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# Ctf4p facilitates Mcm10p to promote DNA replication in budding yeast

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ABSTRACT

Ctf4p (chromosome *t*ransmission *f*idelity) has been reported to function in DNA metabolism and sister chromatid cohesion in *Saccharomyces cerevisiae*. In this study, a *ctf4*$^{S143F}$ mutant was isolated from a yeast genetic screen to identify replication-initiation proteins. The *ctf4*$^{S143F}$ mutant exhibits plasmid maintenance defects which can be suppressed by the addition of multiple origins to the plasmid, like other known replication-initiation mutants. We show that both *ctf4*$^{S143F}$ and *ctf4*Δ strains have defects in S phase entry and S phase progression at the restrictive temperature of 38 °C. Ctf4p localizes in the nucleus throughout the cell cycle but only starts to bind chromatin at the G1/S transition and then disassociates from chromatin after DNA replication. Furthermore, Ctf4p interacts with Mcm10p physically and genetically, and the chromatin association of Ctf4p depends on Mcm10p. Finally, deletion of *CTF4* destabilizes Mcm10p and Pol α in both *mcm10-1* and *MCM10* cells. These data indicate that Ctf4p facilitates Mcm10p to promote the DNA replication.



## 1. Introduction

Eukaryotic DNA replication is tightly regulated to ensure that all chromosomal DNA is replicated exactly once per cell cycle. The stepwise assembly and disassembly of pre-RC at replication origins during the cell cycle are of critical importance for the regulation of DNA replication. Pre-RC assembly requires the recruitment of a number of replication-initiation proteins including Orc1-6p, Noc3p, Cdc6p, Cdt1p and Mcm2-7p onto origins during the M-to-G1 transition [1–3]. Activation of the S phase cyclin dependent kinases and the Cdc7p–Dbf4p kinase leads to the loading Sld2p, Sld3p, Dbp11p, Cdc45p, GINS, Replication Protein A (RPA) and Pol α onto origins to initiate DNA replication [4–9].

Current knowledge about the regulation of DNA replication was largely derived from the studies of replication-initiation proteins and their interactions with replication origins. To further advance our understanding of the complicated mechanism and regulation of replication initiation, we have used a yeast phenotypic screen to identify previously unknown initiation proteins (Ma et al., unpublished) based on the plasmid loss assay that has been reported before [10–12] combined with a yeast colony color assay [13]. We isolated a *ctf4*$^{S143F}$ mutant which exhibited typical plasmid maintenance defects that can be rescued by the presence of multiple replication origins on the plasmid, suggesting that Ctf4p plays a role in DNA replication initiation besides its function in the progression of the replisome [8,14–17].

Ctf4p was originally identified in a genetic screen for mutants affecting chromosome transmission fidelity in budding yeast [18]. Ctf4p is involved in the establishment of sister chromatid cohesion together with Ctf18p to facilitate sister chromatid cohesion in association with replication forks [19]. Moreover, two recent studies in mammalian cells indicated that Ctf4p/And-1 plays essential roles in the assembly of the Cdc45p–Mcm2-7p–GINS complex (CMG complex) and in recruiting Pol α to the chromatin [20,21]; however, these roles have not been reported in budding yeast Ctf4p. Interestingly, deletion of *CTF4* causes synthetic lethality in some known initiation mutants such as *orc2-1*, *orc5-1* and *cdc6-1*, suggesting that Ctf4p may play some roles in initiation of DNA replication [22]. However, the basis for such synthetic lethality is not clear, and the function of Ctf4p in DNA replication has not been clearly demonstrated.

In this study, new evidence for the roles of Ctf4p in DNA replication process is presented. First, plasmid loss rate assays suggested that Ctf4p functions in DNA replication initiation in an ARS-dependent manner. Second, the cell growth and FACS analysis showed that *CTF4* is required for cell viability, S phase entry and S phase progression at 38 °C. Third, fluorescence microscopy analysis and chromatin binding assays showed that Ctf4p localizes in the nucleus throughout the cell cycle but binds onto chromatin mainly during S phase. Furthermore, Ctf4p interacts with Mcm10p physically and genetically, and deletion of *CTF4* destabilizes Mcm10p and Pol12p in both the mutant *mcm10-1* and wild-type *MCM10* strains. Together, these data indicate that Ctf4p facilitates Mcm10p to promote DNA replication in budding yeast.

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## 2. Material and methods

### 2.1. Strains, plasmids, media and antibodies

Yeast strains and plasmids used in this study are listed in Table S1. Strains *ctf4*$^{S143F}$ (YL1100) was obtained by backcrossing the initial candidate from the random mutagenesis for four times to YL34. Constructions of epitope-tagged and gene-deletion strains were performed using methods previously described [23]. YL1105 was constructed by crossing *ctf4*Δ to WCY45 followed by tetrad dissection from the sporulated diploid cells.

Yeast transformation was performed using the lithium acetate method [24]. Unless otherwise specified, cells were grown at 30 °C in YPD medium, synthetic complete medium (SCM), or drop-out medium (SCM minus). The anti-HA (12CA5) and anti-Myc (9E10) antibodies were from Roche Applied Science. Anti-ORC, anti-Mcm2, anti-Cdc45 and anti-Pol12 antibodies were kind gifts from Bruce Stillman.

### 2.2. Plasmid maintenance and quantitative plasmid loss assay

The plasmid maintenance and quantitative plasmid loss assays were performed as reported [12].

### 2.3. Cell synchronization, FACS analysis and chromatin binding assay

Cells were synchronized in G1, S, or G2/M phase with α-factor (5 μg/ml; replenished every 1.5 h), hydroxyurea (HU; 150 mM), or nocodazole (Noc; 15 μg/ml), respectively. Flow cytometry and chromatin binding assay were performed as previously described [12,25,26].

### 2.4. Microscopic analysis of Ctf4-GFP

Cells were collected by centrifugation at 12,000*g* for 5 s and resuspended in pre-cooled (−20 °C) 100% ethanol. Just before observation, cells were washed with 100 μl of 1 × PBS containing 0.5 μg/ml DAPI and resuspended in a small volume (10 μl) of the same solution. Sample images were taken and processed with a Zeiss Axioplan microscope.

### 2.5. Co-immunoprecipitation (co-IP) assay and yeast two-hybrid assay

co-IP analysis and two-hybrid analysis between Mcm10p and Ctf4p was performed as described previously [27].

## 3. Results

### 3.1. Isolation of the *ctf4*$^{S143F}$ replication-initiation mutant from a yeast genetic screen

It is known that all known DNA replication-initiation mutants tested show a high loss rate with a plasmid containing a single ARS and a lower loss rate with a plasmid that bears multiple ARS elements [11,12,28–30]. Based on this, we carried out a sensitive yeast phenotypic screen a colony color sectoring assay [13] to identify replication-initiation proteins using a pair of tester plasmids p1ARS and p8ARSs [12]. After screening over 0.7 million randomly mutagenized yeast colonies, among ~150 mutants in genes that are known or unknown to be related to replication initiation (Ma, et al., unpublished), a *ctf4*$^{S143F}$ mutant was isolated. Both qualitative (Fig. 1A) and quantitative (Fig. 1B) plasmid loss assays indicated that the *ctf4*$^{S143F}$ mutant exhibited a high plasmid loss rate with p1ARS and a much reduced loss rate with p8ARSs, suggesting that Ctf4p is involved in or regulates replication initiation in an ARS-dependent manner. Consistent with a previously report that Ctf4p is involved in chromosome segregation [22], *ctf4*Δ mutant could not stably maintain either p1ARS or p8ARSs. The single point mutation S143F is located in the conservative WD40 domain of Ctf4p (Fig. 1C).

### 3.2. Mutant ctf4 cells are defective in S phase entry and S phase progression at the restrictive temperature of 38 °C

During the construction of the *ctf4*Δ haploid mutant by tetrad dissection, we noticed that *ctf4*Δ cells grew poorly compared to wild-type cells (Fig. S1A). Further testing indicated that *ctf4*Δ mutant cells were lethal at 38 °C (Fig. S1B) and this lethality could be rescued by wild-type *CTF4* gene on the pRS416 vector (data not shown). *ctf4*$^{S143F}$ mutant was also somewhat sensitive to the high temperature (Fig. S1B). These results show that Ctf4p is essential for cell viability in budding yeast at 38 °C.

To further investigate the role of Ctf4p in DNA replication, the DNA contents of *ctf4*$^{S143F}$ and *ctf4*Δ mutants and wild-type control cells in the cell cycle were measured by flow cytometry. Cells were first synchronized in G1 phase with α-factor and then released into growth medium at 25 and 38 °C. Cell samples at various time points after release were harvested for FACS analysis. As shown in Fig. 2A, *ctf4* mutants and wild-type cells could enter and finish S phase at 25 °C, with some delay in the *ctf4* mutants. At 38 °C, while the majority of wild-type cells completed DNA synthesis

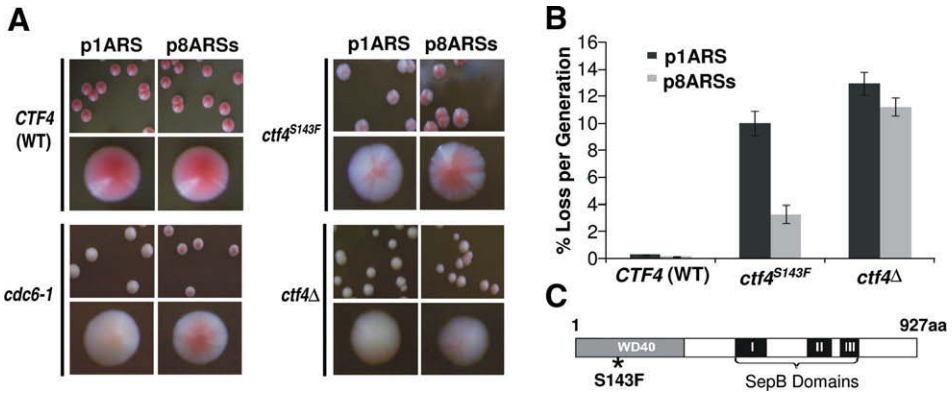


**Fig. 1.** The high plasmid loss rate in *ctf4*$^{S143F}$ mutant can be suppressed by the addition of multiple ARS copies to the plasmid. (A) p1ARS and p8ARSs were separately transformed into *ctf4*$^{S143F}$, *ctf4*Δ, *cdc6-1* and wild-type (WT) cells, and the transformants were grown on YPD plates at 25 °C for 5–7 days, except that the transformants of *cdc6-1* were incubated at 28 °C. (B) Quantitative plasmid loss rates were measured for the *ctf4*$^{S143F}$ and *ctf4*Δ mutants and wild-type (WT) strain. Cells containing either p1ARS or p8ARSs were grown in YPD and SCM-Leu medium for 10–11 generations at 25 °C. Average and standard deviations (error bars) were obtained from three experiments. (C) Schematic representation of the domain structure of Ctf4p and mutation site of *ctf4*$^{S143F}$ (*) in the conservative WD40 domain of Ctf4p.