RESEARCH COMMUNICATION

Chk1 and Claspin potentiate PCNA ubiquitination

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Chk1 is a kinase crucial for genomic integrity and an effector of ATR (ATM and Rad3-related) in DNA damage response. Here, we show that Chk1 regulates the DNA damage-induced ubiquitination of proliferating cell nuclear antigen (PCNA), which facilitates the continuous replication of damaged DNA. Surprisingly, this Chk1 function requires the DNA replication protein Claspin but not ATR. Claspin, which is stabilized by Chk1, regulates the binding of the ubiquitin ligase Rad18 to chromatin. Timeless, a Claspin-associating protein, is also required for efficient PCNA ubiquitination. Thus, Chk1 and the Claspin–Timeless module of replication forks not only participate in ATR signaling, but also protect stressed forks independently of ATR.

Supplemental material is available at <http://www.genesdev.org>.

Received November 8, 2007; revised version accepted March 4, 2008.

The DNA damage and replication stress signaling pathway regulated by the ATR (ATM and Rad3-related) kinase plays crucial roles in the maintenance of genomic stability (Zhou and Elledge 2000). In response to DNA damage or replication stress, ATR phosphorylates numerous proteins involved in DNA repair, DNA replication, and other cellular processes (Matsuoka et al. 2007). One of these substrates is Chk1, a key effector kinase of ATR in this pathway. The phosphorylation of Chk1 by ATR stimulates its kinase activity and regulates its subcellular localization, enabling it to efficiently phosphorylate downstream targets such as Cdc25s. Like ATR, Chk1 is required for sustained cell survival even in the absence of exogenous DNA damage. A number of studies have suggested that the ATR–Chk1 pathway is important for coping with intrinsic replication stresses during the S phase of the cell cycle. Although the signaling from ATR to Chk1 has been studied extensively, it was not clear whether Chk1 is simply a signal transducer of ATR or, like many other ATR substrates, has its own functions in protecting the genome.

Proliferating cell nuclear antigen (PCNA) is an important functional and structural component of DNA replication forks (Moldovan et al. 2007). Upon DNA damage or replication stress, human PCNA is either mono- or

polyubiquitinated. The monoubiquitination of PCNA was suggested to facilitate the interaction of PCNA with several translesion DNA polymerases. These polymerases may enable replication forks to continuously advance on damaged DNA, providing an avoidant mechanism for single-stranded DNA gaps and DNA breaks (Lopes et al. 2006). How the ubiquitination of PCNA is regulated by DNA damage and replication stress is still unclear. Two studies using fission yeast and *Xenopus* extracts suggested that ATR is not required for the ultraviolet light (UV)-induced PCNA ubiquitination (Chang et al. 2006; Frampton et al. 2006). Another study using human cells, however, implicated both ATR and Chk1 in the PCNA ubiquitination induced by the carcinogen B[a]P Di-hydrodiol Epoxide (BPDE) (Bi et al. 2006). Here, we show that Chk1 and the DNA replication proteins Claspin and Timeless, but not ATR, are important for the UV- and Hydroxyurea (HU)-induced PCNA ubiquitination in human cells. Our data suggest a novel ATR-independent function of Chk1 in genome protection, providing a new view of the functional relationships among ATR, Chk1, and the DNA replication machinery.

Results and Discussion

To investigate how the ATR-mediated signaling pathway regulates stressed DNA replication forks, we sought to determine whether ATR and Chk1 are required for the known DNA damage-induced events at replication forks. Like the phosphorylation of Chk1 by ATR, the ubiquitination of PCNA is induced by a variety of DNA-damaging agents that interfere with DNA replication. To assess whether Chk1 is involved in PCNA ubiquitination, we used two independent siRNAs to knock down Chk1 in HeLa or U2OS cells and analyzed the levels of monoubiquitinated PCNA using a monoclonal PCNA antibody as described previously (Kannouche et al. 2004). Both the UV- and HU-induced PCNA ubiquitination was reduced in cells treated with Chk1 siRNA compared with the controls treated with LacZ siRNA (Fig. 1A–C; Supplemental Fig. S1A). To further assess whether the reduced PCNA ubiquitination in Chk1 knockdown cells was indeed due to the loss of Chk1, we generated a cell line stably expressing the siRNA-refractory wild-type Chk1 at the endogenous Chk1 level. Upon treatment with Chk1 siRNA, the endogenous Chk1 in this cell line became undetectable, whereas the siRNA-refractory Chk1 remained unchanged (Fig. 1D). Importantly, the ubiquitination of PCNA in this cell line was largely unaffected by the Chk1 siRNA, demonstrating that compromised PCNA ubiquitination is a specific effect of Chk1 ablation.

Chk1 is not only important for the DNA damage response but also for maintaining genomic stability during the S phase (Syljuasen et al. 2005; Zachos et al. 2005). We therefore asked whether the compromised PCNA ubiquitination in Chk1 knockdown cells was due to a loss of PCNA from replication forks or severely compromised DNA replication. The PCNA foci associated with DNA replication were not affected in Chk1 knockdown cells, including the γ -H2AX-positive cells (Supplemental Fig. S1B). Furthermore, knockdown of Chk1 modestly increased the fraction of cells incorporating BrdU (Supple-

[Keywords: Chk1; Claspin; ATR; PCNA; ubiquitination; replication]

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1632808>.

Figure 1. Chk1 is required for efficient PCNA ubiquitination. (A,B) HeLa cells were transfected with control siRNA (LacZ) or two independent Chk1 siRNAs (Chk1-1 and Chk1-2). The levels of Chk1, PCNA, and monoubiquitinated PCNA (Ub-PCNA) were analyzed by Western blotting 2 h after UV treatment (100 J/m²). (C) Ub-PCNA was analyzed after 2 h of HU treatment (2.5 mM). (D) HeLa cells and the derivative cells stably expressing the siRNA-refractory wild-type Chk1 were transfected with LacZ or Chk1 siRNA. UV-induced Ub-PCNA was analyzed as in A. The relative Ub-PCNA signals were normalized against the corresponding bands of unmodified PCNA in short exposures. The Ub-PCNA signal of the LacZ/UV sample was defined as 1. (E) UV-induced Ub-PCNA was monitored during a 4-h time course.

mental Fig. S1C). These results suggest that the compromised PCNA ubiquitination in Chk1 knockdown cells is not a result of PCNA loss from replication forks or lack of replicating cells. Chk1 has also been implicated in the progression of DNA replication forks (Petermann et al. 2006). To assess whether PCNA ubiquitination was simply delayed in Chk1 knockdown cells, we monitored this modification at different times after UV treatment (Fig. 1E). PCNA was maximally ubiquitinated in control cells within 2 h, but was not ubiquitinated in Chk1 knockdown cells for up to 4 h. Thus, PCNA ubiquitination was diminished rather than delayed in Chk1 knockdown cells.

Since Chk1 is an effector kinase of ATR in DNA damage signaling, we asked whether ATR is also required for PCNA ubiquitination. Using two independent ATR siRNAs, we efficiently knocked down ATR in cells (Fig. 2A). As reported previously, knockdown of ATR resulted in reduced levels of ATRIP and UV-induced Chk1 phosphorylation. The levels of monoubiquitinated PCNA, however, were not significantly affected by ATR knockdown. To confirm these results, we analyzed the ubiquitination of PCNA in the ATR^{flox/-} cells infected with the Cre-expressing adenovirus and in the ATR-deficient Seckel cells. Consistent with the experiments using ATR siRNAs, the UV-induced Chk1 phosphorylation, but not PCNA ubiquitination, was diminished in the ATR-deleted cells (Fig. 2B). Furthermore, the UV-induced PCNA ubiquitination was readily detected in Seckel cells (Supplemental Fig. S2A).

Given the known function of ATR in Chk1 activation, the requirement of Chk1 but not ATR for PCNA ubiqui-

uitation was surprising. Although the checkpoint response to UV and HU is mediated primarily by ATR, a potential role of ATM in Chk1 regulation cannot be excluded. Both the UV- and HU-induced PCNA ubiquitination was observed in AT cells (Supplemental Fig. S2A), suggesting that ATM is not essential for PCNA ubiquitination. To assess whether ATM and ATR function redundantly, we treated control cells and ATR knockdown cells with increasing concentrations of KU-55933, a specific ATM inhibitor (Fig. 2C). Inhibition of ATM did not reduce PCNA ubiquitination in the presence or absence of ATR. Similar results were obtained using KU-55933 and CGK733, an inhibitor of both ATR and ATM (Supplemental Fig. S2B). Furthermore, wortmannin, an inhibitor of DNA-PK, ATM, and ATR, even enhanced PCNA ubiquitination at the concentrations sufficient to completely inhibit Chk1 phosphorylation (Supplemental Fig. S2C). Together, these results suggest that neither ATR nor ATM plays a major role in the regulation of PCNA ubiquitination. Furthermore, consistent with the normal PCNA localization in Chk1 knockdown cells (Supplemental Fig. S1B), they suggest that the effect of Chk1 on PCNA ubiquitination is not due to the replication fork collapse resulting from compromised ATR signaling.

The lack of ATR dependence for PCNA ubiquitination prompted us to assess whether the kinase activity of Chk1 is required for this process. We generated a cell line stably expressing a kinase-defective Chk1 mutant (Chk1-KD) refractory to the Chk1 siRNA. The UV-induced PCNA ubiquitination was partially restored by Chk1-KD when the endogenous Chk1 was depleted (Fig. 2D). Furthermore, UCN-01 and Gö6976, two potent inhibitors of Chk1, did not significantly affect PCNA ubiquitination at the concentrations sufficient to abrogate checkpoint response (Supplemental Fig. S3A,B). These results suggest that the kinase activity of Chk1 is not essential for PCNA ubiquitination.

To further investigate how Chk1 regulates PCNA ubiquitination, we asked whether any of the known functions of Chk1 are ATR-independent. It was shown recently that Chk1 is required for maintaining the steady-state levels of Claspin (Chini et al. 2006), a replication protein functionally linked to Chk1. Another study showed that the levels of Claspin were not significantly affected by ATR knockdown in undamaged cells (Bennett and Clarke 2006). Together, these findings implied that the function of Chk1 in stabilizing Claspin might be ATR-independent. To directly assess this possibility, we used siRNA to knock down Chk1 and ATR in HeLa cells, and compared their effects on Claspin levels. Consistent with the previous studies, knockdown of Chk1 but not ATR reduced the levels of Claspin (Fig. 3A). Interestingly, the levels of Chk1 were also reduced in cells treated with Claspin siRNA (Fig. 3A), indicating that Chk1 and Claspin stabilize each other. In the absence of Chk1, the levels of Claspin gradually declined (Supplemental Fig. S4). The reduction of Claspin was observed in cells treated with two independent Chk1 siRNAs (Fig. 3B) and, furthermore, was partially rescued by the siRNA-refractory wild-type Chk1 and Chk1-KD (Fig. 3C). These results suggest that ATR and the kinase activity of Chk1 are not essential for the function of Chk1 in Claspin stabilization.

We next asked whether the ATR-independent function of Chk1 in stabilizing Claspin contributes to PCNA ubiquitination. The HU- and UV-induced PCNA ubiqui-

Figure 2. ATR is not required for efficient PCNA ubiquitination. (A) HeLa cells were transfected with LacZ siRNA or two independent ATR siRNAs (ATR-1, and ATR-2). Phosphorylated Chk1 (Ser345) and Ub-PCNA were analyzed 2 h after UV treatment. (B) HCT116 (ATR^{+/+}) and its ATR^{lox/-} derivative line were infected with adenovirus expressing Cre. UV-induced Chk1 phosphorylation and Ub-PCNA were analyzed 3 d after infection. (C) Cells transfected with LacZ or ATR siRNA (ATR-1) were treated with 5, 10, 25, or 50 μ M KU-55933 for 1 h prior to UV treatment. Phosphorylated Chk1 (Ser345), Chk2 (Thr68), and Ub-PCNA were analyzed 2 h after UV treatment. (D) HeLa cells and the derivative cells stably expressing the siRNA-refractory Chk1-KD were analyzed as in Figure 1D.

tionation was diminished in cells treated with two independent Claspin siRNAs (Fig. 3D; data not shown). The depletion of Claspin did not significantly alter cell cycle distribution at the time of analysis (Supplemental Fig. S5). Notably, one of the two Claspin siRNAs with a lower efficiency of Claspin knockdown did not significantly affect the levels of Chk1, suggesting that a reduction of Claspin is sufficient to compromise PCNA ubiquitination even in the presence of Chk1 (Fig. 3D). To directly test whether Claspin functions downstream from Chk1 in regulating PCNA ubiquitination, we transiently expressed Claspin in Chk1 knockdown cells and assessed whether it alleviated the defect in PCNA ubiquitination. Indeed, overexpression of Claspin partially rescued PCNA ubiquitination in Chk1 knockdown cells (Fig. 3E), suggesting that Claspin is an important effector of Chk1 in this process.

The involvement of Claspin in PCNA ubiquitination is intriguing because Mrc1, the budding yeast homolog of Claspin, is a component of DNA replication forks (Osborn and Elledge 2003; Gambus et al. 2006). The functions of Mrc1 are tightly linked to Tof1 and Csm3, two other replication fork proteins, suggesting that they act as a distinct module at the forks (Tong et al. 2004). These three proteins function in both Mec1(ATR)-dependent and -independent manners; they not only regulate the signaling of replication stress, but also the progression of replication forks, the stable pausing of stressed forks, and cohesion of sister chromatids (Katou et al. 2003; Xu et al. 2004; Szyjka et al. 2005; Tourriere et al. 2005). To assess whether Claspin associates with replication forks in human cells and functions similarly to Mrc1, we asked whether Claspin interacts with PCNA and Timeless, the human homolog of Tof1. By immunoprecipitating endogenous Claspin and Timeless, we found that these two

proteins associate with each other and with PCNA even in undamaged cells (Fig. 4A,B). Consistent with our results, two independent studies reported recently the interactions of Claspin with PCNA and Timeless (Brondello et al. 2007; Gotter et al. 2007).

In yeast, the Mec1(ATR)-independent functions of Mrc1 are tightly linked to Tof1, raising the possibility that Claspin and Timeless may also function together independently of ATR in human cells. To assess this possibility, we analyzed the effects of Timeless knockdown on PCNA ubiquitination. Knockdown of Timeless by two independent siRNAs reduced the levels of ubiquitinated PCNA by ~50% (Supplemental Fig. S6A). Like in Claspin knockdown cells, the levels of ubiquitinated PCNA on chromatin were reduced in Timeless knockdown cells (Fig. 4C). Timeless is known to form a stable complex with Tipin, the human homolog of yeast Csm3 (Chou and Elledge 2006; Gotter et al. 2007; Unsal-Kacmaz et al. 2007; Yoshizawa-Sugata and Masai 2007). Consistent with previous reports, the levels of Tipin were reduced in Timeless knockdown cells (Supplemental Fig. S6B). In contrast, Chk1 and Claspin were largely intact in these cells. Thus, a reduction of the Timeless–Tipin complex resulted in compromised PCNA ubiquitination.

Timeless and Tipin are important for the rate of DNA replication (Chou and Elledge 2006; Yoshizawa-Sugata and Masai 2007). We therefore asked whether the compromised PCNA ubiquitination in Timeless knockdown cells was an indirect effect of inefficient replication. Mcm2 is a component of the Mcm2–7 helicase complex, and it interacts with Timeless and Tipin in human cells (Chou and Elledge 2006; Gotter et al. 2007). Knockdown of either Mcm2 or Timeless led to a modest accumulation of cells in the S phase at the time of analysis (Supplemental Fig. S6C), indicating a delay in S-phase progression. However, unlike Timeless ablation, knockdown of Mcm2 did not significantly affect PCNA ubiquitination (Fig. 4D). Hence, the effect of Timeless knockdown on PCNA ubiquitination is specific and not simply due to the reduced rate of replication. These results highlight the specific function of the Claspin–Timeless module of replication fork in PCNA ubiquitination.

The level of PCNA ubiquitination is regulated by both the ubiquitin ligase Rad18 and the deubiquitinase Usp1 (Kannouche et al. 2004; Watanabe et al. 2004; Chiu et al. 2006; Huang et al. 2006). The requirement of Claspin for efficient PCNA ubiquitination indicates that it either positively regulates ubiquitin ligation or negatively regulates Usp1 via direct or indirect mechanisms (Fig. 5A). Knockdown of Usp1 led to increased levels of ubiquitinated PCNA, whereas knockdown of Rad18 reduced PCNA ubiquitination regardless of the presence or absence of Usp1 (Fig. 5B–D; Supplemental Fig. S7). If Claspin negatively regulates Usp1, Claspin knockdown

Figure 3. Chk1 stabilizes Claspin independently of ATR. (A) Cells were transfected with siRNAs to LacZ, Claspin, Chk1, or ATR. The levels of Claspin, Chk1, ATR, and ATRIP were analyzed with tubulin as loading controls. (B) The levels of Claspin were analyzed in cells transfected with two independent Chk1 siRNAs. (C) HeLa cells and the derivative cells stably expressing the siRNA-refractory Chk1 or Chk1-KD were transfected with LacZ or Chk1 siRNA. The levels of Claspin were analyzed. (CBB) Coomassie Brilliant Blue. (D) Cells were transfected with LacZ siRNA or two independent Claspin siRNA. The levels of Claspin, Chk1, and Ub-PCNA were analyzed as above. (E) HeLa cells were transfected with LacZ siRNA, Chk1 siRNA, or Chk1 siRNA and a plasmid expressing Claspin. The levels of Claspin, Chk1, and Ub-PCNA were analyzed as above.

should not affect PCNA ubiquitination in the absence of Usp1. On the other hand, if Claspin positively regulates ubiquitin ligation, Claspin knockdown should diminish PCNA ubiquitination even in the absence of Usp1. To assess these possibilities, we analyzed the ubiquitination of PCNA in Usp1 knockdown cells and in Usp1–Claspin double knockdown cells (Fig. 5E). Like that of Rad18, depletion of Claspin diminished PCNA ubiquitination even when Usp1 was simultaneously knocked down, suggesting that Claspin facilitates the establishment of PCNA ubiquitination.

To further pinpoint the function of Claspin in PCNA ubiquitination, we asked whether Claspin regulates the localization of Rad18 or Rad6, the ubiquitin-conjugating enzyme required for PCNA ubiquitination. As reported previously (Yuasa et al. 2006), three distinct forms of Rad18 were detected by immunoblotting, all of which were significantly reduced by multiple independent Rad18 siRNAs (Fig. 5C; data not shown). This observation indicates that Rad18 may undergo modifications or processing in cells. In Claspin knockdown cells, the two slow-migrating forms of Rad18 were reduced, whereas the fastest-migrating form was slightly elevated (Fig. 5F). In addition, the amounts of Rad18 on chromatin were significantly reduced in Claspin knockdown cells in the

presence or absence of DNA damage (Fig. 5F). In contrast, the amounts of a number of chromatin-bound proteins, including PCNA and Rad6, were not affected by Claspin knockdown, suggesting a specific role of Claspin in the binding of Rad18 to chromatin.

Chk1 is a well-characterized effector kinase of ATR in DNA damage response. Our finding that Chk1 but not ATR regulates PCNA ubiquitination reveals that Chk1 is not simply a signal transducer, but also an important regulator of replication forks. Because the function of Chk1 in PCNA ubiquitination is independent of ATR and ATM, it is distinct from the known effector function of Chk1 in the ATR–Chk1 pathway. Given that the ATR–Chk1 pathway is important for stabilizing stressed replication forks, depletion of either ATR or Chk1 would lead to the collapse of some replication forks. However, only the depletion of Chk1 but not ATR reduced the overall levels of ubiquitinated PCNA, suggesting that Chk1 contributes to PCNA ubiquitination independently of its ATR-regulated role in stabilizing stressed forks.

Consistent with a previous report (Chini et al. 2006), we show that Chk1 plays an important role in stabilizing Claspin in human cells. Although not directly involved in DNA synthesis, Claspin is important for the stability of replication forks (Liu et al. 2006). How, exactly, Chk1 stabilizes Claspin is still unclear. We found that Chk1 stabilizes Claspin independently of ATR and its own kinase activity, indicating that it may function through protein–protein interactions. A recent study has suggested that Chk1 and Claspin might interact even in the absence of DNA damage (Chini and Chen 2006). Claspin is degraded in the G2 phase in a Plk1-regulated manner (Gewurz and Harper 2006). Whether the degradation of Claspin in Chk1 knockdown cells is regulated by Plk1 is not known. The absence of Chk1 leads to a decline of the steady-state level of Claspin without reducing S-phase cells, which likely diminishes the function of Claspin during DNA replication.

How does Claspin regulate PCNA ubiquitination?

Figure 4. Timeless but not Mcm2 is required for efficient PCNA ubiquitination. (A) Immunoprecipitation of Claspin from extracts derived from undamaged and UV-treated cells. Coprecipitated Timeless and PCNA were detected by Western blotting. (B) PCNA coprecipitated with both Claspin and Timeless. (C) Cells were transfected with siRNAs to LacZ, Claspin, or Timeless, and were treated with HU for 2 h or left untreated. Chromatin fractions were prepared from cells, and the levels of chromatin-associated Ub-PCNA were analyzed. (D) Cells were transfected with siRNAs to LacZ, Timeless, or Mcm2. The levels of Timeless, Mcm2, and Ub-PCNA were analyzed 2 h after UV treatment.

Figure 5. Claspins are required for the establishment of PCNA ubiquitination. (A) Two possible mechanisms by which Claspin positively regulates PCNA ubiquitination. (B) Usp1 siRNA efficiently knocks down HA-tagged Usp1 in cells infected with adenovirus expressing HA-Usp1. (C) Rad18 siRNAs efficiently knock down three forms of Rad18. (D) Cells were treated with siRNAs to LacZ, Usp1, or both Usp1 and Rad18. Ub-PCNA was analyzed 2 h after UV treatment. (E) Cells were treated with siRNAs to LacZ, Usp1, Claspin, or both Usp1 and Claspin. Ub-PCNA and Claspin were analyzed as above. (F) Soluble and chromatin fractions were prepared from HeLa cells transfected with LacZ siRNA or Claspin siRNA. Cells were treated with 1 mM HU for 16 h (HU1) or 2.5 mM HU for 2 h (HU2), or were irradiated with 100 J/m² UV and harvested after 2 h.

PCNA is ubiquitinated on chromatin after DNA damage. Claspin interacts with PCNA in cells and is important for the association of Rad18 with chromatin, suggesting that Claspin may mediate the interaction between Rad18 and PCNA. Alternatively, Claspin may play a role in configuring replication forks, which is important for Rad18 association and PCNA ubiquitination. A recent study has suggested that PCNA ubiquitination is triggered by the loss of coordination between DNA polymerases and the MCM helicase (Chang et al. 2006). In the yeast *mrc1Δ* and *tof1Δ* mutants, replication proteins including the MCMs, DNA polymerases, and RPA disengage from newly synthesized DNA in the presence of HU (Katou et al. 2003), which may affect the positioning of PCNA and the recruitment of Rad18. In human cells, Claspin and Timeless may function similarly as Mrc1 and Tof1, and are therefore required for PCNA ubiquitination. Further studies are required to assess these models.

We propose that Chk1 may protect replication forks by stabilizing Claspin and maintaining the forks in a state “responsive” to DNA damage. The function of Chk1 in stabilizing Claspin is carried out in the absence of DNA damage, but the effect on PCNA ubiquitination is manifested only after DNA damage. Therefore, a function of Chk1 in unperturbed cells may contribute to DNA damage response. It was reported recently that Chk1 phosphorylates histone H3 at Thr11 in unperturbed cells, which allows cells to repress the transcription of certain genes in a DNA damage-regulated manner (Shimada et al. 2008). Together, these new findings on Chk1 shed new light on the functional relationship between ATR and Chk1, as well as the functions of Chk1 in both unperturbed and damaged cells.

Materials and methods

Cell culture, cell lines, and plasmids

HeLa and U2OS cells were cultured in Dubecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. AT cells (FT-169) and Seckel cells were cultured in DMEM with 15% serum. The ATR^{flax/-} cell line was described in Cortez et al. (2001). The cell lines stably expressing the siRNA-refractory Chk1 or Chk1-KD were derived from HeLa cells. The Chk1 and Chk1-KD plasmids used to generate the cell lines were provided by Dr. K.K. Khanna (Queensland Institute of Medical Research). To confer siRNA resistance to the exogenous Chk1, the target sequence of Chk1-1 siRNA (AAGCGTGCCGTAGACTGTCCA) was mutated to AAGCGAGCGGTTCGATTGCCCT. The Claspin plasmid was provided by Dr. Junjie Chen (Yale University). The adenovirus expressing HA-Usp1 was provided by Dr. Cyrus Vaziri (Boston University).

siRNAs, transfection, and chromatin fractionation

To knock down various target genes, cells were typically transfected twice with siRNA within a 24-h interval, and were directly lysed in SDS sample buffer 2.5–3 d after the first transfection. The sequences of the siRNAs used in this study are listed in the Supplemental Material. In the experiments involving both siRNA and plasmids, cells were first cotransfected with plasmids and siRNA, and then transfected again with siRNA. Chromatin fractionation was conducted as in Zou et al. (2002).

Antibodies

The antibody to PCNA (PC10) was from Chemicon (now Millipore). The antibodies to Claspin and Timeless were from Bethyl Laboratories. The antibody to phospho-Chk1 (Ser345) was from Cell Signaling Technology, and the antibodies to Chk1 and Mcm2 were from Santa Cruz Biotechnology. The antibody to Tipin was a gift from Dr. Stephen Elledge (Harvard University). The antibody to Rad18 was kindly provided by Dr. Satoshi Tateishi (Kumamoto University).

Acknowledgments

We thank Drs. Karlene Cimprich, Junjie Chen, David Cortez, Nick Dyson, Stephen Elledge, Daniel Haber, Wei Jiang, Kum Kum Khanna, Satoshi Tateishi, and Cyrus Vaziri for reagents or discussions. L.Z. is a V Scholar and an Ellison New Scholar. This work was supported by grants from NIH (GM076388), the Susan G. Komen Foundation, and the Breast Cancer Alliance to L.Z. B.S. was partly supported by a fellowship from the Tosteson Foundation.

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Genes Dev. 2008, **22**:

Access the most recent version at doi:[10.1101/gad.1632808](https://doi.org/10.1101/gad.1632808)

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