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Differential activation of intra-S-phase checkpoint in response to tripchlorolide and its effects on DNA replication

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ABSTRACT

DNA replication is tightly regulated during the S phase of the cell cycle, and the activation of the intra-S-phase checkpoint due to DNA damage usually results in arrest of DNA synthesis. However, the molecular details about the correlation between the checkpoint and regulation of DNA replication are still unclear. To investigate the connections between DNA replication and DNA damage checkpoint, a DNA-damage reagent, tripchlorolide, was applied to CHO (Chinese ovary hamster) cells at early- or middle-stages of the S phase. The early-S-phase treatment with TC significantly delayed the progression of the S phase and caused the phosphorylation of the Chk1 checkpoint protein, whereas the middle-S-phase treatment only slightly slowed down the progression of the S phase. Furthermore, the analysis of DNA replication patterns revealed that replication pattern II was greatly prolonged in the cells treated with the drug during the early-S phase, whereas the late-replication patterns of these cells were hardly detected, suggesting that the activation of the intra-S-phase checkpoint inhibits the late-origin firing of DNA replication. We conclude that cells at different stages of the S phase are differentially sensitive to the DNA-damage reagent, and the activation of the intra-S-phase checkpoint blocks the DNA replication progression in the late stage of S phase.

***Keywords*:** intra-S-phase checkpoint, DNA replication, DNA damage, tripchlorolide.

INTRODUCTION

Genomic DNA is duplicated during the S phase of the cell cycle. DNA synthesis initiates at numerous origins that are spatially and temporally defined[1-4]. For example, most actively transcribed genes are replicated early in the S phase, while most inactive genes and repetitive DNA are replicated late in the S phase[1, 2]. It was shown that each stage of the S phase in mammalian cells had a distinct replication pattern that could be visualized with fluorescent microscopy when nuclei were labeled with bromodeoxyuridine (BrdU) and stained with fluorescent antibodies [3, 5]. Generally, pattern I appears in the early stage of the S phase, pattern II mainly appears in the middle stage of the S phase, and patterns III and IV represent the late stage of the S phase ([3-5], also see Fig 1).

Activation of the DNA-damage checkpoint often delays or blocks cell cycle progression in response to DNA damage[6]. During the S phase, the pathway responding to DNA damage is referred to as the intra-S-phase checkpoint. Upon activation by DNA lesions or stalled replication forks, the activated intra-S-phase checkpoint slows down the progression of the S phase and activates the DNA repair system[7, 8]. Increasing data obtained in the past few years have figured the outline of the DNA-damage checkpoint as a complex pathway consisting of sensors, transducers and effectors[6, 9, 10]. Among them, Chk1 kinase is an important transducer in response to DNA damage[9]. It was shown that Chk1 was phosphorylated in response to ion radiation or ultraviolet light in mammals [11, 12]. It was suggested that Chk1 had important functions during the S phase, possibly facilitating DNA replication and preventing premature mitotic entry[9].

DNA replication seems to be required for the activation of the intra-S-phase checkpoint. MMS, an alkylating agent which causes DNA lesions, activated the intra-S-phase checkpoint only when *Xenopus* eggs entered the S phase [13]. Defects on DNA-damage checkpoint activation caused by depletion of primase, which participates in the initiation of DNA replication, can be restored by expression of recombinant wild-type human primase, suggesting primase may play an important role in checkpoint activation[14]. On the other hand, the intra-S-phase checkpoint participates in the regulation of DNA replication. Treatment

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Abbreviations: CHO, Chinese hamster ovary; TC, tripchlorolide; HU, hydroxyurea BrdU, bromodeoxyuridine; DAPI, 4'; 6-diamidino-2-phenylindole; ORC, Origin recognition complex.

of MMS could block the firing of late origins during the S phase[15]. Mutation in Orc2-1, a component of the origin recognition complex (ORC), obligated the activation of the intra-S-phase checkpoint which inhibits the initiation of late origins in response to DNA-damage agents in *S. cerevisiae*[16]. Thus, there are links between the regulation of DNA replication and the response to DNA damage.

Tripchlorolide (TC) is a compound belonging to a family of nature products from extracts of *Tripterygium Wilfordii Hook. f.*, which shows immunosuppressive and antitumor activities[17, 18]. Our recent works revealed that TC could induce DNA damage and result in apoptosis[19, 20]. In the present study, synchronized CHO cells were treated with TC at different stages of the S phase and the effects of TC on the cell cycle and replication were analyzed.

MATERIALS AND METHODS

Cell culture and synchrony

Chinese hamster ovary cell line CHOC400 were cultured in Dulbecco's modified Eagle's medium (Invitrogen life technologies) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 50 μg/ml streptomycin and 100 μM non-essential amino acids (Invitrogen life technologies) in a humidified atmosphere of 5% CO_2 at 37°C. Mitotic cells were obtained by mechanical shake-off after 4-5 h incubation with nocodozole (Sigma Aldrich) at 50 ng/ml as described[21]. G_1/S-phase cells were prepared by releasing mitotic cells in the medium for 4-5 h and then adding 1 mM hydroxyurea (HU) for another 10-11 h.

Flow cytometry

The cells (1×10^6) were harvested and fixed with 70% ethanol, then resuspended in phosphate-buffered saline (PBS) containing 500 μg/ml RNase A (Sigma Aldrich) and incubated at 37°C for 1 h. The cells were stained with 20 μg/ml propidium iodide (Sigma Aldrich) and detected with a FACScan flow cytometer (Becton Dickinson). CellQuest software was utilized for data acquisition and analysis.

BrdU incorporation and fluorescent microscopy

At the indicated time, synchronized cells growing on coverslips were incubated with 30 μg/ml bromodeoxyuridine (BrdU) for 30 min. The cells were fixed in 70% ethanol for 30 min. Incorporated BrdU was detected with antibodies as described[22]. In brief, the cells were sequentially incubated in methanol for 10 min and in 1.5 M HCl for 30 min. Then cells were washed three times with 1×PBS containing 0.5% Tween-20 and incubated with the mouse anti-BrdU primary antibody (Roche Pharmaceuticles) for 1 h. The cells were washed and labeled with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology Associates). The nuclei were simultaneously stained with 10 μg/ml 4' and 6-diamidino-2-phenylindole (DAPI). Cells with different BrdU-incorporation patterns were checked with a conventional fluorescence microscope (ZEISS) or a confocal laser scanning microscope (Bio-Rad Laboratories). Photographs were taken using a Nikon Eclipse TE300 microscope equipped with Radiance 2100 laser scanning system (Bio-Rad Laboratories).

Immunoblotting

The cells were lysed in SDS-sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue) and heated in boiling water for 10 min. Protein concentration was determined by the Lowry method[23]. Equal amounts of cell lysates were separated on SDS-PAGE gels. The gels were transferred to PVDF membranes (Millipore) and incubated with an appropriate dilution of primary antibodies (anti-Chk1, BD transduction laboratories; anti-phospho-Chk1, Cell signaling technology; anti-phospho-H2AX, Upstate; anti-β-actin, Santa Cruz Biotechnology). Immune complexes were detected using the enhanced chemiluminescence system (ECL, Amersham Biosciences).

RESULTS

Replication patterns in the S-phase progression of CHO cells

When the nuclei of mammalian cells were labeled with BrdU during the progression of the S phase, the distinct replication patterns at different stages of the S phase could be visualized[3, 5]. In the present study, CHOC400 cells synchronized at G_1/S board were pulse-labeled by BrdU at different stages of the S phase and stained with anti-BrdU antibody, and also stained with DAPI to show the nuclei. The fluorescent signals were analyzed either by a conventional fluorescent microscope or a confocal laser scanning microscope. We observed four typical replication patterns (patterns I-IV; Fig 1). Pattern I represented the early replication foci throughout the euchromatic regions (Fig 1a). In pattern II, BrdU was incorporated into nuclear periphery and perinucleolar regions of heterochromatin (Fig 1c). Late in the S phase, pattern III was characterized by relatively large foci throughout the nucleus (Fig 1e), which might represent the replication of interior heterochromatin[4]. Pattern IV was defined as replication that took place on several large internal or peripheral foci

Tab 1. Percentage of each replication pattern during S phase progression

Time after HU release	Percentage in BrdU positive nuclei			
	Pattern I	Pattern II	Pattern III	Pattern IV
0.5 h	100	-	-	-
3 h	97.7 ± 13.2	2.3 ± 0.5	-	-
6 h	10.4 ± 2.4	87.5 ± 11.7	2.1 ± 0.8	-
9 h	5.2 ± 2.6	42.6 ± 12.9	23.3 ± 8.0	28.9±10.6

CHOC400 cells were synchronized and BrdU-labeled as in Fig 1. The percentage of each replication pattern of nuclei among BrdU-positive nuclei was noted at the indicated time point. Data from three independent experiments were averaged and expressed as means ± SD.

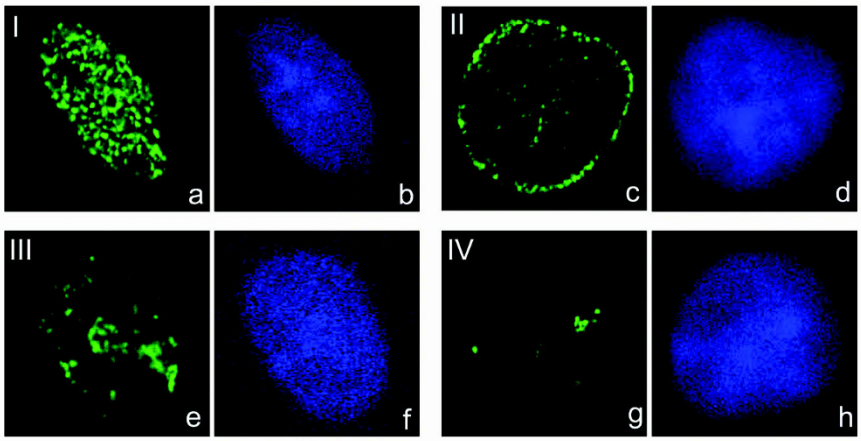

Fig 1. DNA replication patterns in CHO cells. CHOC400 cells were synchronized at G_1/S boundary by HU-block after mitotic shake-off and released into S phase. The cells were pulse-labeled with BrdU at different time points during the progression of S phase and stained with fluorescent antibodies (**a**, **c**, **e**, **g**), or stained with DAPI to show the nuclei (**b**, **d**, **f**, **h**). Photographs were taken using a laser confocal scanning microscope. Four typical relication patterns during S phase were shown (I-IV).

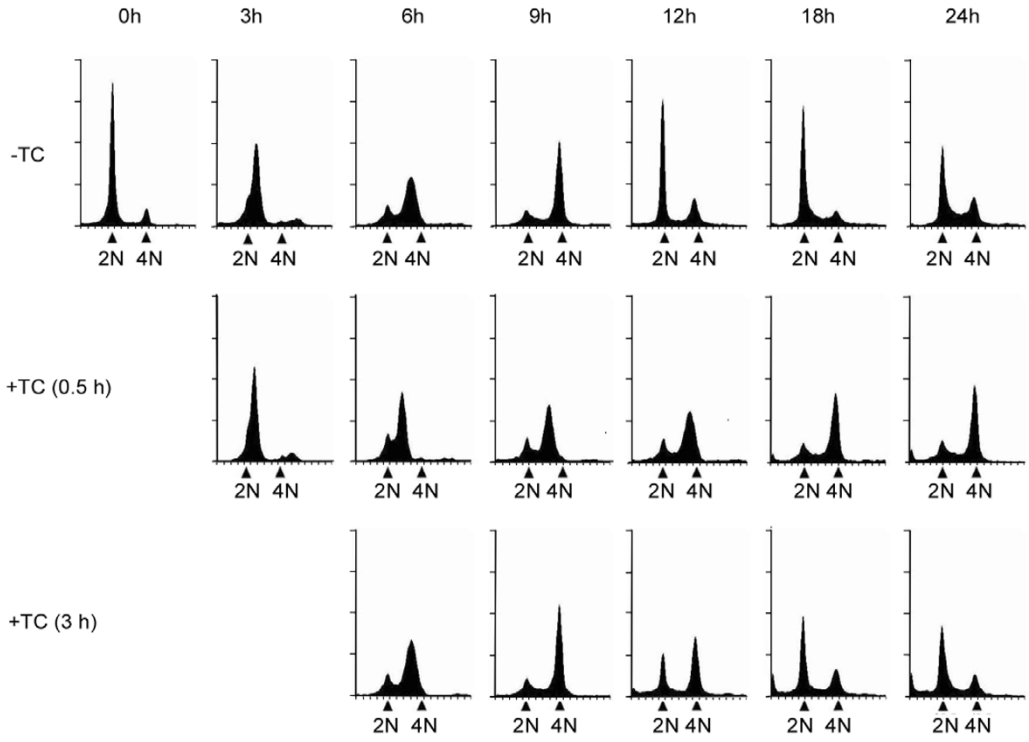

Fig 2. Different effects on cell cycle upon TC treatment at early- or middle-stages of S phase. CHOC400 cells were synchronized at G_1/S boundary by HU-blocking after mitotic shake-off and released into S phase. TC (20 ng/ml) was added at 0.5 h or 3 h after HU-release. Cells were harvested at the indicated time and analysed for DNA content by flow cytometry. Arrowheads indicate 2N and 4N DNA contents.

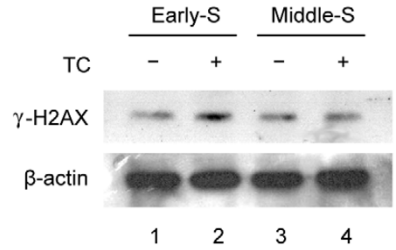

Fig 3. Differential γ-H2AX response in early-S-treated or middle-S-treated cells. CHOC400 cells were synchronized at G_1/S boundary and released into S phase. TC (20 ng/ml) was applied at 0.5 h (early-S) or 3 h (middle-S) after HU-release respectively. Cells were harvested either at 0.5 h and 3 h after HU release (lane 1 and 3) or at 6 h after TC treatment (lane 2 and 4). Equal amount of proteins were loaded on SDS-PAGE and detected by Western blot with specific antibodies for either γ-H2AX or β-actin.

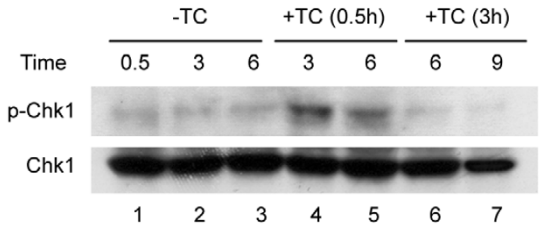

Fig 4. Phosphorylation of Chk1 in CHO cells treated with TC at the early-S phase. The cells were treated as described in the legend of Fig 2 and harvested at indicated time points. Equal amounts of total proteins were subjected to SDS-PAGE and detected by antibodies against Chk1 or phosphorylated Chk1 on Ser345 (p-Chk1).

(Fig 1g). Our results were generally consistent with previous works[3-5].

Statistical analysis revealed that the four patterns appeared sequentially during the S phase (Tab 1). In the early stage of the S phase (in the first 3 h), more than 90% of nuclei of the BrdU-positive cells showed pattern I. During the middle stage (between the 3-6th h), replication patterns shifted from pattern I to pattern II (about 90%). At the late stage of the S phase (between the 6-9th h), more than half of the nuclei showed patterns III and IV. These data confirmed previous observations that the particular replication patterns were tightly correlated with the progression of the S phase[3-5].

Differential activation of intra-S-phase checkpoint by TC treatment

We demonstrated previously that TC induced DNA damage in CHO cells[19]. To elucidate the effects of DNA damage on the S phase of mammalian cell cycle, TC was applied to the different stages of the S phase. CHOC400 were synchronized at G_1/S boundary with HU and released into the S phase by washing out the drug. Then, TC (20 ng/ml) was added at 0.5 h (early-S phase) or 3 h (middle-S phase) after HU-release. As shown in fig 2 (top panel), the duration of the S phase of CHO cells without the drug treatment was about 9 h. However, the S-phase progression of the cells treated at the early-S-phase with TC was delayed significantly, and the majority of TC-treated cells remained in the very late-S phase even 24 h after release from the HU-synchrony (Fig 2, middle panel).

To our surprise, the S-phase progression of the middle-S-phase cells treated with TC only slowed down slightly (Fig 2, low panel; and also see Fig 5b). Taken together, these results suggest that the intra-S-phase checkpoint is activated in the CHO cells treated by TC at early-S phase, but not activated in the cells treated by TC at middle-S phase.

Sensitive to TC-induced DNA damage in early-S-phase cells

Since it is known that TC induces DNA damage in exponentially growing CHO cells[19], the differential activation of the intra-S-phase checkpoint might be due to the early-S-phase cells being sensitive to TC-treatment. We detected the phosphorylation of H2AX in TC-treated CHO cells with a specific antibody for γ-H2AX since the phosphorylated form of H2AX, refered to as γ-H2AX, often serves as an indicator of DNA damage[24, 25]. H2AX is a variant of histone H2A, which responds to DNA damage by being phosphorylated on a serine at carboxyl terminus [26-28]. As shown in Fig 3, phosphorylated H2AX was detected in the early-S-phase cells treated with TC for 6 h (compare lane 2 to lane 1), while the amount of γ-H2AX in the middle-S-phase cells treated with TC for 6 h was similar to that of the control cells (compare lane 4 to lane 3). This result suggests that the early-S-phase CHO cells are more sensitive to TC-induced DNA damage.

It was shown that DNA damage during the S phase activates the intra-S-phase checkpoint and involves transducer kinases such as Chk1/Chk2[9, 10]. Phosphorylation on serine 345 of Chk1 in response to DNA damage is required for Chk1 activation and checkpoint-mediated cell cycle arrest[12, 29, 30]. In the present study, the phosphorylation state of Chk1 in TC-treated cells was analyzed. In the control cells, the phosporylation of Chk1 was quite low (Fig 4, lanes 1-3), in accordance with previous descriptions[30]. Upon treatment with TC in the early S-phase, the phosporylation of Chk1 increased significantly (Fig 4, lane 4 and 5). In contrast, the phosporylation of Chk1 in the cells treated with TC at the middle-S-phase did not increase (Fig 4, lanes 6 and 7). Taken together, these data indicated that the TC-treatment induced DNA

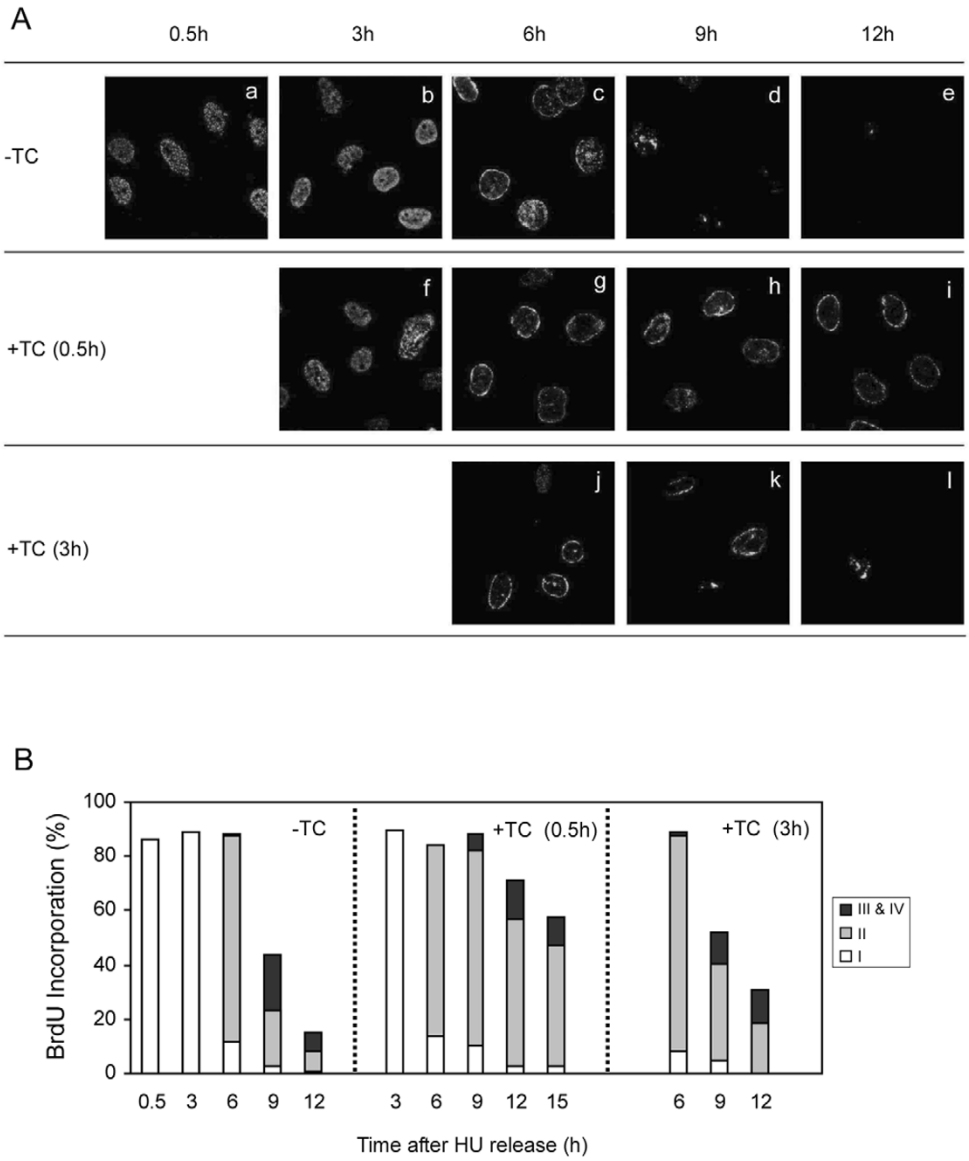

Fig 5. Analysis of DNA replication patterns in TC-treated cells. (**A**) CHOC400 cells grown on coverslips were treated with TC as described in Fig 2, then pulse-labeled with BrdU at the indicated time points and stained with anti-BrdU antibodies. Photographs were taken using a confocal laser scanning microscope. Representative patterns of each time point were shown. (**B**) The percentage of each replication pattern of BrdU-positive nuclei versus total nuclei was calculated. More than one hundred BrdU-positive nuclei for each time point were scored. Similar results were shown in two independent experiments.

damage of the early-S-phase CHO cells and then activated the intra-S-phase checkpoint, whereas the middle-S-phase cells were less sensitive to TC-induced DNA damage and did not activate the intra-S-phase checkpoint.

Identification of replication patterns upon TC treatment

To evaluate the effects of TC-induced DNA damage on the replication process, BrdU incorporation experiments were performed in the synchronized CHO cells as described in Fig 1. In these cells, around 90% of nuclei were BrdU positive within the first 6 h (Fig 5B), indicating that nearly all synchronized CHO cells at G_1/S board were performing DNA replication after release from HU-blocking. However, about 60% of BrdU-positive nuclei remained in the population of the cells treated with TC at the early-S-phase even at 15 h (Fig 5B), suggesting that the activation of the intra-S-phase checkpoint slows down the rate of S-

phase progression, which is in agreement with the data of FACS analysis (Fig 2).

To further analyze the S phase progression under the different drug treatments, the percentage of nuclei containing each replication pattern out of the total number of nuclei was calculated. This quantitative analysis showed that most of the labeled nuclei treated with TC at the early-S phase kept replication pattern II even 15 h after the HU-release, although the replication patterns shifted from pattern I to pattern II within the first 6 h after HU-release (Fig 5). In contrast, the cells either treated with TC or without the drug treatment at the middle-S phase showed the shift of replication patterns during the progression of S phase (Fig 5). These results suggest that the intra-S-phase checkpoint mainly inhibits the firing of late origins of DNA replication.

DISCUSSION

We described here that the application of DNA-damage drug TC to CHO cells at the early stage of S phase activated the intra-S-phase checkpoint and then delayed the S-phase progression severely, whereas the application of TC at the middle-S phase did not activate the intra-S-phase checkpoint. This suggested that cells in the early stage of S phase might be much more sensitive to DNA-damage reagents than cells in the middle stage of S phase. Since TC induced DNA damage in exponentially growing CHO cells and also caused DNA damage and apoptosis after longer exposure in the middle-S-phase cells ([19]; also see Fig 2, 18 h and 24 h), it is also possible that the tolerance to DNA damage at the early and middle stages of S phase is very different. Yeast cells in the S phase were demonstrated to have a much higher threshold of DNA damage required for activation of Rad53p-mediated checkpoint than cells in the G_1 or G_2 phases[31].

There are many DNA replication origins in eukaryotic genomes, which are regulated spatially and temporally[32]. Recent experiments have shown that the activation of the intra-S-phase checkpoint particularly inhibits late-origin firing. The mechanism of this inhibition may involve the Rad53-dependent checkpoint pathway and result from the prevention of the assembling of replication factors such as Cdc45p and ORC proteins[15, 16, 33, 34]. Actions to slow down S phase progression and to inhibit firing of late origins upon activation of the intra-S-phase checkpoint may provide more time for the cells to repair damaged DNA. However, our results showed that DNA replication forks progression continued slowly even without late-origin firing (Fig 2, middle panel, compare 9 h, 12 h, 18 h and 24 h), suggesting that the process of the elongation of DNA replication might be separate from the events of the initiation of the late-origins. Future studies are needed to analyze the relationship between the elongation of DNA replication and the initiation of late-origins when the intra-S-phase checkpoint is activated.

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