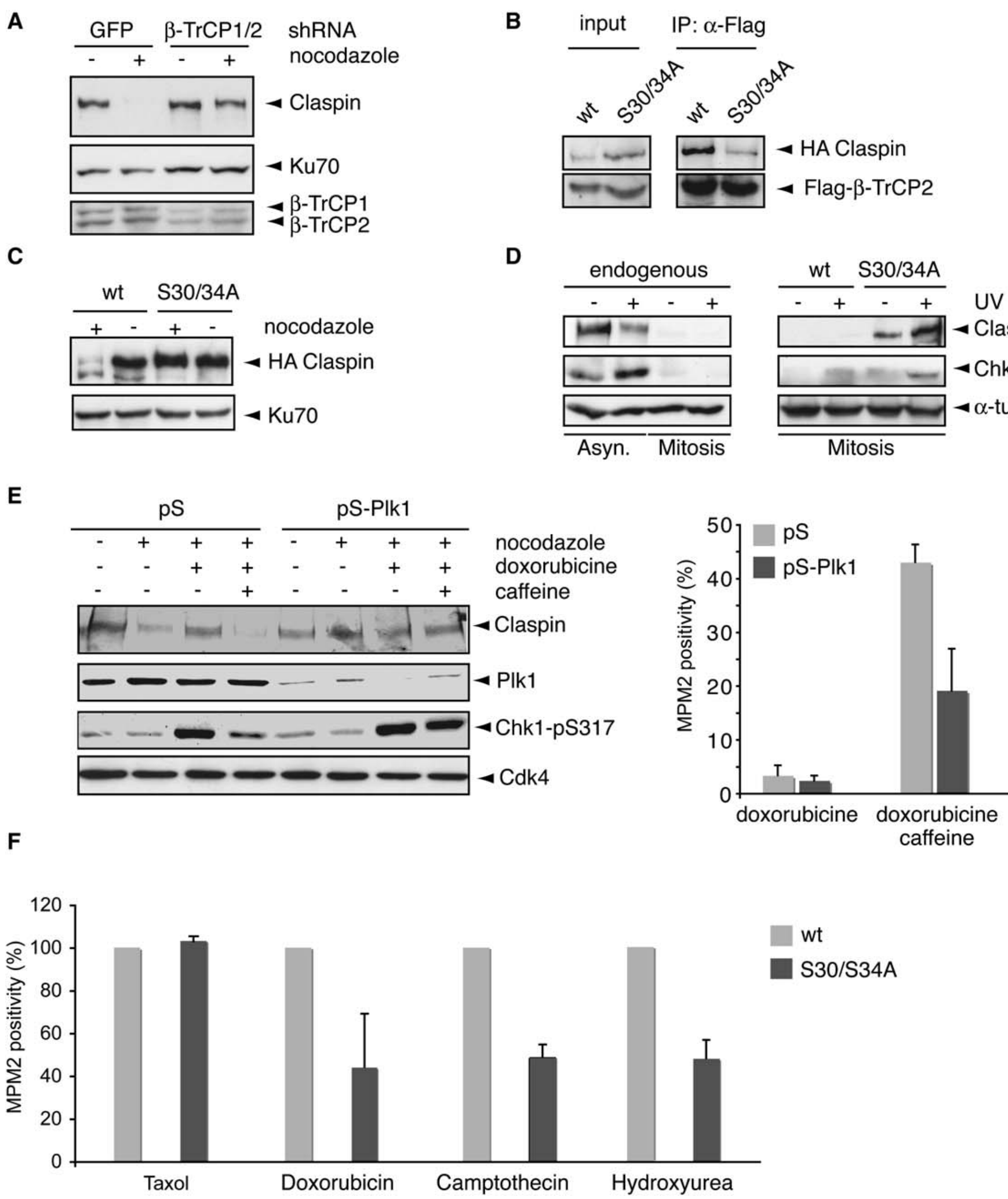

Figure 4. β-TrCP1/2-SCF-Dependent Degradation of Claspin Is Required for Checkpoint Recovery

(A) U2OS cells were transfected with pSuper-GFP or pSuper-β-TrCP1/2 in combination with pBabePuro. Twelve hours after transfection, cells were incubated with puromycin for 24 hr. Cells were then left untreated or incubated with nocodazole for 16 hr. Mitotic cells were collected by gentle shake-off, and WCE were prepared and analyzed by Western blot.

(B) HEK 293T cells were transfected with Flag-β-TrCP2 together with HA-wt-Claspin or HA-S30/34A-Claspin. Immunoprecipitations for Flag were analyzed by Western blot with HA and Flag antibodies.

(C) U2OS cells were transfected with HA-wt-Claspin or HA-S30/34A-Claspin. Cells were treated with nocodazole for 12 hr, then, mitotic cells were collected by gentle shake-off, and WCE were prepared and analyzed by Western blot.

(D) U2OS cells were left untransfected (left panel) or were transfected with either HA-wt-Claspin or HA-S30/34A-Claspin (right panel). Mitotic cells were obtained by treatment of the cells with nocodazole followed by mitotic shake off. Where indicated, cells were treated with UV light (20 J/m2), and WCE were made 90 min later. Claspin and HA antibodies were used for detection of endogenous and exogenous Claspin, respectively.

(E) U2OS cells were transfected with pSuper or pSuper-Plk1 and were synchronized with a 24 hr thymidine block. Eight hours after release, cells were treated for 1 hr with doxorubicin (0.5 μM) or left untreated. Subsequently, nocodazole was added. After 16 hr, all doxorubicin-treated cells were arrested in G2. These cells were left untreated or were treated with 5 mM caffeine for 8 hr. WCE were prepared and analyzed by Western blotting with the indicated antibodies (left panel). In parallel, DNA content and MPM2 positivity was assessed by flow cytometry (right panel).

(F) U2OS cells were transfected with HA-wt-Claspin or HA-S30/34A-Claspin and treated as for Figure 4E. In addition, similar experiments were performed with camptothecin and hydroxyurea. Subsequently, cells were fixed and stained for MPM2. Relative mitotic indices are shown (mitotic indices of HA-wt-Claspin were put to 100%). Recovery after hydroxyurea was assessed in the absence of caffeine.

degradation is a key event in allowing recovery after an arrest by the DNA-damage checkpoint.

Taken together, our observations indicate that the degradation of Claspin serves to silence the DNA-damage checkpoint at mitotic entry. Moreover, we find that expression of nondegradable Claspin inhibits mitotic entry after checkpoint recovery. This effect is seen in cultures that have been arrested in G2 via a variety of DNA damaging agents and that are subsequently induced to enter mitosis by treatment with caffeine. In addition, the effect of nondegradable Claspin on recovery is even more pronounced in cultures that are allowed to recover spontaneously from a hydroxyurea-induced S-phase arrest, representing a physiological condition of checkpoint recovery. In contrast, expression of nondegradable Claspin has no effect on mitotic entry in an unperturbed cell cycle, indicating that Claspin degradation is not required per se for mitotic entry under all conditions. Importantly, expression of a nondegradable Claspin behaves in a similar way as Plk1 depletion, which we previously showed to lead to a stringent block in checkpoint recovery while failing to block mitotic entry in cells that did not activate the G2 DNA-damage checkpoint [10]. Thus, Plk1-induced Claspin degradation appears to positively regulate checkpoint recovery, and interference in this pathway severely limits the capacity of a cell to recover from genotoxic stress.

These results are in good agreement with data from yeast, where the budding yeast polo-homolog Cdc5 is required for the inactivation of the DNA checkpoint kinase Rad53 [11]. Moreover, Polo-like kinase appears to function both in checkpoint adaptation, as was shown in *Xenopus* [3], and in checkpoint recovery, as we demonstrate here in human cells. Nonetheless, there is a clear difference in the mechanism by which Claspin function is controlled in these two different organisms. Plx1-mediated phosphorylation in *Xenopus* leads to displacement of Claspin from the chromatin, without concomitant Claspin degradation [3]. In contrast, in human cells Plk1-mediated phosphorylation causes destruction of Claspin, pointing to a more rigorous, irreversible control mechanism. Interestingly, such a fundamental difference is not without precedent; others have shown that interference with the function of Orc1 occurs through its displacement from chromatin in *Xenopus*, whereas this is mediated via protein destruction in human cells [12]. Also, although binding of Plx1 to xClaspin requires ATR-dependent phosphorylation, it is likely that the priming of Claspin for binding to Plk1 in human cells involves alternative kinases [13]. The identity of these kinases and the mechanism by which Plk1 is reactivated after genotoxic stress and allowed to trigger downregulation of Claspin remain the most interesting questions to resolve because they will help us understand how a cell switches from a checkpoint-arrested state to a state of active recovery. Clearly, our data show that the action of Plk1 during recovery is not restricted to Wee1 but that it also regulates a core checkpoint component that acts at the top of the signaling cascade. In light of our data, one could even speculate that mere activation of Plk1 in DNA-damaged cells might be sufficient to silence the checkpoint and promote checkpoint adaptation.

Supplemental Data

Supplemental Data include four figures and Experimental Procedures and can be found online at: http://www.current-biology.com/cgi/content/full/16/19/1950/DC1/.

Acknowledgments

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Supplemental Data

Polo-like Kinase-1 Controls Proteasome-Dependent Degradation of Claspin during Checkpoint Recovery

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Supplemental Experimental Procedures

Plasmids and siRNA

Expression constructs of GFP-Spectrin, GFP-Histone2B, Plk1-wt, Plk1-kd, pSuper, and pSuper-Plk1 have been described previously [S1]. His-Ubiquitin was expressed from PMT107 and was a gift from Dr. D. Bohmann (Rochester, NY). pcDNA3/HA-Claspin was a kind gift from Dr. P. M. Reaper (Vertex Pharmaceuticals, UK). HA-Claspin S30/34A was generated by PCR-based site-directed mutagenesis according to the manufacturer's instructions (Stratagene), and sequences were verified by sequencing. HA-β-TrCP1 and 2 were kindly provided by Dr. N. Watanabe (Japan) and were described previously [S2]. Flag-β-TrCP1 and 2 contains the full-length human proteins fused to the Flag tag. GST fusions of Claspin were kindly provided by Dr. J. Chen (Mayo Clinic, FL). GST-PBD contains amino acids 345–603 of human Plk1 fused to GST. pSuper-β-TrCP1 and 2 target a common sequence, present in both β-TrCP1 and β-TrCP2, and was provided by Dr. S. Nijman (Netherlands Cancer Institute, the Netherlands). Plasmids expressing Flag-Chk1 and Flag-Chk1-kd (D130A) were kindly provided by Dr. J. Bartek (Institute of Cancer Biology, Copenhagen). Chk1 siRNA experiments were performed with dsRNA oligos (UCGUGAGCGUUUGUUGAACdtdt) from Dharmacon.

Cell Culture

Cells were cultured, transfected, and synchronized as described [S3]. Where indicated, cells were treated with 100 ng/ml nocodazole (Sigma), 0.5 μM doxorubicin (adriamycin), camptothecin (0.5 or 1 μM), and 10 mM hydroxyurea. For double thymidine blocks, cells were treated with 2.5 mM thymidine for 24 hr, released for 12 hr, and blocked in thymidine for another 20 hr. Washing the cells twice with PBS and adding fresh medium with 24 μM deoxycytidine released the cells. MG132 was used at a final concentration of 5 μM when HEK 293T cells were treated, and 10 μM was used with U2OS.

Antibodies

Human Claspin rabbit polyclonal sera were raised against the N terminus (amino acids 85–248), and β-TrCP1 antisera were raised against amino acids 1–300 of the human protein. α-tubulin antibody was from Sigma. Rabbit anti-Ku70 and rabbit anti-Ku80 were kindly provided by Dr. S.P. Jackson (Cambridge, UK). Goat anti-Cdk4 and rabbit anti-Cyclin E were from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-Chk1-pS317 and mouse anti-ubiquitin were from Cell Signaling (Danvers, MA), and rabbit anti-pS10-HistoneH3, mouse anti-MPM2, and rabbit anti-Plk1 were from Upstate Biotechnology (Lake Placid, NY). For mouse anti-HA and mouse anti-Myc, culture medium of hybridomas was used from 12CA5 and 9E10, respectively. Secondary antibodies for immunofluorescence were Alexa-488 and Alexa-568 (Molecular Probes). Peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse were from Dako and Jackson Immunoresearch.

Immunoprecipitations

HEK 293T- or U2OS-transfected cells were collected by trypsinization, washed with ice-cold PBS, and lysed with lysis buffer III (50 mM Tris-HCl [pH 8], 200 mM NaCl, 2 mM EDTA, 0.5% NP40, 1 mM DTT, and 1 mM sodium orthovanadate) supplemented with protease inhibitor cocktail (Roche) and 1 μM microcystin LR (Alexis) for 10 min on ice. Immunoprecipitations were carried out as described [S1] and analyzed on Western blots with the indicated antibodies. Immunoprecipitations for Figure 4B were carried out in lysis buffer III containing 300 mM NaCl.

In Vivo Ubiquitination Assays

Ubiquitinated intermediates in human cells were detected with the (His)6-tagged Ubi method as used by [S4]. HEK 293T cells were transfected with pcDNA3/HA-Claspin either in the absence or in the presence of plasmid encoding His-tagged ubiquitin. Cells were harvested 36 hr after transfection, and His-tagged proteins were purified on Ni-NTA-agarose and subjected to SDS-PAGE. HA-tagged proteins were detected by an immunoblot with HA monoclonal antibody.

Confocal Microscopy and Flow Cytometry

Confocal microscopy and flow cytometry were performed as described [S1].

Supplemental References

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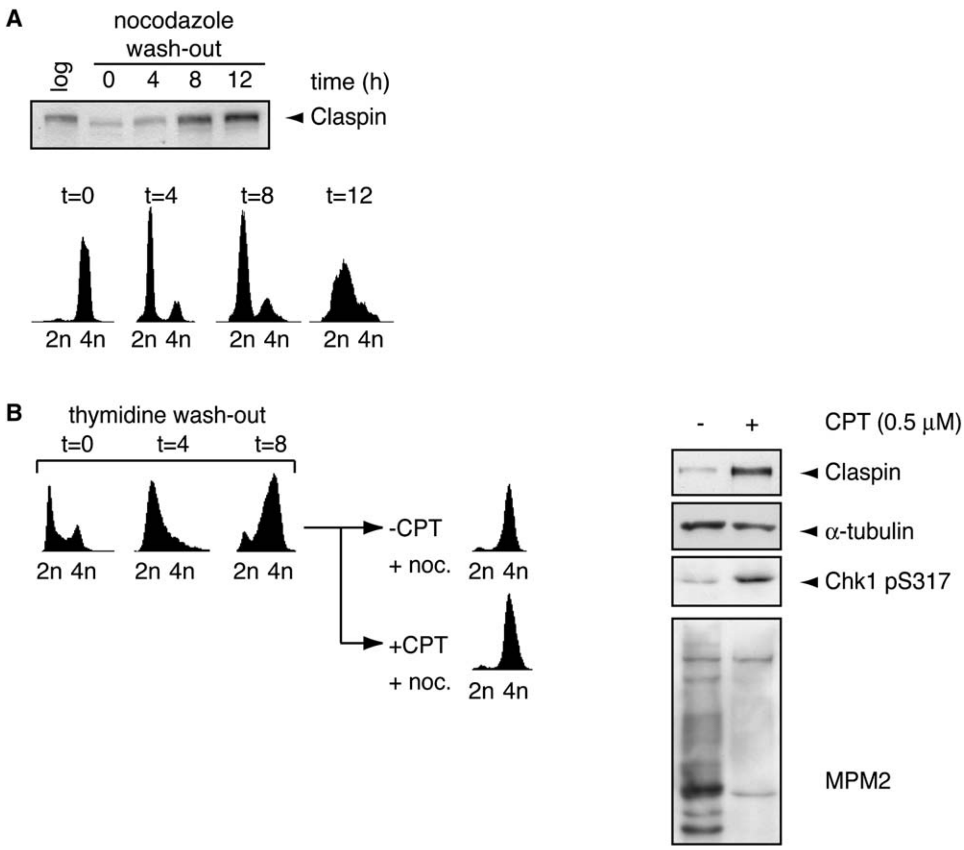


Figure S1. Claspin Regulation during the Cell Cycle and after DNA Damage

(A) U2OS cells were arrested in mitosis by incubation with nocodazole for 16 hr. Mitotic cells were collected by gentle shake-off and subsequently released from this block. Levels of Claspin as well as the DNA content were measure at different time points.

(B) U2OS cells were synchronized by thymidine block and subsequently released. After 8 hr, cells were incubated for 1 hr with 0.5 μM camptothecin (CPT) or left untreated. After the drug was washed away, nocodazole was added for 14 hr. WCE were prepared and analyzed on Western blots. In parallel, DNA content was analyzed by flow cytometry.

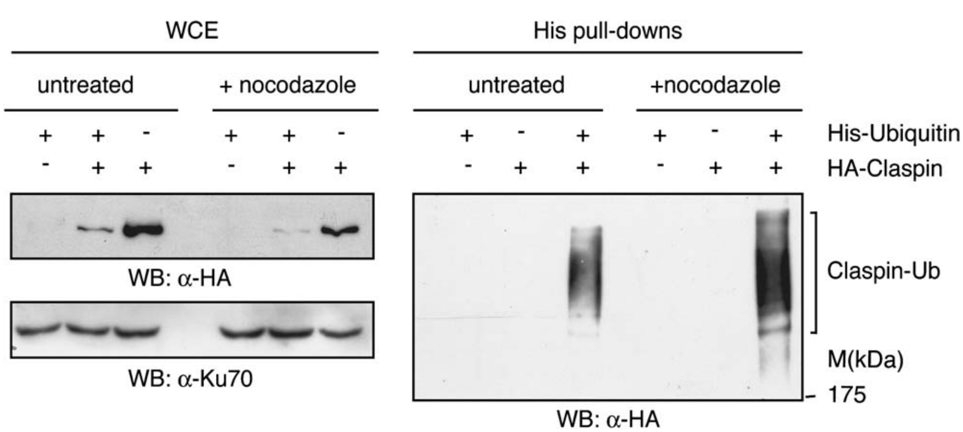


Figure S2. Claspin Protein Levels Are Regulated via Proteasome-Dependent Degradation

HEK 293T cells were transfected with indicated plasmids. Cells were left untreated or were treated with nocodazole. Thirty-six hours after transfection, cells were lysed and extracts were incubated with Ni-NTA beads. After washing, the resin was analyzed for Claspin-ubiquitination.

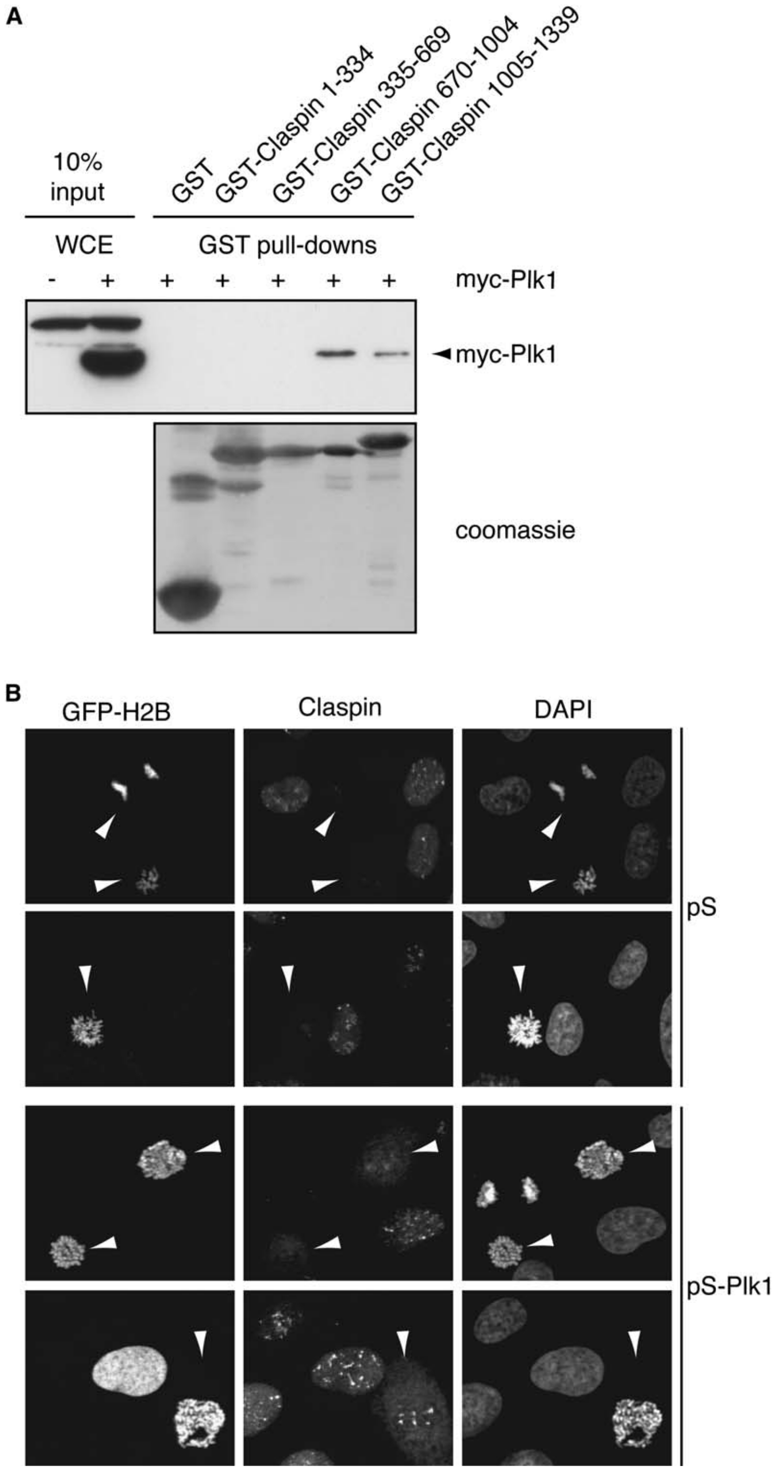
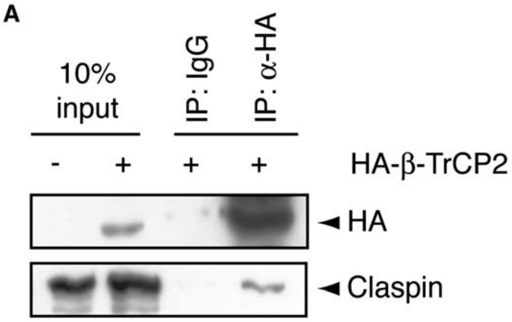


Figure S3. Plk1 Regulates Claspin Levels

(A) GST pull-down assays were performed with the indicated recombinant GST-Claspin fragments, and extracts were made from U2OS cells transfected with Myc-tagged Plk1. The pull-down assays were analyzed by Western blotting with Myc antibodies. Coomassie staining of the GST fragments is shown as a loading control.

(B) U2OS were cotransfected with GFP-H2B in combination with pSuper or pSuper-Plk1. Coverslips were stained with anti-Claspin and DAPI and were analyzed by confocal microscopy. Arrowheads indicate mitotic cells.

A



B

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         DSGXXS
25  DSDSGQGSC  33   X.laevis
27  PSDSGQGSY  35   H.sapiens
27  PVDSGQGSF  35   M.musculus
262 PSDSGQGSC  170  C.familiaris
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Figure S4. β-TrCP1/2-SCF-Dependent Degradation of Claspin

(A) HEK 293T cells transfected with β-HA-TrCP1 were treated with MG132 for 3 hr. Extracts were incubated with HA or control antibodies. Immunoprecipitations were analyzed by Western blotting with HA and Claspin antibodies.

(B) Sequence alignment of the β-TrCP1/2 binding site at the N terminus in *Xenopus* and human Claspin. Identical residues are shown in bold, and numbers represent the residue number in different species.