Analysis of a temperature-sensitive mutation in Uba1: Effects of the click reaction on subsequent immunolabeling of proteins involved in DNA replication

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A B S T R A C T

In our previous study, a Met-to-Ile substitution at amino acid 256 in the catalytic domain of Uba1 was determined in temperature-sensitive CHO-K1 mutant tsTM3 cells, which exhibited chromosomal instability and cell-cycle arrest in the S to G_2 phases with decreased DNA synthesis at the nonpermissive temperature, 39 °C. Mutant cells were also characterized by a significant decrease of Uba1 in the nucleus with decreased ubiquitination activity at 39 °C. Defects of ubiquitination activity in the nucleus resulted in an inappropriate balance between Cdt1 and geminin, a licensing factor of DNA replication and its inhibitor. In the present study, we found that the Cu(I)-catalyzed [3 + 2] cycloaddition (click) reaction inhibits the subsequent indirect immunolabeling of Cdt1 but allows for the detection of PCNA with nascent DNA. Using a procedure without the click reaction, we also demonstrated that Cdt1 remained close to active replication sites in tsTM3 cells at the nonpermissive temperature. Analysis of genome replication by DNA fiber spreading revealed that DNA synthesis continues for at least 10 h after incubation at 39 °C, suggesting that impaired ubiquitination in the nucleus, caused by the defect of Uba1, affected DNA replication only after a long delay.
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1. Introduction

To identify genes responsible for the maintenance of chromosome integrity, Tsuji and colleagues isolated 25 temperature-sensitive (ts) mutants from Chinese hamster wild-type CHO-K1 cells [1]. In that study, a ts mutant, tsTM3, exhibited chromosomal instability and cell-cycle arrest in the S to G_2 phases with decreased DNA synthesis at the nonpermissive temperature, 39 °C. In our previous study, we identified a point mutation in a gene encoding ubiquitin activating enzyme, *Uba1*, isolated from the ts mutant tsTM3 cells, which led to a Met-to-Ile substitution at amino acid 256 in deduced Uba1 protein [2]. The ubiquitination process requires the coordinated action of three enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) [3]. E1 catalyzes the initial step in the ubiquitin

conjugation pathway. In mammalian cells, there are only two E1 enzymes for the entire array of downstream reactions, Uba1 and Uba6, whereas there are dozens of E2s and several hundred E3s [4].

In our previous study, mutant cells at the nonpermissive temperature showed a significant decrease of Uba1 in the nucleus with decreased ubiquitination activity [2]. This defect in ubiquitination led to significant accumulation of geminin and retention of Cdt1, resulting in an inappropriate balance between Cdt1 and geminin. Both Cdt1 and its inhibitor, geminin, are crucial regulators of the licensing of DNA replication and are controlled by the ubiquitin–proteasome system [5]. We thought that the retention of Cdt1 due to the loss of function of Uba1 would contribute to the initiation of DNA synthesis at 39 °C. However, the elongation of DNA synthesis in tsTM3 cells at 39 °C remains unclear.

A useful method to detect DNA synthesis was developed by Salic and Mitchison [6] that is based on the incorporation of a thymidine analog, 5-ethynyl-2′-deoxyuridine (EdU), and its subsequent detection with a fluorescent dye by a Cu(I)-catalyzed [3 + 2] cycloaddition reaction (so-called "click chemistry" [6,7]). The most notable point of this method is that it does not need denaturation of DNA for the detection of EdU, unlike a conventional method such as the immunological detection of incorporated

5-bromo-2′-deoxyuridine (BrdU). However, there are some incompatibilities with other detection systems due to the high reactivity of the click reaction (see the manufacturer's web site: https://www.lifetechnologies.com). In our previous study using sequential double staining for Cdt1 and nascent DNA, we showed that Cdt1 remains in active replication sites even in tsTM3 cells incubated at the nonpermissive temperature [2]. Nascent DNA was labeled by EdU and detected by click reaction with a fluorescent dye. We found that the detection of nascent DNA appeared to inhibit indirect immunolabeling of Cdt1. Two possibilities were raised for this inhibition: the physical distance between Cdt1 and nascent DNA might be too close and the click reaction might be too reactive.

In the present study, we evaluated which of these two possibilities was correct. The effects of the Cu(I)-catalyzed [3 + 2] cycloaddition reaction on indirect immunolabeling were examined with several antibodies. Then, the ts defect of Uba1 on the elongation of DNA replication was analyzed by DNA fiber spreading.

2. Materials and methods

2.1. Cells

The Chinese hamster cell line, CHO-K1, and its ts mutant cell, tsTM3 [1], were grown in Ham's F-12 medium (Wako, Osaka, Japan) containing 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (Gibco/Invitrogen, Carlsbad, CA) at 34 °C. For the analysis of the ts phenotype, cells were shifted up to the nonpermissive temperature (39 °C).

2.2. Indirect immunolabeling and antibodies

Procedures for the indirect immunolabeling and microscopy of labeled cells have been described previously [8]. Images were collected with an Olympus DP30BW digital charge-coupled device camera fitted on an Olympus IX71 microscope (Olympus, Tokyo, Japan) and "contrast-stretched" with Adobe Photoshop CS (Adobe Systems, San Jose, CA). The number of fluorescently labeled nuclei was counted manually with ImageJ 1.42q (http://rsb.info.nih.gov/ij/). Primary antibodies used in this study were rabbit anti-Cdt1 (1:800; Sigma–Aldrich, St. Louis, MO), mouse anti-proliferating cell nuclear antigen (PCNA) (clone PC10, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-mini-chromosome maintenance protein 7 (MCM7) (clone DCS-141, 1:500; Medical and Biological Laboratories, Nagoya, Japan), and mouse anti-α-tubulin (clone DM1A, 1:500; Sigma–Aldrich).

2.3. Labeling of nascent DNA

Cells were grown on glass coverslips at 34 °C or 39 °C for 10 h, incubated with 10 μM EdU (Invitrogen/Molecular Probes, Eugene, OR), washed, and fixed for 20 min at 4 °C with 4% paraformaldehyde (PFA) in 250 mM HEPES. DNA labeled with EdU was detected with Alexa Fluor 488 using the "Click-iT" detection kit according to the manufacturer's instructions (Invitrogen/Molecular Probes).

For introduction of digoxigenin-dUTP (DIG-dUTP) (Roche Diagnostics, Mannheim, Germany) by a hypotonic shift [9], cells were rinsed with hypotonic buffer (10 mM HEPES, 50 mM KCl), treated with 50 μM DIG-dUTP in hypotonic buffer for 10 min at 34 °C or 39 °C, washed and incubated in fresh medium for 30 min at 34 °C or 39 °C, and then fixed for 20 min at 4 °C with 4% PFA in 250 mM HEPES. DNA labeled with DIG-dUTP was detected with rhodamine-conjugated sheep anti-DIG antibody (Roche Diagnostics).

Spreads of extended DNA fibers were made as described previously with some modifications [10–14]. Cells were incubated with 100 μM 5-chloro-2′-deoxyuridine (CldU) (Sigma–Aldrich) for 20 min at 34 °C or 39 °C, washed, and then incubated with 100 μM 5-iodo-2′-deoxyuridine (IdU) (Sigma–Aldrich) for 20 min at 34 °C or 39 °C. Labeled cells were washed and trypsinized and then fixed with methanol:acetic acid (3:1). Fixed cells were spotted onto glass slides, briefly air dried, and then lysed with 0.5% sodium dodecylsulfate in 200 mM Tris (pH 7.5) and 50 mM EDTA for 10 min. Slides were tilted at 15°, allowing a stream of DNA to run slowly down the slide, air dried, and fixed with methanol:acetic acid (3:1). Slides were treated with 2 M HCl for 30 min, washed twice with 100 mM Tris (pH 8.0), and then three times with phosphate-buffered saline. DNA fibers labeled with CldU and IdU were detected indirectly with Alexa Fluor 488 and Alexa Fluor 594, respectively. Primary antibodies used in this analysis were rat anti-BrdU (1:200; clone BU1/75; Serotec, Puchheim, Germany) for CldU and mouse anti-BrdU (1:25; clone B44; Becton Dickinson, San Jose, CA) for IdU. The length of fluorescently labeled DNA fibers was determined with ImageJ 1.42q (http://rsb.info.nih.gov/ij/).

3. Results

3.1. Click reaction inhibits indirect immunolabeling of Cdt1

In our previous study, nascent DNA was labeled by EdU and detected by click reaction with a fluorescent dye. After indirect immunolabeling of Cdt1, detection of EdU was carried out sequentially and showed co-localization between Cdt1 and nascent DNA [2]. However, after fluorescent labeling of nascent DNA, labeling of Cdt1 was significantly decreased. Labeling of nascent DNA appeared to inhibit immunological detection of Cdt1, raising the possibility that the physical distance between Cdt1 and nascent DNA must be very close. Therefore, this possibility was verified by indirect immunolabeling of Cdt1 followed by the click reaction without a fluorescent dye. We found that the click reaction without a fluorescent dye inhibited the immunostaining of Cdt1, suggesting that the fluorescent dye labeling of nascent DNA may not be a problem of inhibition of the immunological detection of Cdt1 (Fig. 1). This result prompted a detailed investigation into the effects of the click reaction on the indirect immunolabeling of other proteins, especially those involved in DNA replication.

3.2. Click reaction allows for the detection of PCNA with nascent DNA

PCNA is involved in the control of eukaryotic DNA replication. It has been shown to function as the sliding clamp during DNA synthesis [15]. PCNA forms nuclear foci representing sites of ongoing DNA replication [16]. Fixation with organic solvents is recommended for immunological detection of PCNA using an antibody such as PC10, a mouse monoclonal antibody raised against PCNA (Supplementary Fig. 1). Interestingly, we found that the click reaction led to indirect immunostaining of PCNA, which gave us results similar to those obtained by methanol fixation. Therefore, we investigated the distribution of PCNA in detail, especially with regard to the link to DNA synthesis, and performed sequential double staining for PCNA and nascent DNA. First, after indirect immunolabeling of PCNA, detection of EdU was carried out sequentially. A mouse monoclonal antibody against PCNA yielded many small and very faint foci in discrete nuclear and cytoplasmic sites (Fig. 2A). These results from PFA fixation are consistent with the recommendation of fixation by an organic solvent such as methanol. Then, after the fluorescent labeling of nascent DNA, PCNA was indirectly immunolabeled. Nascent DNA was again found in many small and some large bright foci in discrete nuclear sites (Fig. 2B). Surprisingly, a mouse monoclonal antibody against PCNA yielded
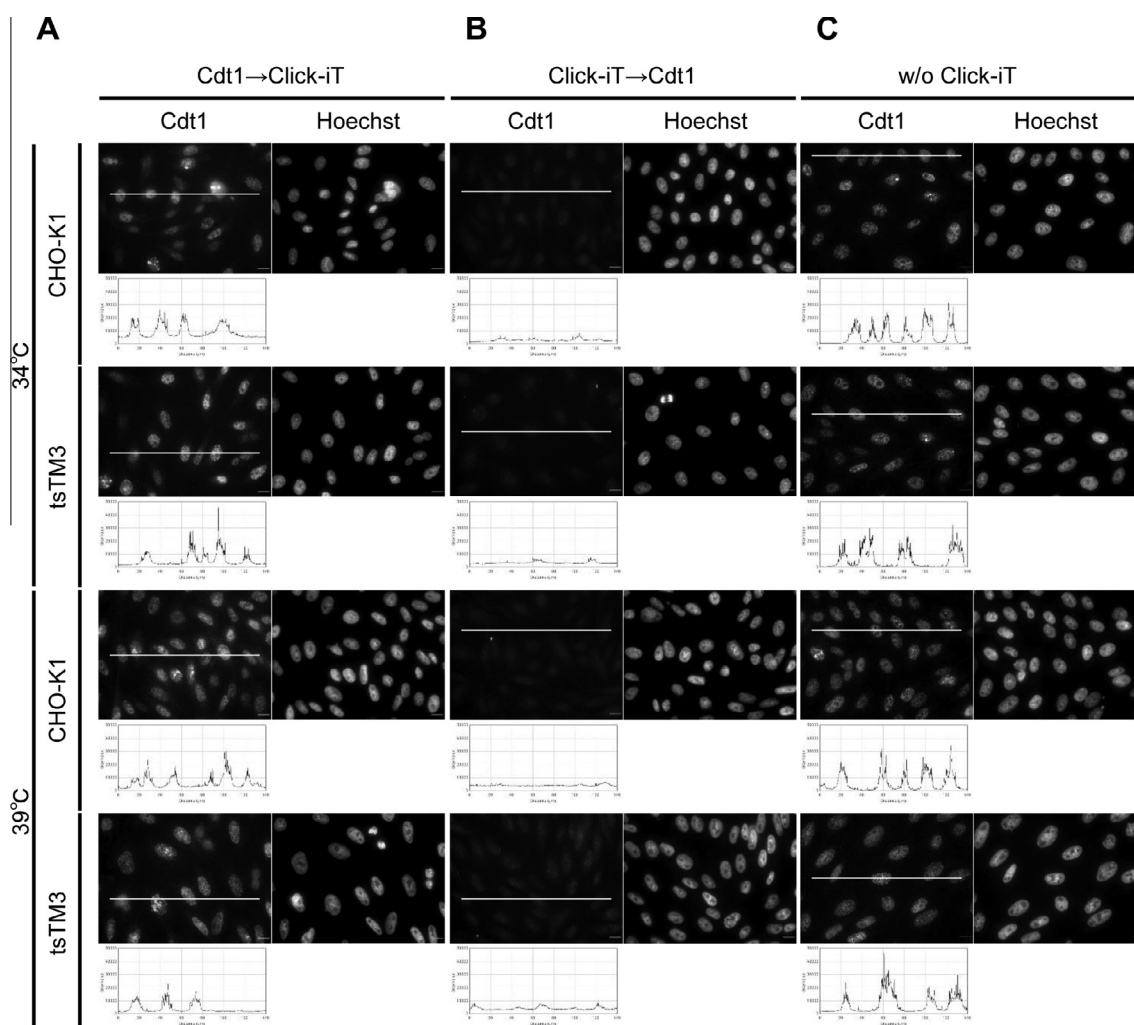
Fig. 1. Indirect immunolabeling of Cdt1 is inhibited by the click reaction. (A) Cells were grown on glass coverslips at 34 °C or 39 °C for 10 h, incubated with 10 μM EdU for 20 min, and then washed and fixed with 4% PFA. Cdt1 was indirectly immunolabeled with Alexa 594. After the click reaction without Alexa 488, cells were counterstained with Hoechst 33342. The intensity of fluorescence in the non-processed image, which is taken at the same microscope setting, is monitored along the white line and is shown below each image of Cdt1. Cdt1 was found in many discrete nuclear sites. (B) After the click reaction without Alexa 488, Cdt1 was stained with Alexa 594. Labeling of Cdt1 was significantly decreased. (C) Cdt1 was stained with Alexa 594 without the click reaction. Bar, 10 μm.

many small and some large bright foci in discrete nuclear sites and produced similar patterns in the wild-type cells at 34 °C and 39 °C and in the mutant cells at 34 °C (Fig. 2B). We found PCNA in the nucleus of tsTM3 cells after 10 h of incubation at 39 °C. Approximately 80% of these cells contained nascent DNA labeled by EdU, which results in the yellow color in the merged images, indicating co-localization between PCNA and nascent DNA (Fig. 2C). These results are consistent with a previous finding that DNA synthesis in tsTM3 cells is still active after 10 h of incubation at 39 °C [1,2], indicating that some PCNAs contribute in active replication sites in tsTM3 cells.

3.3. Effect of click reaction on indirect immunolabeling of MCM7

We found both negative and positive effects of the click reaction on immunological detection with antibodies raised against Cdt1 and PCNA, respectively. We therefore investigated the effects of the click reaction on indirect immunostaining with two other antibodies. MCM7 is essential for eukaryotic genome replication. The hexameric protein complex formed by the MCM proteins is a key component of the pre-replication complex and is involved in the formation of replication forks to recruit other DNA replication-related proteins [17]. A mouse monoclonal antibody against

MCM7 yielded many small and homogeneous foci in discrete nuclear sites and produced similar patterns in the wild-type and mutant cells at 34 °C and 39 °C (Fig. 3A). We found little effect of the click reaction on indirect immunolabeling with an antibody raised against MCM7 (Fig. 3B). Approximately 60%–80% of the MCM7-positive cells contained nascent DNA labeled by EdU, which results in the yellow color in the merged images (Fig. 3C). MCM7 appeared to contribute to the replication in tsTM3 cells incubated at 39 °C.

The α- and β-tubulins represent the major components of microtubules and are highly conserved among species [18]. A mouse monoclonal antibody against α-tubulin yielded a cytoplasmic label representing microtubules and produced similar patterns in the wild-type and mutant cells at 34 °C and 39 °C (Supplementary Fig. 2). Again, we found little effect of the click reaction on indirect immunolabeling with an antibody raised against α-tubulin (Supplementary Fig. 2). These results suggest that the effects of the click reaction appear to differ among antigens.

3.4. Cdt1 remains in active replication sites in tsTM3 cells incubated at 39 °C

The click reaction showed inhibition of the immunological detection of Cdt1 (Fig. 1). We therefore re-verified the effect of
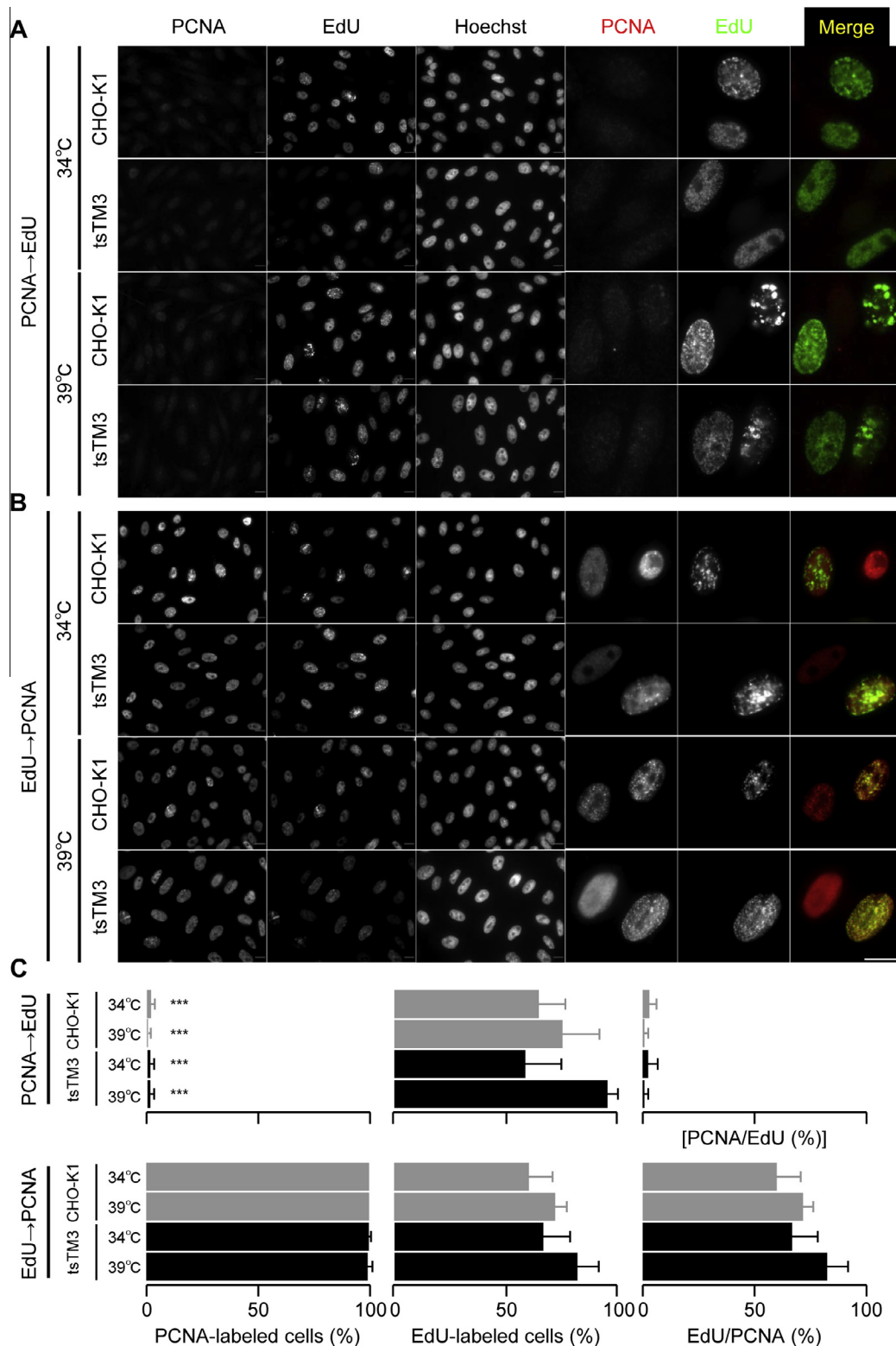
Fig. 2. Click reaction shows significant effect in indirect immunolabeling of PCNA. Cells were grown on glass coverslips at 34 °C or 39 °C for 10 h, incubated with 10 μM EdU for 20 min, washed, and fixed with 4% PFA. (A) PCNA was indirectly immunolabeled with Alexa 594. Next, nascent DNA labeled with EdU was detected with Alexa 488 by the click reaction, and cells were also counterstained with Hoechst 33342. The merged views (right) are composed of PCNA (red channel) and EdU (green channel). Labeling of PCNA was very faint, and little yellow color was found in the merged images. (B) Nascent DNA labeled with EdU was detected with Alexa 488. Next, PCNA was stained with Alexa 594. Surprisingly, PCNA was found in many discrete nuclear sites, and some of these also contained EdU, resulting in the yellow color in the merged images and indicating co-localization between PCNA and nascent DNA. Bar, 10 μm. (C) Quantitative analyses of the numbers of cells expressing PCNA and EdU-incorporated cells. Cells expressing PCNA and cells labeled with EdU, such as those shown in panels (A) and (B), were counted and are expressed as a ratio with standard deviation in the left and middle graphs, respectively ($n > 130$). The proportion of cells labeled with EdU to cells expressing PCNA is shown in the right graph. Because there were none or few of PCNA-positive cells under the condition "PCNA→EdU", the upper right graph shows the proportion of cells expressing PCNA to cells labeled with EdU. P values were calculated by Student t-test. Asterisks indicate a statistically significant difference in the ratio of PCNA-labeled cells between the staining sequences, "PCNA→EdU" and "EdU→PCNA" (***$P < 0.001$).
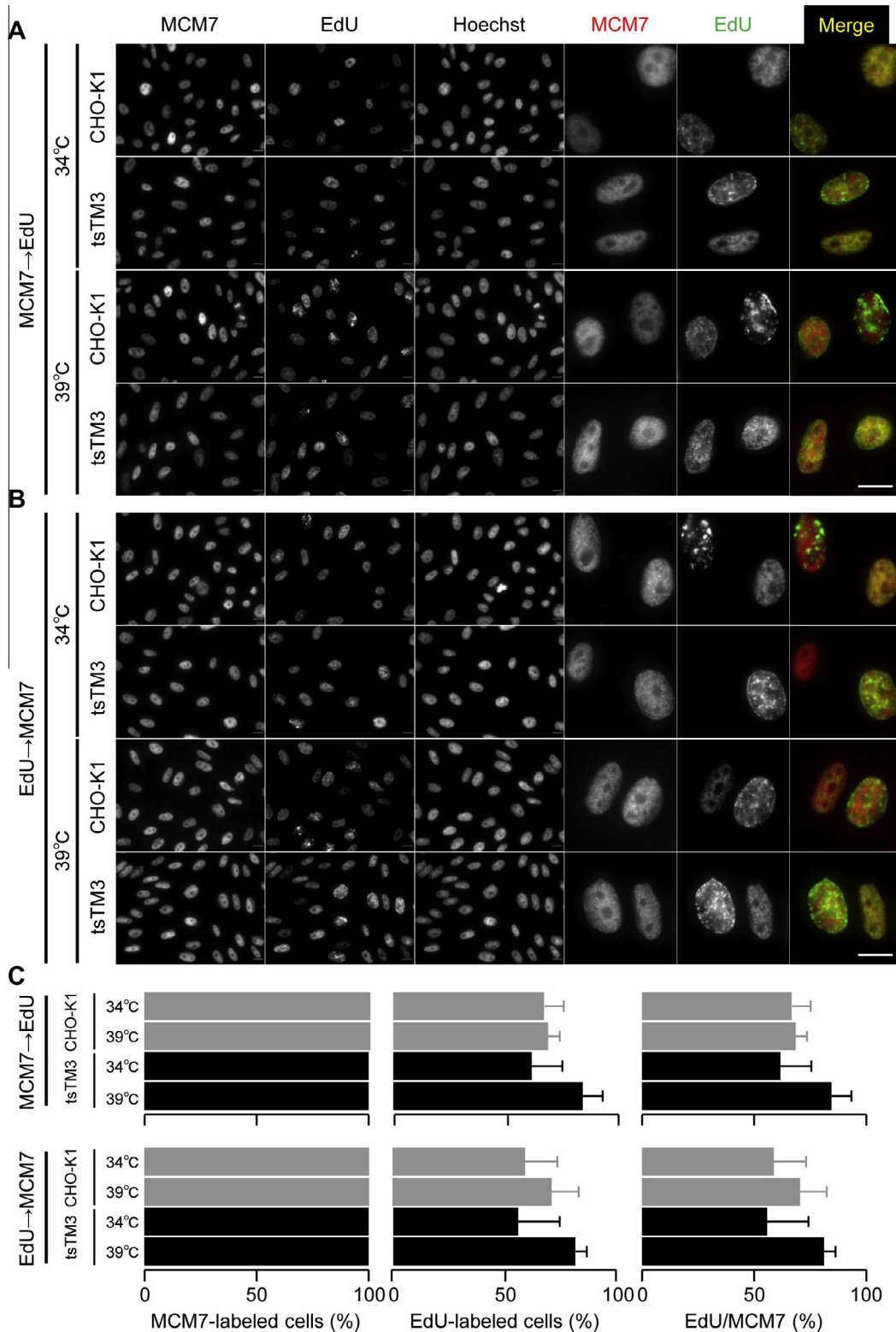
Fig. 3. Effect of the click reaction on indirect immunolabeling of MCM7. Cells were grown on glass coverslips at 34 °C or 39 °C for 10 h, incubated with 10 μM EdU for 20 min, washed, and fixed with 4% PFA. (A) MCM7 was indirectly immunolabeled with Alexa 594. Next, nascent DNA labeled with EdU was detected with Alexa 488 by the click reaction, and cells were also counterstained with Hoechst 33342. The merged views (right) are composed of MCM7 (red channel) and EdU (green channel). (B) Nascent DNA labeled with EdU was detected with Alexa 488. Next, MCM7 was stained with Alexa 594. MCM7 was found in many discrete nuclear sites, and some of these also contained EdU, resulting in the yellow color in the merged images and indicating co-localization between MCM7 and nascent DNA. Bar, 10 μm. The effect of the click reaction on the immunological detection of MCM7 appeared to be small. The pattern of immunostaining for MCM7 appeared to be different from that for PCNA, which represented heterogeneous foci (Fig. 2B). (C) Quantitative analyses of the numbers of cells expressing MCM7 and EdU-incorporated cells. Cells expressing MCM7 and cells labeled with EdU, such as those shown in panels (A) and (B), were counted and are expressed as a ratio with standard deviation in the left and middle graphs, respectively ($n > 160$). The proportion of cells labeled with EdU to cells expressing MCM7 is shown in the right graph.
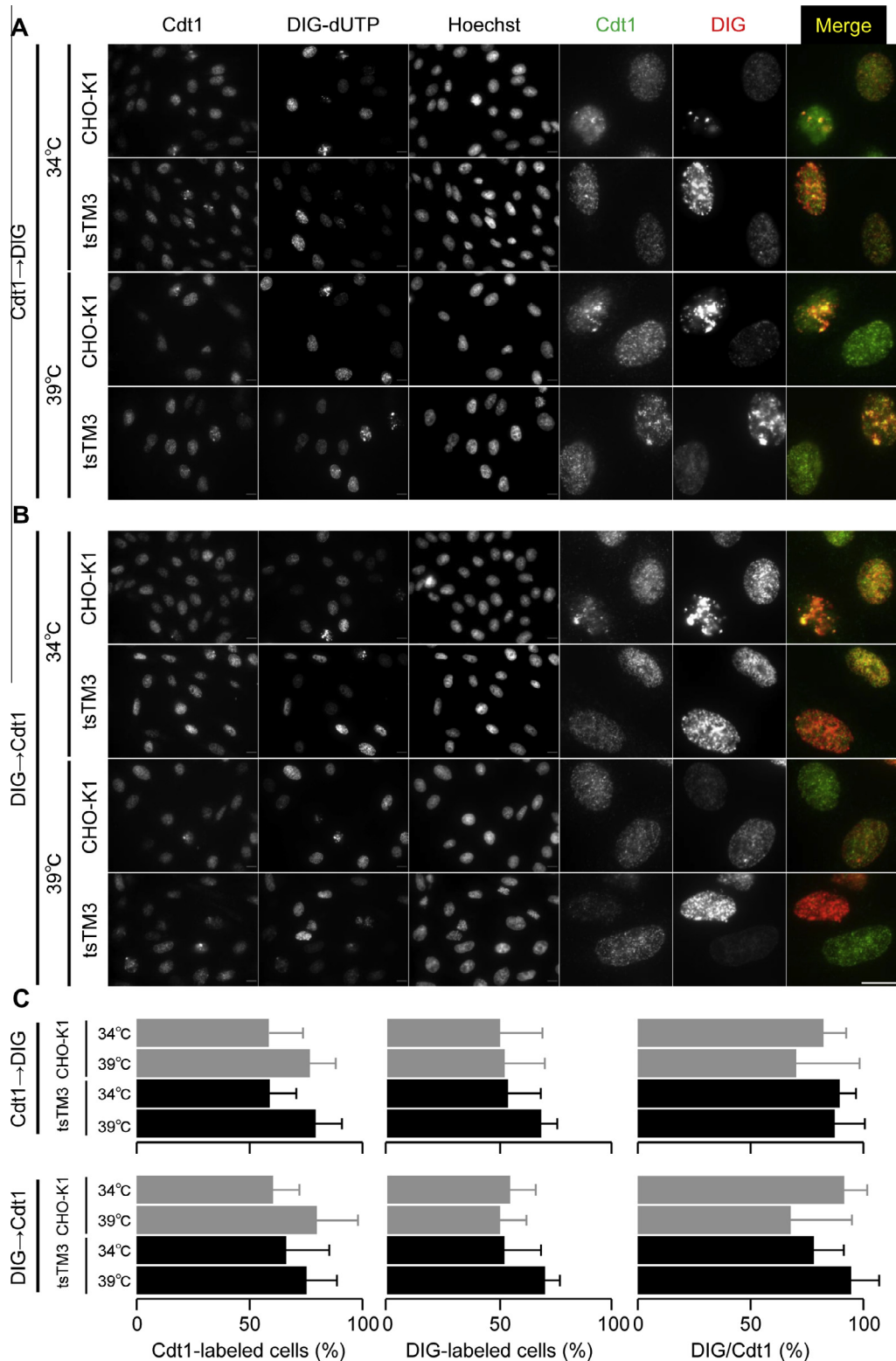
Fig. 4. Cdt1 remained close to active replication sites in tsTM3 cells incubated at 39 °C. Cells were grown on glass coverslips at 34 °C or 39 °C for more than 10 h. DIG-dUTP was introduced into cells by a hypotonic shift to label nascent DNA, and cells were incubated for 30 min, washed, and fixed with 4% PFA. (A) Cdt1 was indirectly immunolabeled with Alexa 488. Next, nascent DNA labeled with DIG-dUTP was detected with rhodamine, and cells were also counterstained with Hoechst 33342. The merged views (right) are composed of Cdt1 (green channel) and DIG-dUTP (red channel). Cdt1 was found in many discrete nuclear sites, and most of these also contained DIG-dUTP, resulting in the yellow color in the merged images, indicating co-localization between Cdt1 and nascent DNA. (B) Nascent DNA labeled with DIG-dUTP was detected with rhodamine. Next, Cdt1 was indirectly immunolabeled with Alexa 488. Cdt1 was again found in many discrete nuclear sites, and most of these also contained DIG-dUTP, resulting in the yellow color in the merged images. Bar, 10 μm. (C) Quantitative analyses of the numbers of cells expressing Cdt1 and DIG-dUTP-incorporated cells. Cells expressing Cdt1 and cells labeled with DIG-dUTP, such as those shown in panels (A) and (B), were counted and are expressed as a ratio with standard deviation in the left and middle graphs, respectively ($n > 150$). The proportion of cells expressing Cdt1 to cells labeled with DIG-dUTP is shown in the right graph.
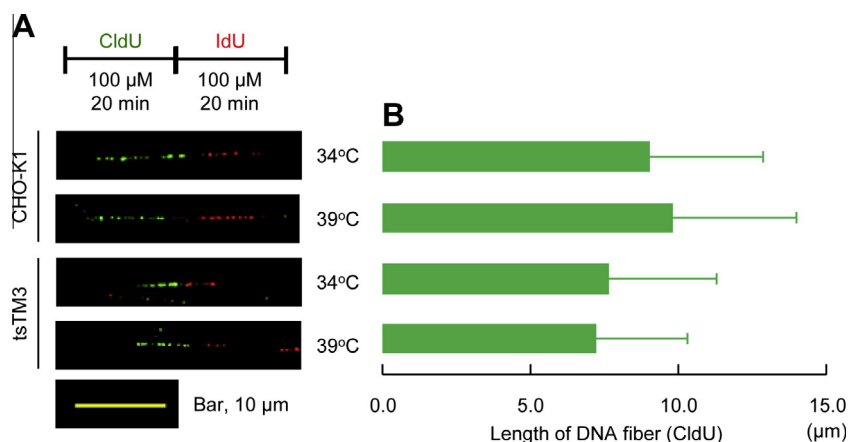
Fig. 5. Analysis of replication by DNA fiber spreading. (A) Cells were grown at 34 °C or 39 °C for 10 h, incubated with 100 μM CldU for 20 min, washed and incubated with 100 μM IdU for 20 min, and then washed and fixed with 4% PFA. After preparation of DNA fibers, nascent DNA labeled with CldU and IdU were indirectly immunolabeled with Alexa 488 and Alexa 594, respectively. Bar, 10 μm. (B) Quantitative analyses of nascent DNA. The length of the labeled DNA fibers was determined by measuring the distance between the initial sites labeled by CldU and IdU (n = 140). The elongation of DNA synthesis appeared to be active in tsTM3 cells after 10 h of incubation at 39 °C.

the retention of Cdt1 with regard to the link to DNA synthesis and performed sequential double staining for Cdt1 and nascent DNA with other detection. Nascent DNA was labeled by a deoxyribonucleotide analog, DIG-dUTP, and detected with fluorescent-labeled antibody raised against DIG. First, after indirect immunolabeling of Cdt1, detection of DIG-dUTP was carried out sequentially. A rabbit polyclonal antibody against Cdt1 yielded many small and some large bright foci in discrete nuclear sites and again produced similar patterns in the wild-type and mutant cells at 34 °C and 39 °C (Fig. 4A). Most of these cells contained nascent DNA labeled by DIG-dUTP, which results in the yellow color in the merged images, indicating co-localization between Cdt1 and nascent DNA (Fig. 4A and C). Next, after fluorescent labeling of nascent DNA, Cdt1 was indirectly immunolabeled. Nascent DNA was again found in many small and some large bright foci in discrete nuclear sites (Fig. 4B). Labeling of Cdt1 was successfully observed as many small and some large bright foci in discrete nuclear sites and produced similar patterns in cells at both 34 °C and 39 °C (Fig. 4B). Labeling of nascent DNA by DIG-dUTP did not appear to inhibit immunological detection of Cdt1. Most of the Cdt1-positive cells contained nascent DNA labeled by DIG-dUTP, resulting in the yellow color in the merged images, indicating co-localization between Cdt1 and nascent DNA (Fig. 4A and C). These results support our finding that Cdt1 remained close to active replication sites in tsTM3 cells incubated at 39 °C.

3.5. Analysis of replication by DNA fiber spreading

We found that the Cdt1 remaining in tsTM3 cells at 39 °C may result in the initiation of DNA replication. However, the elongation of DNA synthesis in tsTM3 cells at 39 °C remains unclear, and DNA fiber spreading analysis was performed to examine it. Cells were grown at 34 °C or 39 °C for 10 h, incubated with 100 μM CldU for 20 min, washed, incubated with 100 μM IdU for 20 min and then washed and fixed with 4% PFA. After preparation of the DNA fibers, nascent DNA labeled with CldU and IdU was indirectly immunolabeled with Alexa 488 and Alexa 594, respectively (Fig. 5A). Nascent DNA was observed in many fibers labeled in tandem with green and red fluorescent dyes in the merged images. Incorporation of thymidine analogs was observed again in tsTM3 cells at 39 °C. Next, we analyzed the elongation speed of DNA synthesis by measuring the labeled DNA fiber. The length of labeled DNA fibers was determined by measuring the distance between the initial sites

labeled by CldU and IdU (Fig. 5B). There was little difference between the length of DNA fiber labeled with CldU prepared from CHO-K1 cells and that from tsTM3 cells even at 39 °C, although the lengths of labeled fibers of tsTM3 cells appeared to be slightly shorter than those of CHO-K1 cells. These results confirmed that the elongation of DNA synthesis appeared to be active in tsTM3 cells after 10 h of incubation at 39 °C.

4. Discussion

4.1. Effects of Cu(I)-catalyzed [3 + 2] cycloaddition reaction on indirect immunolabeling

In this study, we found that the click reaction without a fluorescent dye inhibited the indirect immunolabeling of Cdt1, which may be due to the loss of the properties of the antigen by the reaction. Immunogen of a rabbit polyclonal antibody directed against Cdt1, which was used in the present study, is the synthetic peptide for amino acid position 448–490 of human Cdt1. We suspect that the Cu ion causes enough damage to interfere with the structure of proteins (see the manufacturer's web site: https://www.lifetechnologies.com), although it is not clear whether the entire structure of Cdt1 is affected by the click reaction. We also found that the click reaction leads to immunostaining of PCNA, which produces results similar to those obtained with methanol fixation. The presence of Cu appeared to be important for this effect on the labeling of PCNA, and a similar result was reported in the study of Ligasová and colleagues in which the treatment of cells with Cu ions resulted in the cleavage of DNA allowing the detection of PCNA [19]. Their report showed that oxidative attack at the deoxyribose moiety by Cu(I) in the presence of oxygen resulted in the cleavage of DNA, and the authors termed this action "atomic scissors". Little effect of the click reaction on indirect immunolabeling was shown with antibodies raised against MCM7, suggesting that the effects of the reaction appeared to differ among antigens. It is also possible that the effect of the click reaction on the subsequent immunolabeling is dependent upon the antibody. However, it is interesting that the immunological detection of three proteins involved in DNA replication showed different results. Our results are generally consistent with those addressing compatibility with other detection systems, but at present they are good complementary information that may help in the further investigation of DNA replication with the click reaction.

4.2. Cdt1 remaining close to active replication sites

One of the major purposes of this study was to validate that Cdt1 remains close to active replication sites. In our previous paper, we found co-localization of Cdt1 with nascent DNA using sequential double staining [2]. However, the click reaction inhibits the detection of Cdt1. We therefore wanted to show co-localization of Cdt1 with nascent DNA by another method in the present study. DIG-dUTP, a deoxyribonucleotide analog, was selected as a precursor of DNA because replication labeling by means of a hypotonic shift has been established [9]. Immunostaining with an antibody raised against DIG showed co-localization of Cdt1 with nascent DNA, even when DIG labeling was followed by Cdt1 labeling. There may be no physical inhibition to the detection of Cdt1 by the immunolabeling of nascent DNA. We conclude that Cdt1 remained close to active replication sites in tsTM3 cells at 39 °C, suggesting the possibility of the over-replication of DNA in tsTM3 cells.

4.3. A ts mutation of Uba1 and its relation to DNA replication

We analyzed the activity of DNA synthesis in tsTM3 cells at 39 °C by DNA fiber spreading. Measuring the labeled DNA fragments with CldU and IdU allowed us to examine the elongation of DNA replication. There were no significant differences in the elongation of DNA replication between CHO-K1 and tsTM3 cells even after 10 h of incubation at 39 °C, which is consistent with previous findings showing the DNA synthesis activity in mutant cells [1]. The Cdt1 remaining in tsTM3 cells at 39 °C may result in the initiation of DNA replication. Analysis of nascent DNA with fiber spreading suggested that the elongation occurred following the initiation of replication. Furthermore, double staining of EdU with PCNA and MCM7 provided support for DNA synthesis activity in tsTM3 cells up to at least 10 h at 39 °C. This implies that DNA is over-replicated in tsTM3 cells. Over-replication may cause one of the phenotypes of tsTM3 cells, as an induction of chromosome aberration.

We conclude that the Cu(I)-catalyzed [3 + 2] cycloaddition reaction appeared to inhibit the subsequent indirect immunolabeling of Cdt1 but allowed for the detection of PCNA with nascent DNA. Labeling of nascent DNA with DIG-dUTP incorporated into cells by hypotonic shift and subsequent immunodetection led us to the conclusion that Cdt1 remained close to active replication sites in tsTM3 cells, a ts Uba1 mutant, for at least 10 h at the nonpermissive temperature. The elongation activity of DNA synthesis in the mutant cells remained at least after 10 h of incubation at 39 °C. Taken together with previous observations, the effects of impaired ubiquitination in the nucleus by the ts defect of Uba1 appear slowly in DNA replication.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.02.004.

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