MCM7 Ubiquitination Regulates CMG Helicase Disassembly in Human

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Research

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Abstract

Background

The disassembly of the replisome plays an essential role in maintaining genome stability at the termination of DNA replication. However, the mechanism of replisome disassembly remains unknown in human. In this study, we screened E3 ligases and deubiquitinases (DUBs) for the ubiquitination of minichromosome maintenance protein (MCM) 7 and provided evidence of this process driving CMG helicase disassembly in human tumor cells.

Methods

SILAC-MS/MS was analyzed to identify ubiquitinated proteins in HeLa cells. The ubiquitination/deubiquitylation assay *in vitro* and *in vivo* were detected by Western blot. Thymidine and HU were implied to synchronized cell cycle and detect the role of ubiquitinated MCM7 in cell cycle. Cell fractionation assay was used to detect the function of ubiquitination of MCM7 in chromatin and non-chromatin. Aphidicolin-Etoposide-ICRF-193 and IR were applied to cause replication fork stalling. MG-132 and NMS-873 were used to inhibit the proteasome degradation and p97 segregase. Flow cytometer and FlowJo flow cytometry software were used to cell cycle analysis.

Results

In our study, we found that the ubiquitin ligase RNF8 catalyzes the k63-linked poly-ubiquitination of MCM7 both *in vivo* and *in vitro*, and lysine 145 of MCM7 is the primary ubiquitination site. Moreover, the poly-ubiquitination of MCM7 mainly exists in the chromatin, which is dynamically regulated by the cell cycle, mainly occurs in the late S phase. And DNA damage can significantly reduce the poly-ubiquitylation of MCM7 in the late S phase. Furthermore, the proteasome, p97 segregase, USP29 and ATXN3 are required for the removal of MCM7 ubiquitination to promote the disassembly of CMG on chromatin.

Conclusions

In the late S phase of cell cycle, RNF8 catalyzes the poly-ubiquitination of MCM7, and then initiates the disassembly of CMG helicase from chromatin, which is mediated by p97, proteasome, USP29 and ATXN3 in human. We reveal the novel function of the poly-ubiquitylation of MCM7, which is a regulatory signal to control CMG complex unloading at replication termination sites.

Introduction

Replication in eukaryotic cells is precisely regulated. All chromosomes need to be duplicated only once in each cell cycle. The loading of the MCM2-7 DNA helicase into chromatin origins occurs during late mitosis and in the G1 phase, and the helicase is recruited by the origin recognition complex (ORC), Cdc6, and Cdt1 to assemble the pre-replicative complex (pre-RC)^[1, 2]. During the S phase in eukaryotes, MCM2-7 recruits Cdc45 and GINS to form the active replication fork helicase CMG, which unwinds double-stranded

DNA^[3, 4] and functions in initiation and elongation during replication^[5]. The termination of replication forks occurs when replication forks converge from neighboring origins in opposite directions. The removal of the replisome from fully duplicated DNA is the last stage of replication fork termination^[6].

In organisms, the polyubiquitylation of MCM7 is responsible for CMG complex unloading at replication termination sites. In late S phase, the K48-linked polyubiquitylation of MCM7 in *Saccharomyces cerevisiae* (by SCF^{Dia2})/*Xenopus* (by Cul2^{LRR1}) and *Caenorhabditis elegans* embryos (by Cul2^{LRR1}) drives the disassembly of the terminated CMG complex, which requires the activity of p97/VCP segregase^[7–10]. In mitosis, TRAIP catalyzes MCM7 ubiquitylation with K6- and K63-linked ubiquitin chains, which promote replisome disassembly to remove any replisome from chromatin before cell division in *Xenopus laevis* egg extract^[11]. When two replisomes converge at an interstrand DNA crosslink (ICL), TRAIP ubiquitylates MCM7 of CMG to activate CMG unloading and entry into the Fanconi anemia (FA) pathway in *Xenopus* egg extracts^[12]. A recent study in mouse embryonic stem cells found that ubiquitin ligases CUL2LRR1 and TRAIP control p97-dependent replisome disassembly during DNA replication termination and mitosis, respectively^[13]. However, the mechanism of human chromosomal replication dissociation is incompletely understood.

The E3 ubiquitin ligase RNF8 is a member of the RING finger family, which maintains genome integrity through participation in DNA damage repair. RNF8 functions in histone H2A and H2AX ubiquitination, which mediates the recruitment of 53BP1 and BRCA1 at sites of DNA damage to promote the DNA damage repair pathway^[14–21]. RNF8 physically interacts with TPP1 to generate K63-linked polyubiquitin chains that stabilize TPP1 at telomeres, which is essential for protection of telomere end integrity^[22]. In the late S/G2 phase, RNF8-mediated K63polyubiquitylation of tankyrase 1 promotes its stability and association with telomeres and then facilitates the resolution of sister telomere cohesion^[23]. RNF8 also regulates mitotic exit, and overexpression of RNF8 causes aberrant mitosis and unresolved cytokinesis^[24–26].

It was reported that human MCM7 can be polyubiquitylated in HEK293T cells, which is catalyzed by HPV-18E6 protein *in vivo* and *in vitro*^[27]. INT6 overexpression was found to increase polyubiquitylation of MCM7 on chromatin^[28]. We further explored the processes of MCM7 ubiquitination and the role of MCM7 ubiquitination in regulating CMG complex disassembly during replication.

Experimental Procedures

Plasmids, Antibodies, and Cell Culture

Full-length MCM7 amplified from human cells was subjected to RT-PCR and cloned into the Flag-pcDNA3.1 and myc-pRK5 vectors. Plasmids harboring the K28R and K145R mutations in MCM7 were generated by PCR-mediated mutagenesis. RNF8, deubiquitinating enzymes (DUBs) and shRNA of DUBs were cloned into plasmids as previously described^[29–31].

The antibodies used in the Western blotting assay were as follows: β -actin, FLAG, RNF8 (B-2), MCM7 (141.2), c-Myc (9E10), PCNA (PC10), and DO-1 from Santa Cruz Biotechnology; H2B, HA, GAPDH (D16H11), phospho-histone γ H2AX (Ser139), and Ubiquitin from Cell Signaling Technology; MCM2 from Sangon Biotech; CDC45 from Bimake; USP29 from ABclonal; and ATXN3 from Proteintech.

HEK293T, H1299, HeLa and U2OS cells were cultured in DMEM with 10% fetal bovine serum (Gibco). SiRNA transfection using Lipofectamine 3000 (Invitrogen) was performed according to the manufacturer's protocol. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The sequence of USP29 siRNA#1:5'-CCCAUCAAGUUUAGAGGAUTT-3'; #2:5'-GGAUAUAGCUGAAGGAAAUTT-3'. ATXN3 siRNA#1:5'-GCAGGGCUAUUCAGCUAAGTT-3'; #2:5'-GCAGAUGAUUAGGGUCCAATT-3'. USP50 siRNA#1:5'-CAACACAUGCUGCGUGAAUTT-3'; #2:5'-CUACCCAGCAUUUACGAAATT-3'. MINDY2 siRNA#1:5'-CCGAUGAUGGAAAUCAUAATT-3'; #2:5'-GCACCAUGACCAAAUACAATT-3'; #3:5'-GCUGGUUAGUGAAGGCUUUTT-3'. USP53 siRNA#1:5'-GAGCCAACAUCACUUAGAATT-3'; #2:5'-GUGCGGUACAUUUCUACAATT-3'; #3:5'-GUGCUCUGAAAGCUAUUGATT-3'.

Silac-ms/ms To Identify Ubiquitinated Proteins In Hela Cells

HeLa cells were labeled with "heavy" or "light" isotopic lysine using a SILAC Protein Quantitation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, two cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum and with either the "heavy" (H) form of Lys⁸Arg¹⁰ or "light" (L) Lys⁰Arg⁰ for eight passages before being used in the assay. Cells, either without or with IR treatment (10 Gy, 2 h), were harvested and lysed in RIPA buffer (40 mM Tris, pH 8.0, 200 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 1% SDS) on ice for 20 min. Equal amounts of protein from cells in the no-IR group and IR group were mixed. The proteins were digested with trypsin. To enrich di-GG-ubiquitinated peptides, the tryptic peptides were incubated with anti-di-GG agarose beads (PTM Biolabs Inc., Chicago, IL, USA) at 4°C for 4 h with gentle shaking. The beads were washed four times and the bound peptides were eluted from the beads with 1% trifluoroacetic acid. HPLC/MS/MS analysis was performed. The ratio of H/L peptides was normalized by eliminating the impact of protein level change. The results are shown in Supplementary Table 1.

Western Blotting Analysis

Cells were lysed in radioimmune precipitation assay (RIPA) buffer (20 mM Tris-HCl pH7.3, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.05% SDS, 5 mM EDTA, and freshly supplemented proteinase inhibitor mixture) on ice for 30 minutes, followed by centrifugation for 15 minutes at 15 000 rpm and 4°C. The supernatant was used for total protein detection.

M2 Beads Pull Down

Cells were transfected with Flag-tagged plasmids and harvested after 24-48 h of transfection. Cells were lysed in cold BC500 buffer (20 mM Tris-HCl pH7.3, 500 mM NaCl, 1.5 mM MgCl₂, 10 mM KCl, 0.5% Triton, 20% glycerol), sonicated for 5 minutes and incubated with ANTI-FLAG M2 Agarose (Sigma) at 4°C overnight. After washing the beads twice in BC500 buffer and three times in BC100 buffer (20 mM Tris-HCl pH7.3, 100 mM NaCl, 1.5 mM MgCl₂, 10 mM KCl, 0.1% Triton X-100, 20% glycerol), the proteins bound to the beads were eluted with FLAG peptide to obtain active proteins or eluted by boiling in 2× SDS-PAGE sample loading buffer for 5 minutes to obtain denatured proteins.

Cell Synchronization

Cells were synchronized at the G1/S boundary by incubation with thymidine or hydroxyurea (HU). For thymidine blockade, thymidine (2 mM) was added to the cells at 30% confluence, and the cells were incubated at 37°C for 14 h. Then, thymidine was removed by washing the cells with prewarmed PBS, prewarmed (37°C) fresh medium was added, and the cells were incubated for 10 h. Subsequently, a second round of thymidine (2 mM) was added, and the cells were incubated for another 14 h. At this point, the cells were synchronized at the G1/S boundary. Cells were released by washing with prewarmed PBS and incubating cells with prewarmed fresh medium. For HU blockade, HU (2 mM) was added to the cells at 40% confluence, and the cells were incubated at 37°C for 18 h. Then, the HU was removed by washing the cells with prewarmed PBS, and prewarmed fresh medium was added for release. Cells were harvested at the indicated time after block release for Western blotting analysis and flow cytometry.

Chromatin Fractionation Analysis

Cells were harvested and subsequently were lysed with CSK buffer (10 mM HEPES pH 7.9, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100) containing 300 µg/ml RNase A. Then, the lysates were centrifuged at 5 000 rpm for 5 minutes at 4°C. The supernatant was collected as the non-chromatin fraction. The pellet enriched with chromatin-bound proteins was resuspended in CSK buffer, and the above procedures were repeated three times. The supernatant and the pellet were lysed with the indicated buffer, sonicated and centrifuged at 15 000 rpm for 10 minutes at 4°C. The protein concentration was measured by the Bradford assay, and the sample was then used for the Ni-NTA pull down assay or M2 beads pull down assay.

Ni-nta Pull Down

Cells were transfected with the indicated plasmids and harvested after 24-48 h of transfection. The cells were lysed in buffer P1 (6 M guanidinium-HCl, 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 10 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 5 mM imidazole, and 0.2% Triton X-100), followed by sonication for 5 minutes and incubation with Ni-NTA agarose overnight at 4°C. The beads were washed once with buffer P1, twice with buffer P2 (8 M urea, 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 10 mM Tris-HCl pH 8.0, 10 mM β-

mercaptoethanol, 5 mM imidazole, 0.2% Triton X-100), and three times with buffer P3 (8 M urea, 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄, 10 mM Tris-HCl pH 6.3, 10 mM β-mercaptoethanol, 5 mM imidazole, 0.2% Triton X-100). Proteins were eluted by boiling the beads in 2× SDS-PAGE sample loading buffer for 5 min. The eluted proteins were analyzed by immunoblotting.

In Vitro Ubiquitination Assay

An *in vitro* ubiquitination assay was performed with 20 ng of recombinant human ubiquitin-activating enzyme (UBE1; Boston Biochem), 50 ng of recombinant human Ubch5c/UBE2D3 (Boston Biochem), 2 μg of recombinant human ubiquitin (Boston Biochem), purified E3 ubiquitin ligase and substrate in ubiquitylation buffer (40 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 2 mM ATP, 2 mM DTT). Reactions were carried out in a final volume of 30 μl for 1 h at 37°C and terminated by the addition of 4× SDS sample loading buffer. The reaction products were analyzed by immunoblotting.

In Vitro Deubiquitylation Assay

An *in vitro* deubiquitylation assay was performed by DUBs and ubiquitin-MCM7 purified from HEK293T cells. Reactions were carried out in deubiquitylation buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, and 5% glycerol) for 1 hour at 37°C.

Flow Cytometry

Cells were digested with trypsin, resuspended in PBS, and fixed in ethanol (75%) at -20°C overnight. Then, the cells were washed three times with PBS, and RNase A (100 ng/μl) was added for 15 min. The nuclei were stained with propidium iodide for 10 min. The DNA content was determined on a flow cytometer and analyzed by FlowJo flow cytometry software.

Results

1. MCM7 is ubiquitylated by RNF8 in vivo and in vitro

To detect protein ubiquitination in the ionizing radiation (IR) induced DNA damage response pathway, we applied stable isotope labeling with amino acids in cell culture (SILAC) and mass spectrometry (MS) analysis in HeLa cells, which showed that the ubiquitination of MCM7 was obviously decreased, typically at Lys 145 (**Supplementary Table 1**). Due to E3 ligase RNF8 playing an essential role in DSB repair, we hypothesized that whether RNF8 can catalyze MCM7 ubiquitination. To test this hypothesis, H1299 cells were transiently transfected with plasmids expressing His-ubiquitin (Ub), myc-MCM7, and HA-RNF8. Western blotting analysis showed that polyubiquitin-conjugated MCM7 was bound to Ni-NTA resin (Figure 1A). Additionally, we observed the same result in HEK293T cells (Figure 1B). To confirm that RNF8 ubiquitylates MCM7, we performed a FLAG-M2 agarose immunoprecipitation assay. HEK293T cells were transiently transfected with plasmids expressing Flag-MCM7, HA-Ub, and RNF8, which showed that

MCM7 was ubiquitinated by antibodies against Flag and Ub (Figure 1C). To further verify that RNF8 ubiquitylates MCM7 *in vitro*, we purified Flag-RNF8 from HEK293T cells as the E3 ligase, pET-Flag-MCM7 from the *Escherichia coli* strain Rosetta (Figure 1D) and Flag-MCM7 from HEK293T cells (Figure 1E) as the substrate. *In vitro* ubiquitination assay showed that RNF8 catalyzed the polyubiquitylation of MCM7 directly (Figure 1D-1E), but not the RING-inactive mutant RNF8 C403S (Figure 1F). According to the HPLC-MS/MS results, the ubiquitination of MCM7 occurred mainly at Lys 28 and Lys 145. Next, we generated plasmids harboring site-specific mutations in MCM7, namely, MCM7-K28R, MCM7-K145R and MCM7-K28R+K145R. The results showed that RNF8 catalyzes the polyubiquitylation of MCM7 specifically on Lys 145 *in vivo* (Figure 1G) and *in vitro* (Figure 2A). Correspondingly, knockdown of RNF8 suppressed the polyubiquitylation of MCM7 in U2OS cells (Figure 2B). Together, we identified that the E3 ligase RNF8 contributes for the polyubiquitylation of MCM7. Interesting, we also detected that MCM7 is highly ubiquitinated in the absence of RNF8 overexpression, which may be catalyzed by endogenous RNF8 or other E3s.

2. RNF8 dependent polyubiquitylation of MCM7 occurs on chromatin in the late S phase of the cell cycle

In the late M and G1 phases, the MCM2-7 DNA helicase is loaded at chromatin origins and gradually dissociates from chromatin during DNA replication in the S phase, although the total amount of MCM7 protein in the nucleus remains relatively constant^[32]. To identify the distribution of ubiquitinated MCM7, we performed a chromatin fractionation assay with CSK buffer. We found that polyubiquitylation of MCM7 catalyzed by RNF8 occurred mainly in chromatin (Figure 2C-2E). Given that the association of MCMs with chromatin is cell cycle regulated, we synchronized HeLa cells at the G1-S boundary with double thymidine block, which showed that cells entered S phase during 2-6 h and the G2-M phase during 8-10 h after block release respectively (Figure 2F). Then, we isolated chromatin extraction from synchronized HeLa cells by double thymidine block and performed M2 beads pull down analysis, which showed that polyubiquitylation of MCM7 increased greatly but transiently after 6 h of release (Figure 3A), while the total amount of chromatin-bound MCM7 gradually diminished from the G1 phase to late M phase of the cell cycle (Figure 3A). In synchronized HeLa cells blocked by HU, polyubiquitylation of MCM7 was transiently increased after 5 h of release (Figure 3B), suggesting that ubiquitylation of MCM7 also occurs in the late S phase. This is slightly different to thymidine double block, which showed polyubiquitylation of MCM7 was transiently increased after 6 h of release. This may be caused by the difference of cell cycle progression after block and release by thymidine and HU. In conclusion, we showed that polyubiquitylation of MCM7 mediated by RNF8 occurs only transiently *in vivo* and is mainly involved in the final stages of DNA replication. This is consistent with previous reports in *S. cerevisiae* and *Xenopus*^[7, 8]. Moreover, the polyubiquitylation of MCM7 also occurred at the G1-S boundary and in the late M phase, suggesting an unknown function in the cell cycle, which remains to be further investigated.

3. MCM7 is polyubiquitylated by RNF8 with K63-linked ubiquitin chains

Differently linked ubiquitin chains have distinct topologies and cellular functions. In *S. cerevisiae* (by SCF^{Dia2}) and *X. laevis* (by Cul2^{LR1}), MCM7 is modified with K48-linked ubiquitin chains, which leads to replisome disassembly in the late S phase^[7, 8]. MCM7 is catalyzed by TRAP1 to form K6- and K63-linked ubiquitin chains, which promote the mitotic disassembly pathway in *X. laevis*^[11]. To uncover the novel function of ubiquitinated MCM7 in human tumor cells, we detected the formation of the ubiquitin chains of MCM7 mediated by RNF8. To determine the half-life of MCM7, U2OS cells were treated with cycloheximide (CHX), an inhibitor of eukaryotic protein synthesis, while the total amount of MCM7 remained stable (Figure 3C). To determine whether proteasome activity influences the lifespan of MCM7, U2OS cells were treated with MG-132, while we did not detect obvious degradation (Figure 3D). OTU DUBs (ovarian tumor-associated proteases domain-containing proteins) recognize and hydrolyze specific ubiquitin chain types and can be used to identify the linkage types on a ubiquitinated substrate^[33]. To investigate linkage-specific polyubiquitin conjugation of MCM7 catalyzed by RNF8, we performed OTU DUBs to cleave the ubiquitin chain of MCM7 *in vitro*. The results showed that OTUD1 greatly cleaved the polyubiquitylation chains of MCM7 (Figure 3E). OTUD1 is highly active and specifically cleaves the K63-linked ubiquitin chain^[33], suggesting that MCM7 ubiquitination catalyzed by RNF8 forms the K63-linked ubiquitin chain. Consistent with this notion, the K63-linked ubiquitin chain of MCM7 was enriched by K63-UIM in U2OS cells (Figure 3F). In HEK293T cells, the overexpression of His-Ub mutant K63R plasmid decreased polyubiquitylation of MCM7 mediated by RNF8 (Figure 3G). In conclusion, MCM7 is polyubiquitylated by RNF8 with K63-linked ubiquitin chains.

4. Rnf168 And Brca1 Promote The Polyubiquitylation Of Mcm7

At double-strand breaks (DSBs) induced by IR, RNF8 is responsible for the initiation of K63-linked ubiquitylation in the DNA damage response, which is subsequently amplified by RNF168 for the further recruitment of BRCA1, which is the regulator of the DSB HR response^[14–21]. During ICL repair-mediated unloading in *Xenopus*, BRCA1 acts upstream of MCM7 polyubiquitylation and recruits p97 to promote CMG unloading^[34, 35]. Therefore, we determined whether RNF168 and BRCA1 promote the ubiquitylation of MCM7. We used HeLa cells for overexpression of plasmids containing myc-MCM7, His-Ub and Flag-RNF168 or Flag-BRCA1 and found that both promoted the ubiquitylation of MCM7 *in vivo* (Figure 4A and 4C). However, we did not detect RNF168 mediated polyubiquitylation *in vitro* (Figure 4B), suggesting the function of RNF168 may be not directly regulated. In chromatin fractionation, the results showed that polyubiquitylation of MCM7 mediated by RNF168 and BRCA1 occurred on chromatin (Figure 4D). In synchronized HeLa cells blocked by HU, polyubiquitylation of MCM7 promoted by RNF168 was at the G1/S boundary and slightly increase after 6-7h of block release (Figure 4E). In addition, polyubiquitylation of MCM7 catalyzed by BRCA1 was transiently increased after 7h of release (Figure 4F), suggesting that ubiquitylated MCM7 occurs mainly in the late S phase or at the S/G2 boundary. Together, these results show that RNF168 and BRCA1 play a role in promoting polyubiquitylation of MCM7 in chromatin during termination of DNA replication. Consistent with the result for RNF8, the polyubiquitylation of MCM7

mediated by RNF168 and BRCA1 also occurs at the G1-S boundary and in the late M phase. The molecular basis of this regulation remains to be determined.

5. Inhibition of replication by DNA damage obviously reduces polyubiquitylation of MCM7 mediated by RNF8

DNA damage caused by physical genotoxic agents and chemical agents can induce genome instability, which causes fork replication stalling or collapse, disturbs replication fork progression, and triggers cell cycle arrest. Aphidicolin is an inhibitor of DNA polymerase α that blocks DNA replication in the S phase^[36]. IR can induce single-strand breaks (SSBs), DSBs, and base damage^[37]. Doxorubicin (Adriamycin) HCl blocks DNA synthesis by inserting itself into DNA and inhibiting DNA topology isomerase II^[38]. Actinomycin D inhibits the initiation of DNA replication in mammalian cells^[39]. DNA topoisomerase II (Top2) modulates the topological state of double-stranded DNA and allows the completion of DNA replication^[40]. Both etoposide and ICRF-193 inhibit the activity of Top2, blocking replication fork termination between replisomes and the accumulation of blocked forks on chromatin in the late S phase^[41, 42].

To determine whether the polyubiquitylation of MCM7 occurs in the final stage of DNA synthesis, we treated HeLa cells with HU to synchronize the cell cycle. After 2.5 h of release, HeLa cells were treated with various physical genotoxic agents and chemical agents, causing early-S phase and late-S phase damage in DNA replication. Because CMG unloading involves polyubiquitylation of CMG's MCM7 subunit, we detected the ubiquitinated MCM7 and CMG disassembly on chromatin after 4-7 h of block release. The results showed that DNA damage significantly reduces the polyubiquitylation of MCM7 but increases the amount of MCM2 and MCM7 in chromatin extraction (Figure 5A-5F), suggesting prolonged association of replicative CMG helicase with chromatin. At the same time, flow cytometric analysis also showed that DNA damage caused S phase blockade (Figure 6A). In particular, the Top2 inhibitor ICRF-193 arrested cells at the late S phase (Figure 6A), consistent with the decreased ubiquitination of MCM7 (Figure 5F), which further verified that the ubiquitination of MCM7 occurred at the end of DNA replication. These results suggest that DNA damage can significantly reduce the polyubiquitylation of MCM7 in late S phase, suggesting that polyubiquitylation of MCM7 occurs only when DNA replication can be completely duplicated.

6. Proteasome and Cdc48/p97 segregase are required for MCM7 deubiquitylation and CMG disassembly

In *X. laevis* and *S. cerevisiae*, MCM7 deubiquitylation during replication termination depends on p97/Cdc48/VCP segregase but not proteasomal degradation^[7, 8]. MCM7 polyubiquitylation acts as the signal for p97-mediated extraction and unloading of the CMG complex from chromatin^[7, 8, 10, 13]. To investigate the mechanism of MCM7 deubiquitylation in human tumor cells, we inhibited proteasomal degradation with MG-132 treatment, which showed increased ubiquitination of MCM7 and prolonged CMG association on chromatin in the late S phase (Figure 6B). Blocking p97-mediated polyubiquitylation segregation by NMS-873 also resulted in increased polyubiquitylation of MCM7 and prolonged

association of the helicase components MCM2, CDC45 and PCNA with chromatin (Figure 6C). Moreover, flow cytometry analysis showed that cells treated with MG-132 or NMS-873 were arrested in the S phase (Figure 6D). Together, these results suggest that deubiquitylation of MCM7 mediated by p97 and proteasomal degradation could drive the disassembly of the replicative helicase during termination. Consistent with the results for *S. cerevisiae*, *X. laevis*, *C. elegans* embryos and mouse embryonic stem cells^[7, 8, 13], we identified that polyubiquitylation of MCM7 plays a conserved role in regulating replisome disassembly from chromatin in humans.

7. USP29 and ATXN3 promote MCM7 deubiquitylation and CMG disassembly

Substrate release from the p97 complex requires the cooperation of a DUB, which trims polyubiquitin to an oligoubiquitin chain that is then translocated through the pore^[43]. To further explore the mechanism of CMG disassembly, we systematically screened the DUBs that are responsible for deubiquitylation of MCM7 *in vitro* and *in vivo*. We purified ubiquitinated MCM7 and 77 DUBs for the *in vitro* deubiquitylation assay, which showed that USP7, USP8, USP12, USP15, USP18, USP19, USP20, USP28, USP29, USP30, USP31, USP33, USP36, USP37, DUB3, USP45, OTUD1, ATXN3, JOSD2, and OTUD6A can deubiquitylate MCM7 (**Figure S1A**). Therefore, we designed shRNAs targeting these DUBs for knockdown experiments *in vivo*. Due to the lack of partial DUB plasmids, we also designed shRNAs targeting MINDY1, MINDY2, MINDY3, MINDY4, USP50, USP53 and ZRANB1. Then, HEK293T cells were used to package lentivirus, and U2OS cells were infected. The results showed that USP29, USP50, USP53, MINDY2 and ATXN3 could remove the polyubiquitylation of MCM7 *in vivo* (**Figure S1B**). To further identify the DUBs of MCM7, we knocked down USP29, USP50, USP53, ATXN3 and MINDY2 in U2OS cells with the indicated siRNAs and found that USP29 and ATXN3 were more efficient at catalyzing the deubiquitylation of MCM7 (Figure 7A). In U2OS cells synchronized by HU, knockdown of USP29 and ATXN3 increased the polyubiquitylation of MCM7 and prolonged its accumulation on chromatin. Moreover, we detected the inhibition of replisome disassembly (Figure 7B). Correspondingly, knocking down RNF8, USP29 and ATXN3 perturbed the cell cycle and arrested S phase progression (Figure 7C-7D). Therefore, USP29 and ATXN3 promote MCM7 deubiquitylation and CMG disassembly (Figure 7E).

Discussion

CMG helicase disassembly is conserved across evolution from yeasts to humans. Here, we identified that MCM7 of the MCMs protein family is controlled by ubiquitination and deubiquitylation. We proposed a model in which at the end of DNA replication, RNF8 is recruited to catalyze the polyubiquitylation of MCM7, which is the signal for the p97 segregase, proteasome, USP29 and ATXN3 to promote the disassembly of terminated replisomes by removing the ubiquitination of MCM7. However, whether the effect of CMG disassembly is due to direct regulation of ubiquitinated MCM7 is unclear.

Given that the RNF8-mediated polyubiquitylation of MCM7 occurs mainly at Lys 145, we attempted to construct cell lines with mutation of MCM7 at Lys 145 to directly detect the role of polyubiquitylation of MCM7 in the disassembly of CMG. We applied adenine base editing by xCas9-ABE for A·T to G·C

conversion, which can theoretically mutate the codon AAG of MCM7 at Lys 145 to the codon GAG/GGG/AGG. After clonal cell culture and gene sequencing of the PCR products, we screened nearly 100 cell lines, but no mutant clone was established. One possible explanation is that mutation of MCM7 at Lys 145 severely disrupted cell survival or that the mutation efficiency of xCas9-ABE base editing at Lys 145 was not sufficient. Therefore, we lack direct evidence to prove that polyubiquitylation of MCM7 drives the disassembly of CMG.

In the ubiquitin-proteasome system, p97 induces the initial unfolding of ubiquitylated substrates to prepare them for subsequent proteasomal degradation^[44]. p97 extracts and segregates multimeric complexes that are well folded or located in cell membranes or chromatin, such as the ring-shaped protein complex Ku70/80 (Ku) and CMG complex^[7, 8, 45–47]. In *S. cerevisiae/Xenopus* and *C. elegans* embryos, the p97-Ufd1-Npl4 complex disassembles the entire MCM complex by extracting the ubiquitin-Mcm7 subunit, while the proteasome plays a minor role^[47, 7, 8]. We found that both the proteasome and p97 segregase modulate the deubiquitylation of MCM7 and the CMG disassembly pathway. MCM7 is catalytically attached to a long polyubiquitin chain in human tumor cells, suggesting that p97 may play a role in ubiquitin chain unfolding to facilitate the subsequent deubiquitylation of MCM7 and proteasome degradation, which remains to be verified. In our study, we did not directly prove that MCM7 is a substrate of proteases and p97 in the late S phase. Proteases and p97 usually have many substrates. Therefore, the process of proteases and p97 regulating the deubiquitination of MCM7 is not clear. It was shown that the inhibition of proteasome and p97 also led to increased total levels of RNF8 and ubiquitinated RNF8, which may cause the increase of ubiquitinated MCM7^[48]. Therefore, it will be important in subsequent studies to characterize the coordination of p97 segregase, proteasome and DUB in promoting MCM7 deubiquitylation and CMG helicase disassembly.

We identified that RNF8, RNF168 and BRCA1 are required for the polyubiquitylation of MCM7. The difference of catalyzation process mediated by RNF8, RNF168 and BRCA1 remains to be determined. These E3s are necessary for the DSB-induced ubiquitination cascade. Moreover, TRAIP ubiquitin ligase-mediated ubiquitin-MCM7 promotes the mitotic pathway of replisome disassembly and certain forms of DNA damage, such as ICL^[11, 12]. Therefore, we hypothesize that replication termination may utilize a similar mechanism to displace the CMG complex from chromatin, such as DNA damage repair. In our study, we found that the polyubiquitylation of MCM7 mediated by RNF8 and BRCA1 also occurred in the late M phase (Figure 3A and Figure 4F), indicating that human tumor cells may have a mitotic pathway for CMG helicase disassembly, analogous to the process of mitotic CMG disassembly that was originally observed in *C. elegans* embryos and *X. laevis* egg extracts^[10, 11]. However, the polyubiquitinated MCM7 at the G1-S boundary (Figure 3A and 4F) have not been reported, which remains to be further investigated. In mouse embryonic stem cells, study found that ubiquitin ligases CUL2LRR1 and TRAIP control p97-dependent replisome disassembly during DNA replication termination and mitosis, respectively^[13], which may also regulate the ubiquitination of MCM7 in Human, and is worth for further investigation.

In conclusion, in the late S phase of cell cycle, RNF8 catalyzes the poly-ubiquitination of MCM7, and then initiates the disassembly of CMG helicase from chromatin, which is mediated by p97, proteasome, USP29 and ATXN3. Altogether, our founding provides insight into the mechanism of replisome disassembly during DNA replication termination in human. We reveal the novel function of the poly-ubiquitylation of MCM7, which is a regulatory signal to control CMG complex unloading at replication termination sites.

Abbreviations

CDC45: Cell division cycle 45

CDK: Cyclin-dependent kinases

CMG: CDC45-MCM2-7-GINS

DSBs: Double-strand breaks

MINDYs: Motif interacting with Ub-containing novel DUB family

MS: Mass spectrometry

MCM: Mini-chromosome maintenance protein

HU: Hydroxyurea

UIM: Ubiquitin interacting motif

SILAC: Stable isotope labeling with amino acids in cell culture

SCF: Skp, Cullin, F-box containing complex

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: The authors are consent for publication.

Availability of data and material: All data and material are available on reasonable request.

Competing interests: The authors declare that they have no conflict of interest.

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Figures

Figure 1

MCM7 is ubiquitinated by RNF8 in vivo and in vitro. A. RNF8 promoted MCM7 ubiquitination in vivo. H1299 cells were transfected with plasmids encoding His-Ub, myc-MCM7 and HA-RNF8 at a ratio of 1:1:2, and were subject to Ni-NTA pull down assay. Western blotting assays were performed with MCM7 antibody. The cell lysate extracted by RIPA buffer was probed with RNF8, myc and Actin antibodies. B. HEK293T cells were transfected and analyzed as described in Figure 1A. C. HEK293T cells were

transfected with plasmids encoding HA-Ub, Flag-MCM7 and RNF8 at a ratio of 1:1:2. After 24-48 hours of transfection, cells were harvested and lysed. Flag tagged proteins were purified with the M2 beads pull down assay from the lysates. Western blotting assays were performed with MCM7 and Ub antibodies. The cell lysate was probed via Western blotting using anti-RNF8 and anti-Flag antibodies. D. RNF8 catalyzed MCM7 ubiquitination in vitro. Ubiquitylation assays were performed at 37°C for 1 h in the presence of UBE1, E2 (UbcH5c), Ub, Flag-RNF8, and pET-Flag-MCM7 purified from the E. coli strain Rosetta. Reaction mixtures were analyzed by Western blotting with MCM7, Ub and RNF8 antibodies. E. Flag-MCM7 purified from HEK293T cells was used as the substrate in vitro ubiquitination assay, and analyzed with MCM7 antibody. F. Reactions were performed as described in Figure1D. Flag-RNF8 and Flag-RNF8 C403S were purified from HEK293T cells. G. HEK293T cells were transfected with plasmids encoding MCM7, MCM7-K28R, MCM7-K145R and MCM7-K28R+K145R. cells were harvested and lysed. Flag tagged proteins were purified with the M2 beads pull down assay from the lysates. The polyubiquitylated form of MCM7 was detected with an anti-ubiquitin antibody.

Figure 2

RNF8 dependent polyubiquitylation of MCM7 occurs on chromatin in the late S phase of the cell cycle. A. An in vitro ubiquitylation reaction was carried out using purified Flag-MCM7, Flag-MCM7-K28R, Flag-MCM7-K145R and Flag-MCM7-K28R+K145R from HEK293T cells as substrates. The polyubiquitylated form of MCM7 was detected with an anti-MCM7 antibody. B. RNF8 knockdown decreased MCM7 ubiquitylation. U2OS cells were transfected with His-Ub and a control or RNF8 siRNA. The proteins were

purified and enriched by Ni-NTA agarose and probed with antibodies against MCM7. C. RNF8 dependent polyubiquitylation of MCM7 occurs on chromatin. HeLa cells were co-transfected with plasmids encoding Flag-MCM7, RNF8 and HA-Ub and lysed in CSK buffer. The Flag-tagged proteins of the non-chromatin and chromatin fractions were enriched by M2 beads pull down assay, and detected with Ub and MCM7 antibodies. D. HeLa cells were co-transfected with plasmids encoding myc-MCM7, HA-RNF8 and His-Ub, and fractioned in CSK buffer. The His-tagged proteins of the non-chromatin and chromatin fractions were enriched by Ni-NTA pull down assay, and detected with MCM7 and myc antibodies. E. HEK293T cells were co-transfected with myc-MCM7, HA-RNF8 and His-Ub plasmids and lysed in CSK buffer. Samples were purified and blotted as shown in Figure 2D. F. HeLa cells were synchronized by double thymidine block and harvested at the indicated increasing time intervals after block release. The DNA content was measured by propidium iodide staining and flow cytometry.

Figure 3

Figure 3

MCM7 was polyubiquitylated by RNF8 with K63-linked ubiquitin chains. A. HeLa cells were co-transfected with Flag-MCM7, RNF8 and HA-Ub plasmids. After 24 hours of transfection, the cells were treated with double thymidine block for synchronization. Chromatin-bound proteins were extracted and subjected to M2 beads pull down assay, which was detected with Ub and MCM7 antibodies. Chromatin extraction in RIPA buffer were probed with γ -H2AX, MCM2, MCM7, PCNA, and CDC45 antibodies. B. HeLa cells were co-

transfected with Flag-MCM7, RNF8 and HA-Ub and synchronized by HU blockade. Chromatin-bound proteins were extracted and blotted as shown in Figure 3A. A. To determine the half-life of MCM7, U2OS cells were treated with CHX (10 µg/ml) and lysed in RIPA buffer. Western blotting was carried out with MCM7 antibody, and DO-1 anti-p53 antibody was used as a positive control. B. To inhibit the proteasome, U2OS cells were treated with the proteasome inhibitor MG-132 (20 µM) and lysed in RIPA buffer to detect MCM7 by Western blotting analysis. C. OTUD1 removed polyubiquitylation of MCM7. HEK293T cells were transfected with plasmids of OTU DUBs to purify active DUBs, and transfected with HA-Ub, Flag-MCM7, and RNF8 for the purification of ubiquitinated MCM7. An in vitro deubiquitylation assay was performed and analyzed with Ub antibody. D. MCM7 was polyubiquitylated with K63-linked ubiquitin chains. U2OS cells were transfected with the indicated plasmids and harvested for the K63-UIM pull down assay[30], analyzed with MCM7 and myc antibodies. E. HEK293T cells were transfected with indicated plasmids, and were subject to Ni-NTA pull down assay, which was detected with MCM7 antibody.

Figure 4

Figure 4

RNF168 and BRCA1 promote the polyubiquitylation of MCM7. A. RNF168 increased the polyubiquitylation of MCM7. HeLa cells were transfected with plasmids encoding myc-MCM7, His-Ub and Flag-RNF168. The cells were harvested and probed with a Ni-NTA pull down assay, and the results were analyzed with MCM7 antibody. B. Ubiquitylation assays were performed in the presence of UBE1, E2(UbcH5c), Ub, and Flag-RNF168 purified from HEK293T cells, pET-Flag-MCM7 purified from Escherichia coli strain Rosetta.

Reaction mixtures were analyzed by western blot with MCM7 and Ub antibody. C. BRCA1 increased the polyubiquitylation of MCM7. HeLa cells were transfected with plasmids encoding myc-MCM7, His-Ub and Flag-BRCA1. Samples were purified and blotted as shown in Figure 4A. D. The polyubiquitylation of MCM7 promoted by RNF168 and BRCA1 occurred on chromatin. HeLa cells were transfected with Flag-MCM7, His-Ub, RNF8, RNF168 and BRCA1 and lysed in CSK buffer. The non-chromatin and chromatin fractions were extracted and analyzed by a Ni-NTA pull down assay and probed with MCM7 antibody. E. The polyubiquitylation of MCM7 mediated by RNF168 was cell cycle related. HeLa cells were co-transfected with plasmids encoding myc-MCM7, Flag-RNF168 and His-Ub, and were treated with HU for synchronization. Ni-NTA pull down analysis of chromatin-bound proteins was performed and probed with MCM7 antibody. F. The polyubiquitylation of MCM7 mediated by BRCA1 was cell cycle related. HeLa cells were transfected with myc-MCM7, Flag-BRCA1 and His-Ub, synchronized and blotted as described in Figure 4E.

Figure 5

Figure 5

Inhibition of replication by DNA damage obviously reduces polyubiquitylation of MCM7 mediated by RNF8. A-F. DNA damage obviously reduced polyubiquitylation of MCM7. HeLa cells were co-transfected with plasmids encoding Flag-MCM7, HA-Ub and HA-RNF8 and blocked with HU (2 mM) for 18 hours. After 2.5 hours of release, HeLa cells were treated with aphidicolin (1 μ M), IR (10 Gy), Adriamycin HCl (0.5 μ g/mL), Actinomycin D (5 nM), Etoposide (10 μ M) and ICRF-193 (5 μ M), and subsequently harvested at

the indicated times after block release. Cells were lysed in CSK buffer to obtain chromatin-bound fractions and were immunoblotted with the M2 beads pull down to detect polyubiquitylated MCM7 with Ub antibody. Chromatin extraction in RIPA buffer were probed with γ -H2AX, MCM2, MCM7, PCNA, and CDC45 antibodies.

Figure 6

Figure 6

Proteasome and Cdc48/p97 segregase are required for MCM7 deubiquitylation and CMG disassembly. A. HeLa cells were synchronized by HU (2 mM) for 18 hours. After 2.5 hours of release, HeLa cells were treated with aphidicolin (1 μ M), Adriamycin HCl (0.5 μ g/mL) and ICRF-193 (5 μ M), and subsequently harvested at the indicated increasing time intervals after block release. The DNA content was measured by propidium iodide staining and flow cytometry. B. Proteasome is required for MCM7 deubiquitylation and CMG disassembly. HeLa cells were co-transfected with plasmids encoding Flag-MCM7, HA-Ub and HA-RNF8, and synchronized by HU (2 mM). After 2.5 h of release, cells were treated with MG-132 (20 μ M) and harvested at the indicated time after block release. M2 beads pull down assay of chromatin-bound proteins was performed to detect the polyubiquitylation of MCM7. Chromatin extraction lysed in RIPA buffer was probed with γ -H2AX, MCM2, MCM7, PCNA, and CDC45 antibodies. C. HeLa cells were treated with NMS-873 (5 μ M) after 2.5 hours of release and were detected as described in Figure 6B. D. HeLa cells were synchronized and treated as described in Figure 6B-6C. Cells were harvested at the indicated increasing time intervals after block release and analyzed for DNA content by propidium iodide staining and flow cytometry.

Figure 7

Figure 7

USP29 and ATXN3 promote MCM7 deubiquitylation and CMG disassembly. A. U2OS cells overexpressing His-Ub plasmids were transfected with USP29-, USP50-, USP53-, ATXN3-, MINDY2- and control-siRNAs. The ubiquitin proteins were enriched by a Ni-NTA pull down assay and detected by Western blotting with the MCM7 antibody. B. U2OS cells were co-transfected with plasmids encoding Flag-MCM7, HA-Ub and HA-RNF8, treated with USP29-, ATXN3- or control-siRNAs, and synchronized by HU block. Cells were

harvested at the indicated time after block release. The M2 beads pull down assay of chromatin-bound proteins was performed to detect ubiquitinated MCM7. C. U2OS cells were treated with RNF8- or control-siRNAs and incubated in medium containing thymidine (2 mM) for 16 hours. Subsequently, the cells were released into cell cycle progression, fixed in 75% ethanol, and stained with propidium iodide before flow cytometry analysis. D. U2OS cells were treated with USP29-, ATXN3- or control-siRNAs. The cells were detected as described in Figure 7C. E. Model describing the regulation of MCM7 ubiquitylation and the CMG helicase disassembly during DNA replication termination in human.

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