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Dual Functions of DNA Replication Forks in Checkpoint Signaling and PCNA Ubiquitination

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Abstract

During cell proliferation, DNA damage inflicted by intrinsic or extrinsic genotoxic stresses impose a thereat to DNA replication. The stability of the DNA replication forks that encounter DNA damage is crucial for genomic integrity. Both the ATR-regulated checkpoint pathway and the translesion DNA synthesis mediated by the ubiquitinated PCNA are important for continuous replication of damaged DNA. We have recently shown that Chk1, a key effector kinase of ATR in checkpoint response, is required for efficient PCNA ubiquitination after DNA damage. Surprisingly, the ubiquitination of PCNA is independent of ATR, but regulated by Claspin, a replication protein that mediates the activation of Chk1 by ATR. Like Claspin, Timeless and Rad17, two other Chk1 regulators at stressed replication forks, are also implicated in PCNA ubiquitination. These findings suggest that while ATR signaling and PCNA ubiquitination are two independent processes, they are mediated by a common group of proteins including Chk1 and it regulators at replication forks. Furthermore, these data raise the possibility that Chk1 and its regulators may constitute a functional module at replication forks to enable multiple stress responses.

Keywords

DNA damage; checkpoint; replication fork; PCNA; ATR; Chk1; ubiquitination

Introduction

The stability of DNA replication forks is crucial for genomic stability and cell survival. When DNA replication forks encounter DNA damage in the genome, they rely on several signaling and DNA repair/recombination mechanisms to overcome the impediment. Among these mechanisms are the DNA damage-signaling pathway regulated by the Ataxia Telangiectasia-mutated and Rad3-related (ATR) kinase, which is often referred to as the ATR checkpoint [1, 2], and the process of translesion DNA synthesis (TLS) mediated by the ubiquitinated form of PCNA [3]. Both the ATR pathway and TLS are important for continuous replication of damaged DNA and avoidance of fork collapse. The ATR pathway is critical for stabilizing stressed replication forks, whereas TLS allows replication forks to progress through certain types of DNA lesions.

The activation of the ATR pathway is regulated by specific DNA structures and DNA damage sensor proteins at stressed DNA replication forks [2]. Several components of normal replication forks are known to play important roles in mediating the signaling from ATR to its effector kinase Chk1. Like the signaling of ATR pathway, TLS is also induced by DNA replication

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stress. PCNA, a key structural and functional component of replication forks, is ubiquitinated in response to replication interference. The mono-ubiquitination of PCNA facilitates its association with several translesion DNA polymerases and may have additional functions [4–6]. Recent studies by others and us have revealed that ATR activation and PCNA ubiquitination are two independent processes but, surprisingly, are regulated by a common group of proteins at stressed DNA replication forks [7]. These studies have shed new lights on how multiple protective mechanisms are orchestrated by specific proteins and protein-DNA structures at stressed replication forks.

PCNA ubiquitination in human cells

In response to DNA damage, PCNA is either mono- or poly-ubiquitinated in yeast. In human cells, PCNA is predominantly mono-ubiquitinated after treatment with ultraviolet light (UV) or hydroxyurea (HU), an inhibitor of DNA replication. The mono-ubiquitination of PCNA is primarily mediated by the E2-E3 complex composed of Rad6 and Rad18[4, 5]. In cells, PCNA is ubiquitinated on chromatin after DNA damage. In an in vitro assay using purified proteins, PCNA is ubiquitinated by Rad6-Rad18 only when it is loaded onto DNA by RFC [8]. These results suggest that the loading of PCNA onto DNA is a prerequisite for its mono-ubiquitination. In response to UV damage, Rad18 and PCNA colocalize in nuclear foci [5], suggesting that the accumulation of Rad18 at stressed replication forks may be an important step for the induction of PCNA mono-ubiquitination.

The levels of PCNA mono-ubiquitination are also regulated by the de-ubiquitinase Usp1[9]. Depletion of Usp1 in human cells led to accumulation of mono-ubiquitinated PCNA even in the absence of DNA damage (Fig. 1A), suggesting that PCNA mono-ubiquitination may occur during the normal cell cycle, and that the steady-state levels of PCNA mono-ubiquitination are determined by the balance between Rad18 and Usp1. Mono-ubiquitinated PCNA has been detected in Xenopus extracts during unperturbed DNA replication [10]. Depletion of Usp1 also elevated the levels of ubiquitinated PCNA after DNA damage (Fig. 1A), suggesting that Usp1 counteracts Rad18 at stressed forks. Moreover, poly-ubiquitinated PCNA became readily detectable when Usp1 was removed (Fig. 1A), suggesting that Usp1 limits the poly-ubiquitination of PCNA in human cells. It has been shown that Usp1 is degraded after UV damage through autocleavage [9], providing another possible mechanism for the damage induction of PCNA ubiquitination.

Independent ATR activation and PCNA ubiquitination

The potential link between the ATR checkpoint and PCNA ubiquitination was first assessed in yeast. In fission yeast, neither Rad3 nor Tel1, the respective homologous of ATR and ATM (a kinase with overlapping functions with ATR in DNA damage signaling), is required for damage-induced PCNA ubiquitination [11]. Furthermore, elimination of the ubiquitination site of PCNA did not affect the phosphorylation of a Rad3 substrate, suggesting that PCNA ubiquitination is not needed for Rad3 activation [11]. In Xenopus extracts, inhibition or depletion of the ATR-ATRIP complex did not affect PCNA ubiquitination [12]. Consistent with these findings, we found that neither ATR nor ATM is required for UV- or HU-induced PCNA ubiquitination [2]. Together, these results strongly suggest that ATR activation and PCNA ubiquitination are two independent processes from yeast to humans.

Parallels between the ATR pathway and PCNA ubiquitination

1. RPA and ssDNA

While ATR signaling and PCNA ubiquitination are independent of each other, they are induced by a similar spectrum of DNA damage and replication stress, suggesting that they may share

common mechanisms for activation. In response to UV-induced DNA damage or replication inhibitors such as HU and aphidicolin, DNA synthesis on the leading and/or lagging strands of replication forks is interrupted, and the coordination between DNA helicase and DNA polymerases at replication forks is compromised [13]. These changes at stressed replication forks lead to accumulation of increased amounts of single-stranded DNA (ssDNA) at or behind the forks [14, 15]. The ssDNA induced by replication stress is rapidly coated by RPA, resulting in the RPA-ssDNA complex. RPA-ssDNA is directly recognized by ATRIP, the regulatory partner of ATR, thereby providing a landing pad for the ATR-ATRIP complex at stressed forks or ssDNA gaps [16, 17]. Like ATR activation, PCNA ubiquitination is also induced by uncoupling DNA helicase and polymerase in Xenopus extracts [12]. Rad18 is capable of binding ssDNA under mild conditions in vitro through its SAP domain [18]. However, depletion of RPA from human cells compromises PCNA ubiquitination [6, 19], suggesting that RPA contributes to Rad18 recruitment in vivo. Remarkably, RPA directly associates with Rad18 and stimulates its binding to ssDNA, providing a key mechanism by which Rad18 is recruited to stressed forks [20]. Thus, ATR and Rad18, the two central players for the ATR signaling pathway and the ubiquitination of PCNA, respectively, are both recruited by the same protein-DNA structure at stressed forks, RPA-ssDNA.

2. Rad17

During the process of ATR activation, RPA-ssDNA not only recruits the ATR-ATRIP kinase complex, but also its key regulators and substrates. One of the regulators of ATR is the RFC-like Rad17 complex [21, 22]. Once recruited to stressed forks by RPA-ssDNA, the Rad17 complex recognizes the junctions between single- and double-stranded DNA and loads the PCNA-like 9–1–1 (Rad9-Rad1-Hus1) complex onto DNA. The 9–1–1 complex interacts with TopBP1, a protein that directly stimulates the ATR-ATRIP kinase [23], providing a means for ATR activation at stressed forks. We found that the levels of ubiquitinated PCNA in cells treated Rad17 siRNA were reduced compared to those in control cells, suggesting that Rad17 and perhaps 9–1–1 contribute to PCNA ubiquitination (Fig. 1B). Interestingly, it was recently shown that the yeast homologue of human Rad9, like PCNA, is ubiquitinated by Rad6-Rad18[24]. These findings suggest that the Rad17 and 9–1–1 complexes may function in parallel with the RFC and PCNA complexes to recruit Rad6-Rad18 to stressed forks. Given that the loading of 9–1–1 by Rad17 is greatly stimulated by DNA damage, one interesting possibility is that 9–1–1 plays a role in recruiting Rad18 or delivering Rad18 to PCNA. Further studies are needed to assess this model.

3. Chk1 and Claspin

Chk1 is a key effector kinase of ATR that is required for stabilizing stressed replication forks. The phosphorylation of Chk1 by ATR not only requires the recruitment and activation of the ATR kinase, but also a number of replication proteins including Claspin, Timeless, and Tipin. Using siRNA to deplete Chk1 from human cells, we found that Chk1 is required for efficient PCNA ubiquitination [7]. Surprisingly, the compromised PCNA ubiquitination in Chk1-depleted cells was partially rescued by a kinase-defective Chk1 mutant, suggesting that the kinase activity of Chk1 is not essential for its function in PCNA ubiquitination. This novel function of Chk1 is at least in part attributed to its role in stabilizing the replication protein Claspin. Depletion of Claspin also leads to compromised PCNA ubiquitination in human cells, whereas overexpression of Claspin partially rescues PCNA ubiquitination in Chk1-depleted cells. Depletion of Timeless, a Claspin-associated protein, also results in reduced PCNA ubiquitination.

How do Chk1, Claspin, and Timeless contribute to PCNA ubiquitination? We found that depletion of Claspin from human cells significantly reduced the amounts of Rad18 on chromatin [7]. In addition, we showed that both Claspin and Timeless associated with PCNA in

human cells. The stability of Chk1 and Claspin relies on each other even in the absence of DNA damage [7], suggesting that they exist in a complex. It was recently reported that Chk1 interacts with PCNA through a PCNA-interacting-protein (PIP) box, and that the Chk1-PCNA interaction is important for both DNA replication and checkpoint response [25]. These findings suggest that Chk1, Claspin, and Timeless may associate with PCNA as a complex and facilitate the recruitment of Rad18 to PCNA. Furthermore, Tipin, a protein stably associated with Timeless, has been shown to bind RPA [26, 27]. This Tipin-RPA-ssDNA interaction may allow the complex above to contribute to the recruitment of Rad18 to RPA-ssDNA.

The functions of Chk1, Claspin, and Timesless in PCNA ubiquitination could also be explained by their roles in configuring replication forks. Mrc1 and Tof1, the budding yeast homologues of Claspin and Timeless, respectively, are important for maintaining stressed replication forks at the sites of DNA synthesis [28]. In the absence of Mrc1 or Tof1, replication forks disengage from nascent DNA strands when stressed by HU. If such an event occurs at sites of DNA lesion, the separation of replication proteins from the termini of impeded DNA strands would present a problem for TLS, and may affect the positioning of PCNA or the recruitment of Rad18. Mrc1 is also known to interact with the MCM helicase, Cdc45, and DNA polymerase ε (Pol ε), the polymerase functions on the leading strand of replication forks [29]. The lack of Mrc1 may compromise the coordinated movement of helicase and polymerase, or the coordination between the proteins on leading and lagging strands. These structural changes in replication forks may in turn affect the interaction between PCNA and its ubiquitination ligase. These possibilities remain to be assessed by further studies.

A multi-functional module at replication forks

The involvement of RPA, Rad17, Claspin, Timeless, and Chk1 in PCNA ubiquitination has revealed that these proteins play multiple roles in different stress responses at replication forks. Among these proteins, RPA and Rad17 recognize specific DNA structures induced at stressed replication forks. Both RPA-ssDNA and the DNA-bound 9–1–1 complexes loaded by Rad17 are important protein-DNA structures upon which the checkpoint-signaling complex is assembled [2]. Claspin, Timeless, and Chk1 function downstream of RPA and Rad17 in checkpoint signaling, suggesting that they may be part of the signaling complex organized by RPA-ssDNA, Rad17, and 9–1–1[30]. The interactions of Claspin, Timeless, and Chk1 with PCNA and their functions in PCNA ubiquitination suggest that PCNA may also contribute to the organization of this complex (Fig. 2). Furthermore, the interaction between Chk1 and PCNA, the role of Chk1 in stabilizing Claspin, and the involvement of Chk1 in fork progression in unperturbed cells have linked Chk1 to normal DNA replication [7, 25, 31, 32].

Yeast genetic studies have strongly suggested that Mrc1, Tof1, and Csm3 (the yeast homologue of Tipin) constitute a multi-functional module at replication forks [33]. These proteins are not only involved in checkpoint signaling, but also in replication fork progression, stable replication pausing, and sister chromatid cohesion [28, 34–38]. While these proteins clearly interact with each other, some functional differences between Mrc1 and Tof1 have been reported [37, 39, 40], suggesting that this module is not a static complex. Like their yeast homologues, human Claspin, Timeless, and Tipin have also been implicated in checkpoint signaling and fork progression [26, 41, 42]. Our finding that Claspin and Timeless contribute to PCNA ubiquitination independently of ATR suggests that similar multi-functional modules may exist in both yeast and human cells. In human cells, the stability of Clapsin and Chk1 is dependent on each other, thus linking Chk1 to this module [7]. Likewise, Timeless and Tipin rely on each other to be stable [7, 42, 43]. These findings suggest that the human module may be consisted of two complexes: Claspin-Chk1 and Timeless-Tipin. The presence of this pre-assembled module at replication forks may have important functions during normal replication,

and enable the forks to response swiftly to different stresses (Fig. 2). Further analyses of normal and stressed DNA replication forks both in vivo and in vitro are needed to test this model.

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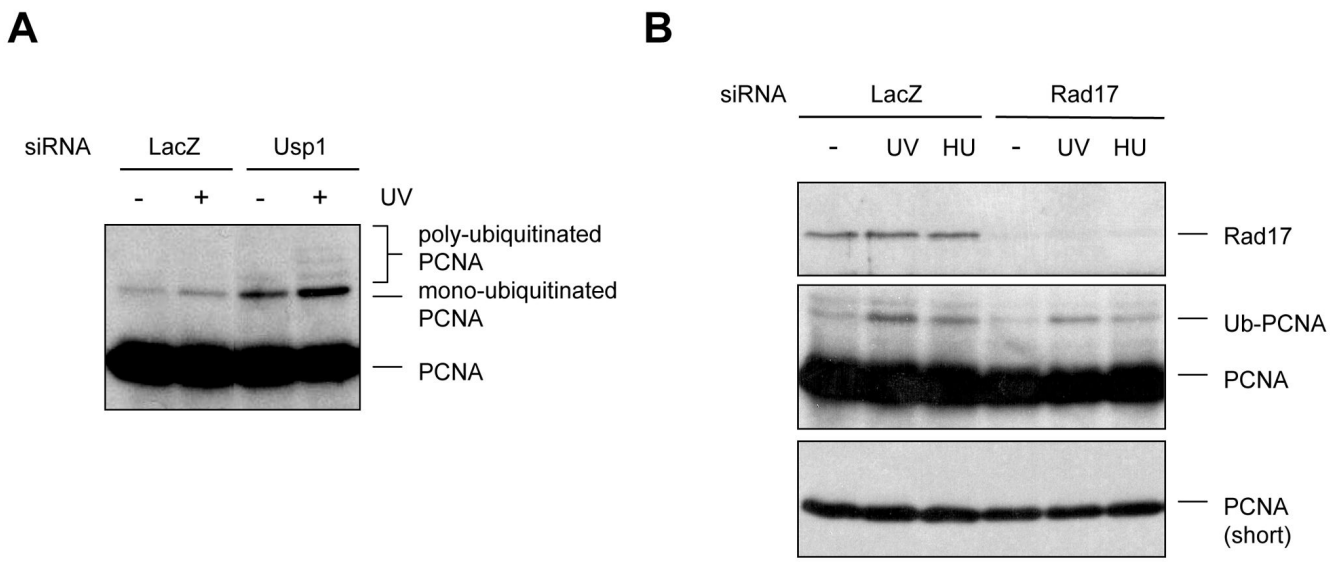

Figure 1.

Regulation of PCNA ubiquitination by Usp1 and Rad17. (A) Depletion of Usp1 leads to elevated levels of ubiquitinated PCNA in undamaged and damaged cells. HeLa cells transfected with Usp1 siRNA or control siRNA were treated with UV or left untreated. The unmodified and ubiquitinated PCNA was detected with an anti-PCNA antibody. (B) Depletion of Rad17 leads to reduced levels of ubiquitinated PCNA in UV- and HU-treated cells. Cells transfected with Rad17 siRNA or control siRNA were treated with UV and HU as indicated, and were analyzed by immunoblotting as above.

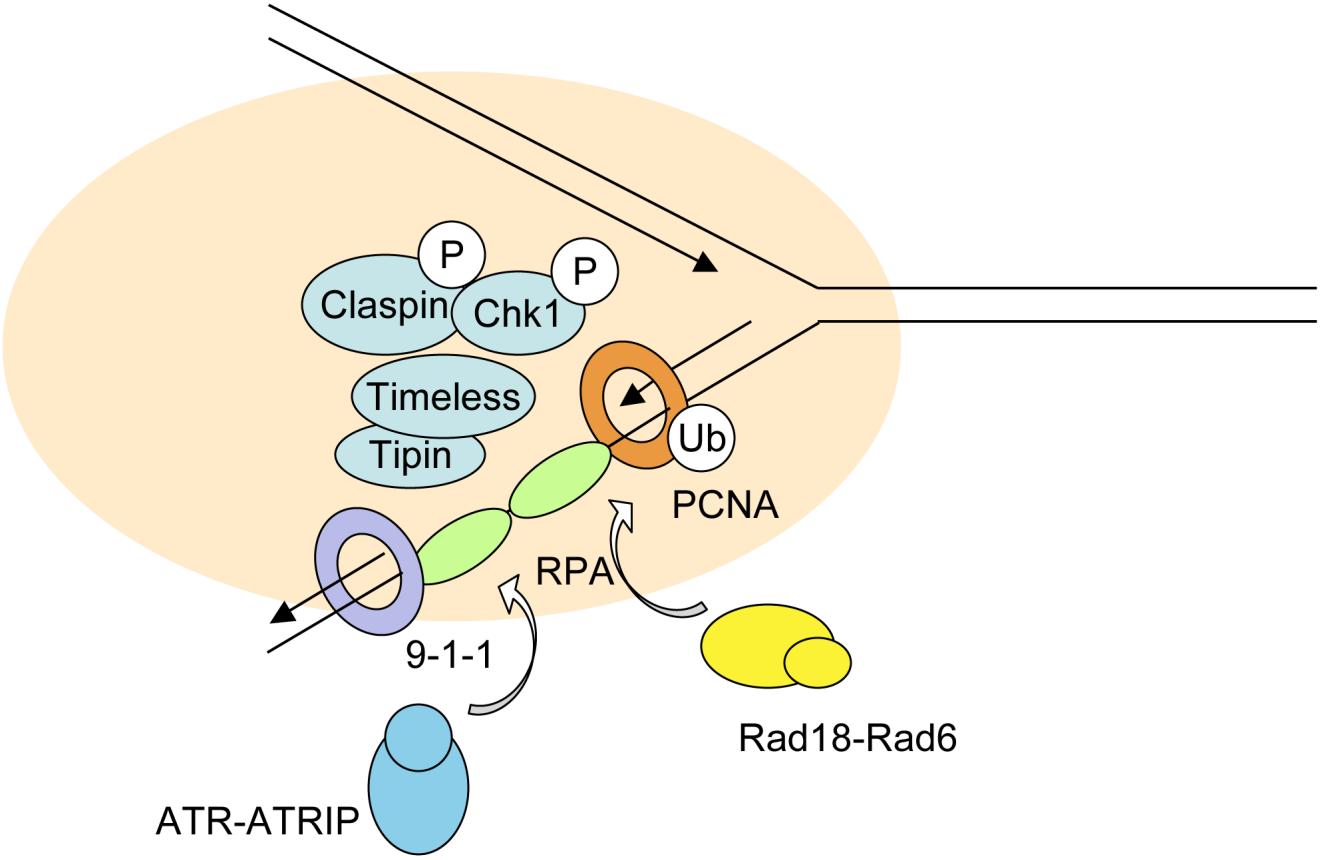

Figure 2.
A model for a multi-functional module at DNA replication forks. Chk1, Claspin, Timeless, and Tipin may constitute a functional module at replication forks. Through its interactions with RPA-ssDNA, 9–1–1, and PCNA, this module may play dual roles in ATR checkpoint signaling and PCNA ubiquitination.