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Analysis of Origin Recognition Complex in *Saccharomyces cerevisiae* by Use of Degron Mutants

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Origin recognition complex (ORC), a six-protein complex (Orc1p-Orc6p), may deeply involve in initiation of chromosomal DNA replication. However, since most temperature-sensitive *orc* mutants of *Saccharomyces cerevisiae* show the accumulation of cells with nearly 2C DNA content, the exact stage at which ORC acts is not fully understood. In this study, we constructed a heat-inducible degron mutant for each ORC subunit. As well as each targeted subunit, other subunits of ORC were also rapidly degraded under non-permissive conditions. In the *orc5* degron mutant, incubation under the non-permissive conditions caused accumulation of cells with nearly 2C DNA content, and phosphorylation of Rad53p. When Orc5p (ORC) is depleted, this inhibits G1/S transition and formation of a pre-replicative complex (pre-RC). For pre-RC to form, and G1/S transition to proceed, Orc5p (ORC) must be present in late G1, rather than early G1, or G2/M. Block and release experiments revealed that Orc5p (ORC) is not necessary for S and G2/M phase progression. We therefore propose that ORC is necessary for the G1/S transition and pre-RC formation, and accumulation of cells with nearly 2C DNA content seen in various *orc* mutants is due to inefficient pre-RC formation, and/or induction of checkpoint systems.

Key words: DNA replication, heat inducible degron mutant, ORC, Orc5p, pre-replicative complex.

Abbreviations: α-factor, α mating factor; DHFR, dihydrofolate reductase; FACS, fluorescence-activated cell sorter; HA, haemagglutinin; HU, hydroxyurea; MCM, mini-chromosome maintenance complex of proteins; ORC, origin recognition complex; pre-RC, pre-replication complex.

The initiation of chromosomal DNA replication is tightly regulated to replicate the genome just once per cell cycle. To reveal the underlying molecular mechanism for this regulation, it is important to understand the initiator of chromosomal DNA replication. In *Escherichia coli*, DnaA is the initiator of chromosomal DNA replication: temperature-sensitive *dnaA* mutants show defects in initiation of DNA replication, and an *in vitro* chromosomal DNA replication system, reconstituted from purified enzyme, is dependent on DnaA (*1*). In eukaryotes, origin recognition complex (ORC) is the most likely initiator. ORC was originally identified as a six-protein complex that specifically binds to *Saccharomyces cerevisiae* (*S. cerevisiae*) origins of chromosomal DNA replication (*2*) (in this manuscript, 'ORC' refers to *S. cerevisiae* ORC). ORC homologues have been found in various eukaryotic species, including humans (*3*). Although there is only weak homology in amino acid sequence between ORC and DnaA, these two factors share a number of functions; (i) both bind to each origin of chromosomal DNA replication (*1*, *2*); (ii) both bind to ATP and ADP, they have intrinsic ATPase activity, and adenine nucleotides bound to them regulate their activities (*4–10*) (iii) both proteins interact with replicative DNA helicase and recruit it to each origin of DNA replication (*1*, *10*, *11*). These observations strongly suggest that ORC is the initiator of chromosomal DNA replication. However, an origin-dependent *in vitro* chromosomal DNA replication system has not yet been developed for eukaryotes, so the dependency of replication on ORC has not been formally proved.

Most temperature-sensitive S. cerevisiae orc mutants show the accumulation of cells with nearly 2C DNA content at non-permissive temperatures (*12–16*), suggesting that ORC may be involved in the G2/M progression rather than the G1/S transition. This observation argues against the idea that ORC initiates DNA replication. On the other hand, the contribution of ORC to the initiation has been suggested by using temperature-sensitive orc mutants and block and release experiments, microscopic observation and 2D gel experiments (*13*, *16–19*). Thus, exact role of ORC in vivo is not yet fully understood. There are significant problems in interpreting results with such temperature-sensitive mutants; some mutant proteins show gain-of-function, and the mutant protein may maintain some functions even at non-permissive temperatures.

To address this issue, we used genetic systems that cause rapid and conditional elimination of the target protein. In yeast, we generally use a genetic shut-off

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system, for example, locating the target gene under the *GAL* promoter and changing the carbon source of culture medium from galactose to glucose to suppress transcription. However, in this system, the target protein is depleted according to its natural half-life. Therefore, stable proteins such as ORC are depleted slowly, and thus other approaches seem necessary (*15*).

Creating a heat-inducible degron mutant is a very useful way to achieve rapid conditional degradation of a target protein. The N-terminal of the target protein is fused with the N-terminal fragment of mutated (temperature-sensitive) mouse dihydrofolate reductase (DHFR) gene, the so-called 'heat-inducible degron'. When cells are incubated at the non-permissive temperature (37°C), the fused protein is easily ubiquitinated, resulting in its rapid degradation by ubiquitin-proteasome system (*20*). Diffley and co-workers (*21–23*) improved this method by *GAL* promoter-regulated simultaneous overproduction of Ubr1p, the E3 ubiquitin ligase, which stimulates this degradation. This improvement has proved useful for genetic analysis of DNA replication-related proteins, such as Cdc45p, and the mini-chromosome maintenance complex of proteins (MCM). Kanemaki *et al*. (*24*) clearly showed that Mcm4p can be rapidly depleted in a heat-inducible degron mutant with the overproduction of Ubr1p, but not in cells with a genetic shut-off system. In this study, we constructed a heat-inducible degron mutant for each ORC subunit. Under non-permissive conditions (incubation at 37°C in the presence of galactose), each targeted subunit, and also other subunits of ORC, were rapidly degraded. All *orc* degron mutants showed a temperature-dependent galactose-stimulated growth defect. Block and release experiments using the *orc5* mutant showed that Orc5p (ORC) is necessary at late G1, rather than early G1 and G2/M phases, for the G1/S transition and pre-replicative complex (pre-RC) formation, suggesting that ORC functions as the initiator of chromosomal DNA in budding yeast.

MATERIALS AND METHODS

Strains, Plasmids and Medium—*S. cerevisiae* strains are listed in Table 1 (*25*). Cells were cultured in YP medium (1% yeast extract and 2% Bacto-peptone) containing 2% galactose, raffinose or glucose. Plasmids, pPW66R and pKL54, were gifts from Dr. Diffley. Plasmids, pRS404, pFA6a-3HA-*TRP1* and pFA6a-13Myc-*TRP1*, were from our laboratory stock.

The degron mutant for each *ORC* gene was constructed as described previously (*21*, *23*). The plasmid pKL54 (*GAL-UBR1*) was digested with *Pme*I and transformed into W303-1A. The construct of the resultant strain (YYM101) was confirmed by colony PCR. DNA encoding the N-terminal region of each ORC subunit was then amplified by PCR and the amplified DNA was ligated into the *Hin*dIII-*Xho*I region of pPW66R. The resultant plasmid was digested with a single-cutting restriction enzyme and transformed into YYM101. The construction of resultant strains (YYM102–107) was confirmed by colony PCR.

Modification of the *ORC2* gene with 13Myc, or of the *ORC6* gene with 3HA (haemagglutinin, HA) was performed as described previously (*26*). PCR was performed using pFA6a-3HA-*TRP1* or pFA6a-13Myc-*TRP1* plasmid as template, with primers to the C-terminal region of the *ORC6* or *ORC2* genes, respectively. The amplified DNA was transformed into W303-1A, YYM104 or YYM106. The construction of resultant strains (YYM108–113) was confirmed by colony PCR.

Table 1. **Yeast strains**

Strain	Genotype	Reference
W303-1A	*Mat**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Thomas and Rothstein (*25*)
YYM101	W303-1A *ubr1*△*::GAL-Myc-UBR1::HIS3*	This study
YYM102	YYM101 *orc1-td*	This study
YYM103	YYM101 *orc2-td*	This study
YYM104	YYM101 *orc3-td*	This study
YYM105	YYM101 *orc4-td*	This study
YYM106	YYM101 *orc5-td*	This study
YYM107	YYM101 *orc6-td*	This study
YYM108	W303-1A *ORC2-13Myc::TRP1*	This study
YYM109	W303-1A *ORC6-3HA::TRP1*	This study
YYM110	YYM104 *ORC2-13Myc::TRP1*	This study
YYM111	YYM104 *ORC6-3HA::TRP1*	This study
YYM112	YYM106 *ORC2-13Myc::TRP1*	This study
YYM113	YYM106 *ORC6-3HA::TRP1*	This study
YYM114	W303-1A *bar1*△*::TRP1*	This study
YYM115	YYM106 *bar1*△*::TRP1*	This study

Disruption of the *BAR1* gene in W303-1A and YY106 was performed as described previously (*27*). PCR of the *BAR1* gene was performed using pRS404 (a plasmid with *TRP1*) as template, and primers to the 5′ upstream region of the N-terminal or 3′ downstream region of the C-terminal. The amplified DNA was transformed into W303-1A or YYM106. The construct of resultant strains (YYM114–115) was confirmed by colony PCR.

Fluorescence-Activated Cell Sorter (FACS) Analysis—Samples were prepared as previously described (*15*) with some modifications. Cells were pelleted by centrifugation, washed with sterilized water and fixed in 70% ethanol for 12 h. Cells were again pelleted, re-suspended in 50 mM sodium citrate, sonicated for 1 min, treated with 0.25 mg/ml RNase A (SIGMA) for 1 h at 50°C and then with 1 mg/ml Proteinase K (Wako) for 1 h at 50°C. DNA was stained with 50 μg/ml of propidium iodide (SIGMA) and 20,000 cells from each sample were scanned with a FACSCalibur (Becton Dickinson).

Chromatin-Binding Analysis—Yeast spheroplasts were lysed with Triton X-100 and samples were processed into soluble (supernatant) and chromatin (insoluble precipitate) fractions by centrifugation (*15*). Equivalent amounts (total protein) of chromatin fractions were electrophoresed on 7.5% polyacrylamide gels containing SDS, transferred to PVDF membrane–and probed with monoclonal antibodies against Orc3p (SB3), Orc5p (SB5), Mcm2 (Mcm2–28), HA (12CA5) or Myc (9E10) (*28–31*).

Block and Release Experiments for Cell Cycle Progression—Yeast cells were cultured at 24°C to early log phase and cell cycle progression was blocked at the G1, S or G2/M phase by incubation with 0.05–5 μg/ml with α mating factor (α-factor) (SIGMA), 0.1 M

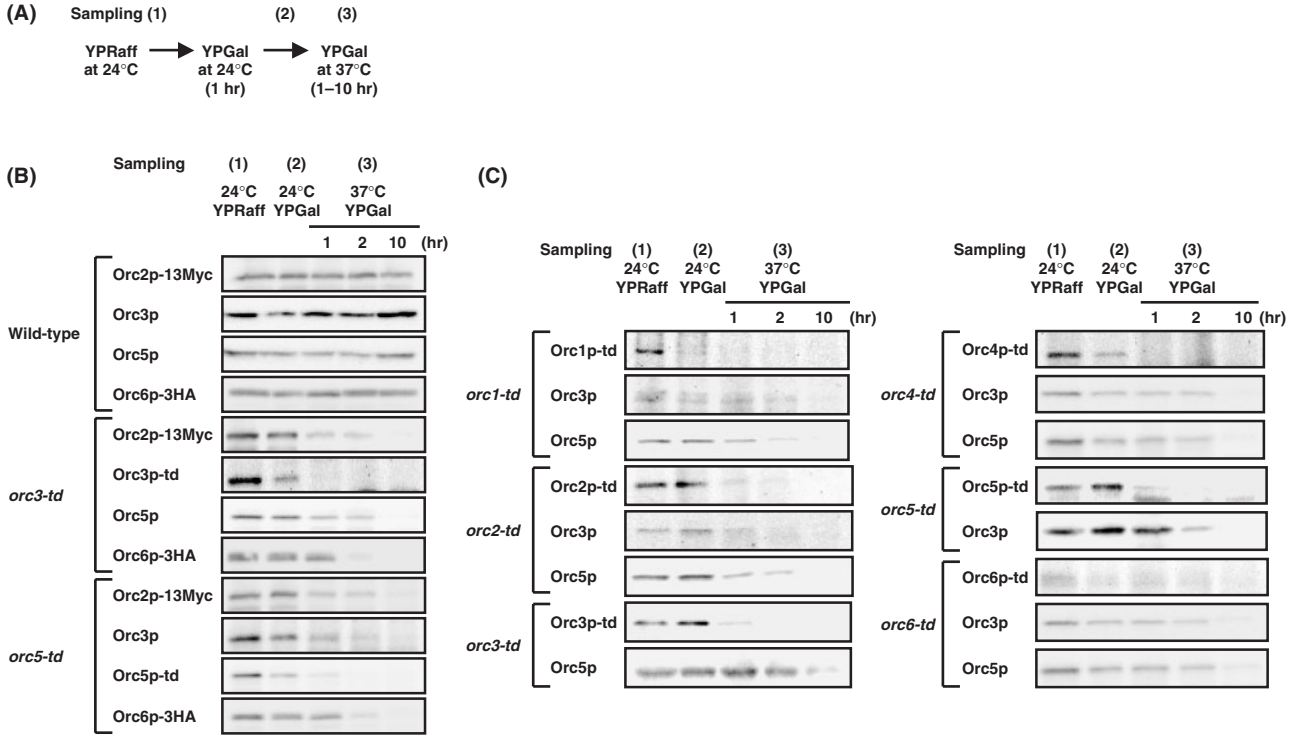

Fig. 1. **Degradation of ORC subunits in *orc* degron mutants.** (A) Experimental outline and timing of sampling. (B) W303-1A, YYM108, YYM109 (wild-type); YYM104, YYM110, YYM111 (*orc3-td*); and YYM106, YYM112, YYM113 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C, and then cultured in YP medium with 2% galactose (YPGal) for 1 h at 24°C. Cells were further incubated at 37°C in the same medium, and a small portion of culture was taken when indicated. Chromatin fractions were prepared and analysed by immunoblotting using monoclonal antibodies specific for Orc3p, Orc5p, Myc (for Orc2p-13Myc) and HA (for Orc6p-3HA and each td-subunit). (C) YYM102-107 (*orc1-td*, *orc2-td*, *orc3-td, orc4-td orc5-td* and *orc6-td*) cells were cultured and analysed as above.

hydroxyurea (HU) (SIGMA) or 3 μg/ml nocodazole (SIGMA), respectively. Cells were released from the block by washing and re-suspending in fresh medium without the blocking agents.

RESULTS

Construction of a Heat-inducible Degron Mutant for Each ORC Subunit—To achieve temperature- and galactose-dependent rapid degradation of the targeted subunit of ORC, we inserted the 'heat-inducible degron' sequence at the N-terminal of each subunit of ORC, and transformed a plasmid containing the *UBR1* gene under the *GAL* promoter. Pre-incubation of *orc* degron mutants at 24°C for 1 h in YP medium with galactose (Fig. 1A) stimulated the subsequent degradation of the targeted subunit at 37°C (data not shown), so we routinely performed this pre-incubation. As shown in Fig. 1B, in the *orc3* degron mutant (*orc3-td*), Orc3p disappeared within 1 h of temperature shift (from 24°C to 37°C). Furthermore, following Orc3p-degradation, other subunits (myc-tagged Orc2p, Orc5p, HA-tagged Orc6p) also disappeared (Fig. 1B). Degradation of Orc1p and Orc4p could not be tested, as antibodies against Orc1p and Orc4p did not work under our experimental conditions, and we could not insert any tag into either the *ORC1* gene or the *ORC4* gene. Similar results were obtained with the *orc5* degron mutant (*orc5-td*); rapid degradation of Orc5p and subsequent degradation of other subunits (myc-tagged Orc2p, Orc3p, HA-tagged Orc6p; Fig. 1B). We confirmed that in wild-type cells the amount of each subunit of ORC is constant under the experimental conditions used (Fig. 1B).

For other heat-inducible *orc* degron mutants (*orc1-td, orc2-td, orc4-td* and *orc6-td*), there was rapid degradation of each targeted subunit and subsequent degradation of other subunits (Orc3p and Orc5p; Fig. 1C). Based on these results, we concluded that we had constructed heat-inducible degron mutant for every ORC subunit. From the results in Fig. 1, it can be suggested that ORC becomes unstable when any one of its subunits is degraded.

Growth Phenotypes of the Heat-Inducible orc Mutants—The effect of incubation temperatures and galactose on growth of each *orc* degron mutant strain was tested on YP agar plates (Fig. 2A). Compared to wild-type cells, the growth of all mutants was slow at 37°C, especially with galactose. At 24°C, growth of mutants was indistinguishable from that of wild-type cells (Fig. 2A). Since the transformation of *UBR1*-expression plasmid (*GAL-UBR1*) into the wild-type strain did not affect growth (Fig. 2A), the inhibition seen in mutants must be due to degron-dependent degradation of the targeted subunit and other subunits.

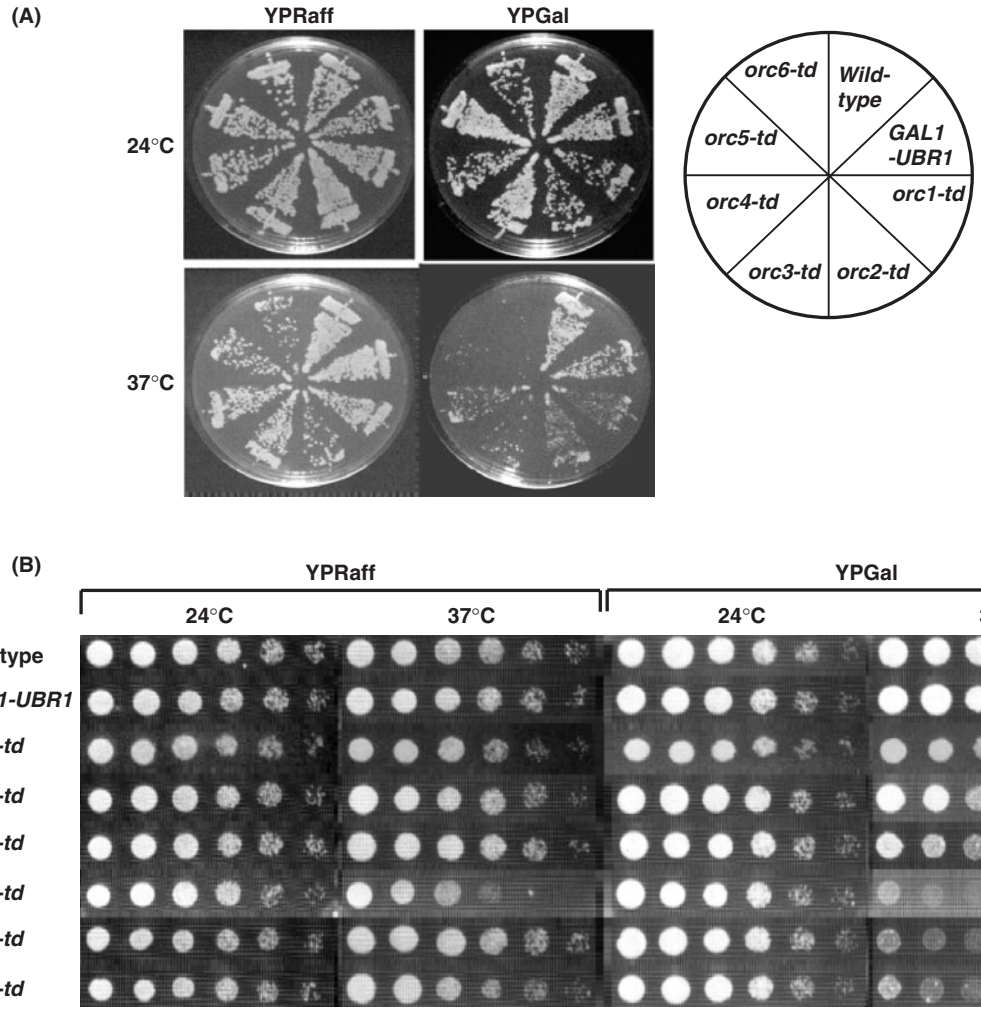

Fig. 2. **Growth phenotypes of ORC degron mutants.** W303-1A (wild-type), YYM101 (*GAL-UBR1*) and YYM102-107 (*orc1-td*, *orc2-td*, *orc3-td, orc4-td orc5-td* and *orc6-td*) cells were streaked on YP agar plates with 2% raffinose (YPRaff) or galactose (YPGal) and incubated at 24°C or 37°C for 2 days (A). Cell suspensions of each strain ($O.D._{600} = 1$, 0.25, 0.13, 0.068, 0.034, 0.017; from left to right) were dropped onto YPRaff or YPGal and incubated at 24°C or 37°C for 2 days (B).

For quantitative monitoring of the growth of *orc* degron mutants, we performed a dilution assay. Every mutant showed a temperature-dependent and galactose-stimulated growth defect (Fig. 2B), which is consistent with the results in Fig. 2A. The extents of growth defects differed among the various *orc* degron mutants; *orc4, orc5* and *orc6* mutants showed relatively clear phenotype. We used some not all *orc* degron mutants in the following experiments.

Cell Cycle Progression in the Heat-Inducible orc Mutants—The results in Fig. 2 indicate that under the non-permissive conditions the mutants have defects in cell cycle progression. To determine which phase of the cell cycle is blocked, *orc3* and *orc5* mutants and wild-type cells were asynchronously grown at 24°C in YP medium with raffinose, then with galactose, the incubation temperature was shifted to 37°C, and their DNA contents were determined by FACS analysis (Fig. 3A). Compared to the wild-type cells, the proportion of cells with nearly 2C DNA content increased over time in *orc3* and *orc5* mutants (Fig. 3B), suggesting that most cells were blocked in late S phase or G2/M phase. This accumulation was observed earlier in the *orc5* mutant than in the *orc3* mutant, which is consistent with the greater growth defect in *orc5* mutant (Fig. 2B). The transformation of *UBR1*-expression plasmid into wild-type cells did not affect the cell cycle progression (Fig. 3B), suggesting that it is the degron-dependent degradation of ORC, which is responsible for this defect of cell cycle progression.

Eukaryotic cells have checkpoint systems, which detect DNA damage and defects in DNA replication and stop cell cycle progression (*32*, *33*). Phosphorylation of Rad53p plays an important role in the checkpoint systems, and in some temperature-sensitive *orc* mutants Rad53p was phosphorylated at non-permissive temperatures (*32–34*). Thus, checkpoint systems may become induced in *orc* degron mutants under non-permissive conditions. We monitored Rad53p phosphorylation in the *orc5* mutant by immunoblotting under the same conditions as the FACS analysis. As shown in Fig. 3C, after incubation in

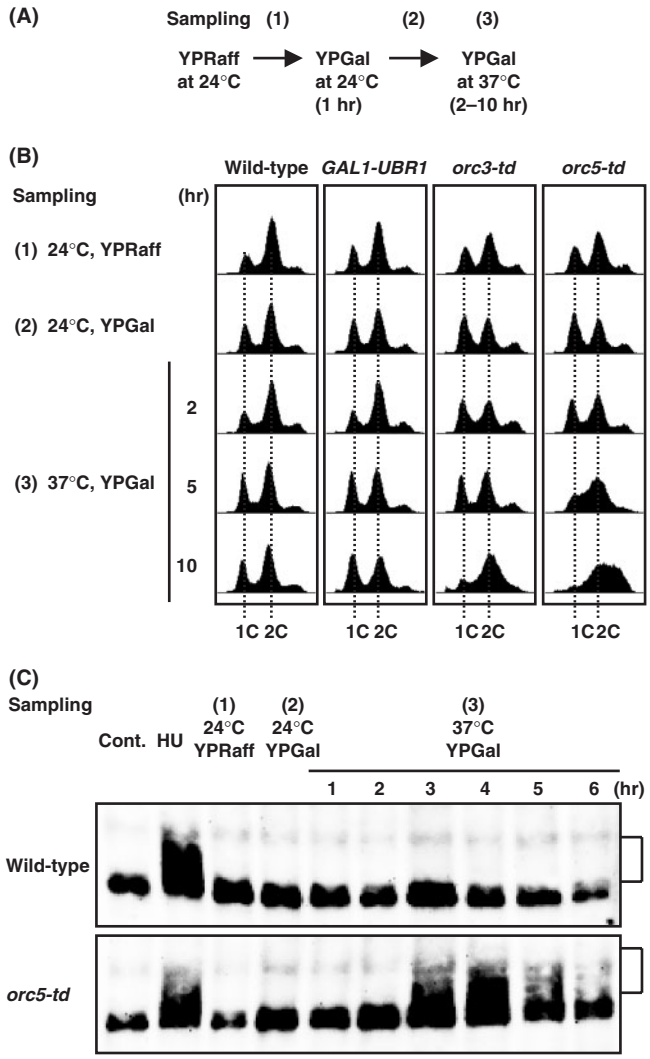

Fig. 3. **Cell cycle progression and phosphorylation of Rad53p in the *orc5* degron mutant.** W303-1A (wild-type), YYM101 (*GAL-UBR1*), YYM104 (*orc3-td*) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and then cultured in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium at 37°C. A small portion of culture was taken when indicated. (A) Experimental outline and timing of sampling. (B) Cellular DNA contents analysed by FACS. (C) Whole-cell extracts were analysed by immunoblotting with monoclonal antibody for Rad53p. For control experiments, wild-type cells were cultured for 2 h at 24°C in YPRaff with hydroxyurea (HU) or without it (Cont.). Phosphorylated forms of Rad53p are shown by an asterisk.

non-permissive conditions, the upper-shifted band of Rad53p increased in the *orc5* mutant, but not in wild-type cells. Similar increases were observed in wild-type cells treated with HU. These results suggest that checkpoint systems are induced when Orc5p (ORC) is degraded, and the defect of cell cycle progression in the *orc5* mutant (Fig. 3B) may involve checkpoint systems.

Progression of Distinct Cell Cycle in orc5 Mutants—We performed block and release experiments to examine the effect of the degradation of Orc5p (ORC) on cell cycle progression. Asynchronously cultured *orc5* mutant and wild-type cells were synchronized at G1, S or G2/M phase by incubation with α-factor, HU or nocodazole, respectively. In the *orc5* mutants, Orc5p was supposed to be degraded by incubating cells in YP medium with galactose at 24°C, then at 37°C in the presence of each blocking agent, then cells were released into fresh YP medium with galactose (Fig. 4A). As shown in Fig. 4B, both S phase progression (from HU block) and G2/M phase progression (from nocodazole block) were the same in the *orc5* mutants and wild-type cells. G1/S transition (from α-factor block) was slightly slower in the *orc5* mutants (Fig. 4B). Furthermore, in nocodazole and HU block, although the entry into G1 phase was indistinguishable, the following G1/S transition was delayed in the mutant, suggesting that Orc5p (ORC) is necessary for this step. In *orc5* mutants, the unclear inhibition of G1/S transition (from α-factor block) is probably due to inhibition of the ORC degradation at late G1 phase (at the point of α-factor block); ORC degradation is less in cells blocked with α-factor than in cells blocked with nocodazole, or asynchronously growing cells (data not shown).

To test whether Orc5p (ORC) is necessary for G1/S transition, *orc5* mutant cells, and wild-type cells synchronized at G2/M phase, were incubated at 37°C in YP medium with galactose to degrade Orc5p (ORC), then released into fresh medium with α-factor, to be re-synchronized at late G1 phase, and finally released again into fresh medium to start S phase (Fig. 5A). As shown in Fig. 5B, the G1/S transition was clearly inhibited in *orc5* mutants. In wild type, cells with 2C DNA content peaked within 30 min, and cells with 1C DNA content re-appeared within 100 min of the final release (Fig. 5B). On the other hand, in *orc5* mutants, the peak of DNA content moved gradually from 1C to 2C and cells with 1C DNA content did not reappear (Fig. 5B). These results suggest that when Orc5p (ORC) is depleted from G2/M to late G1, cells can enter G1 normally, but subsequent entry into S phase is inhibited. Thus, ORC must be required for G1/S transition.

Formation of the Pre-RC in the orc5 Mutant—In vivo foot-printing analysis revealed that a protein complex, called pre-RC, is formed in late M or G1 on each origin of chromosomal DNA replication (*35–39*). Pre-RC includes at least ORC, Cdc6p, Cdt1p and MCM (*40*). We here examined the formation of pre-RC in *orc5* degron mutants. We used a chromatin-binding assay to examine the loading of Mcm2p onto chromatin. (Mcm2p is a subunit of MCM). Wild-type cells and *orc5* mutants were synchronized at G2/M, followed by ORC degradation, and finally cells were released into fresh medium (Fig. 6A). As shown in Fig. 6B, in the wild-type cells, Mcm2p bound to chromatin 40 min after the release, and the intensity of band decreased gradually (may be due to cell cycle progression into S and G2/M), consistent with our previous results (*41*). On the other hand, in *orc5* mutants, Mcm2p did not bind chromatin at least until 90 min after the release (Fig. 6B). Thus, when ORC is depleted from G2/M to late G1, pre-RC does not form efficiently. This inefficiency is perhaps what inhibits G1/S transition in the *orc5* mutants (Fig. 5B).

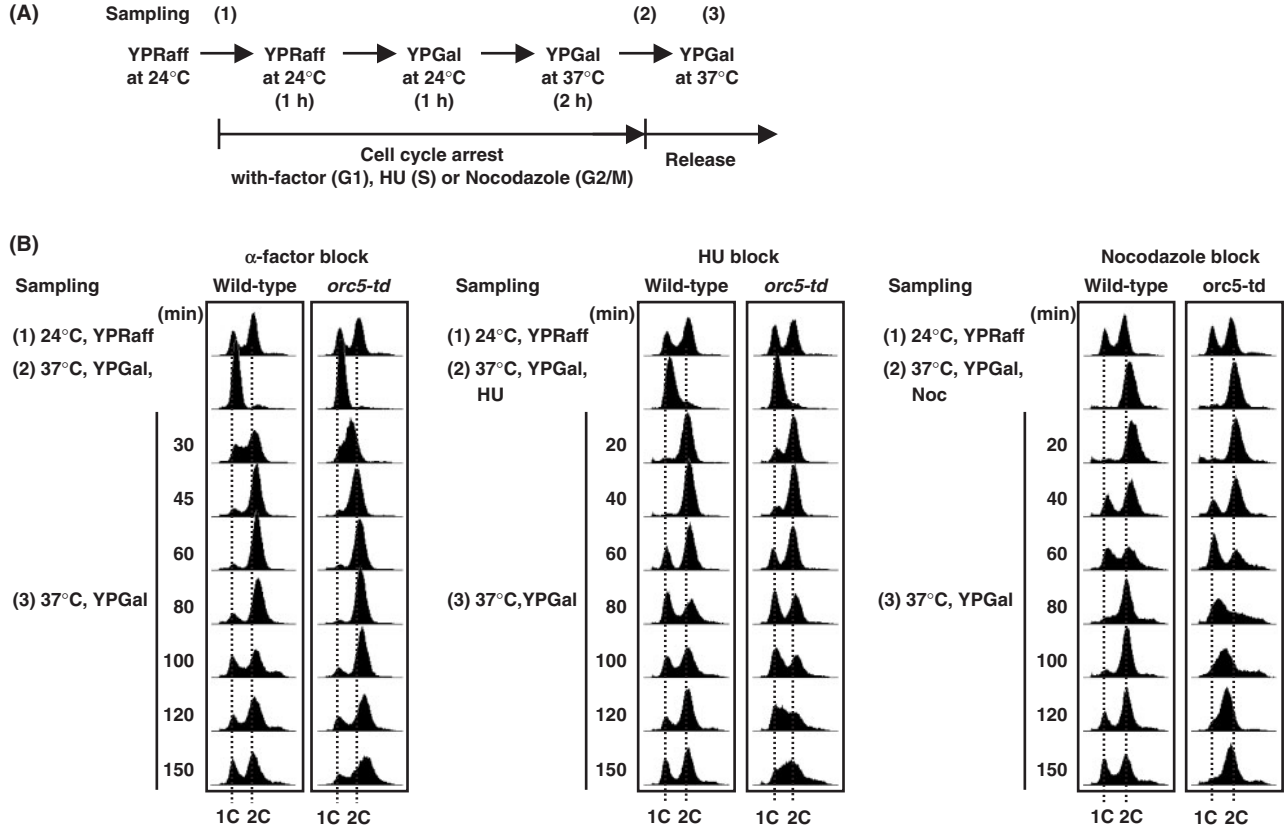

Fig. 4. **Block and release analysis for cell cycle progression in the *orc5* degron mutant.** W303-1A (wild-type) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G1, S or G2/M phase by incubation with α-factor, HU or nocodazole, respectively. Cells were cultured first in YPRaff for 1 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C, and finally in the same medium for 2 h at 37°C in the presence of each arrest agent. Cells were washed and released into the fresh YPGal and cultured at 37°C. (A) Experimental outline and timing of sampling. (B) FACS analysis was performed as described in Fig. 3.

ORC is Required at Late G1 Rather Than at Early G1 and G2/M, for Cell Cycle Progression and Pre-RC Formation—In experiments shown in Figs 5 and 6, ORC was absent in both G2/M and the following G1 phases. To define precisely when ORC is required for cell cycle progression (G1/S transition) and pre-RC formation, we designed experiments shown in Fig. 7A; wild-type cells and *orc5* mutants synchronized at G2/M phase, were first incubated in medium with galactose at 37°C to degrade Orc5p (ORC); then released into fresh medium with α-factor to be re-synchronized at G1 phase; then incubated at 24°C in medium with glucose plus α-factor (to restore ORC), or at 37°C in medium with galactose plus α-factor (to keep ORC absent); and finally released into fresh medium to examine cell cycle progression. We used glucose instead of raffinose after the final release, because under these conditions, even wild-type cells grow slowly with raffinose (data not shown). As shown in Fig. 7D, Orc5p was restored by the incubation at 24°C in YP medium with glucose (note the band of Orc5p-td at 60–120 min in the lower right panel in Fig. 7D). Under these conditions, cell cycle progression (G1/S transition) after the final release was identical in *orc5* mutants and wild type (right panel, Fig. 7B) and cells with nearly 2C DNA was not accumulated, even in *orc5* mutants (right panel, Fig. 7B). On the other hand, with continued absence of Orc5p (left panel, Fig. 7B), inhibition of G1/S transition and accumulation of cells with nearly 2C DNA content were observed in the *orc5* mutants, as in Fig. 5B.

We also monitored pre-RC formation under these conditions (Fig. 7C). Following incubation in YP medium with galactose and α-factor at 37°C, both Orc5p and Mcm2p were loaded on chromatin in the wild-type strain, but not in the *orc5* mutant (Fig. 7D, sampling time 3). When *orc5* mutants were subsequently incubated in YP medium with glucose at 24°C to restore Orc5p, both Orc5p and Mcm2p were loaded on chromatin, (lower right panel, Fig. 7D). On the other hand, if the incubation conditions were not shifted (to keep Orc5p absent), neither Orc5p-td nor Mcm2p was loaded on chromatin (lower left panel, Fig. 7D). Thus, for cell cycle progression (G1/S transition) and pre-RC formation, Orc5p (ORC) is required at late G1 (the point of α-factor block), but not at early G1 and G2/M.

Finally, we examined whether pre-RC is degraded after ORC degradation in α-factor-blocked cells. After formation of pre-RC in α-factor-blocked cells, ORC was tried to be degraded by incubation of the *orc5* mutants at 37°C in YP medium with galactose and the level of MCM (Mcm2p) was monitored (Fig. 8A and B). As shown in

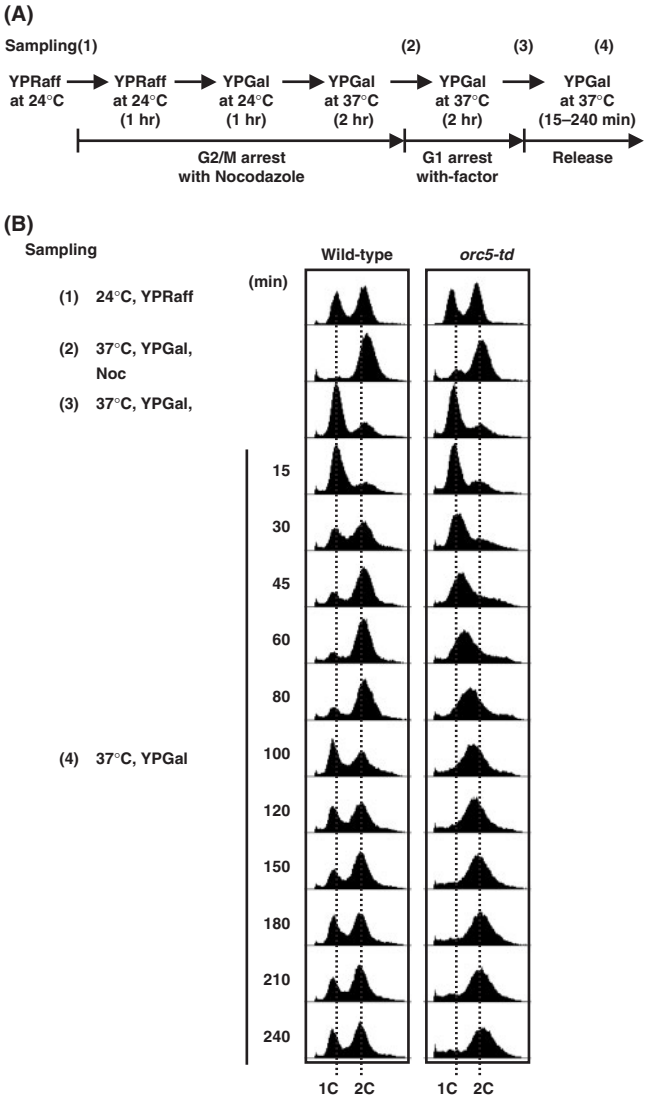

Fig. 5. **Requirement of ORC for G/S transition.** W303-1A (wild-type) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were cultured first in YPRaff for 1 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium for 2 h at 37°C in the presence of nocodazole. Then cells were washed and released into the fresh YPGal containing α-factor and cultured for 2 h at 37°C. Finally, cells were released into the fresh YPGal. (A) Experimental outline and timing of sampling. (B) FACS analysis was performed as described in the legend of Fig. 3.

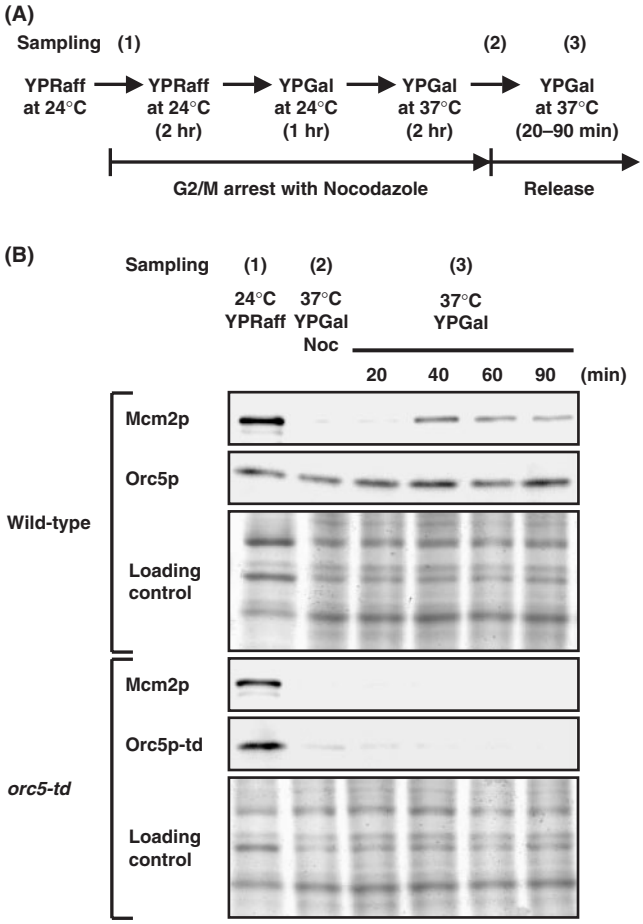

Fig. 6. **The pre-RC formation in the *orc5* degron mutant.** W303-1A (wild-type) and YYM106 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were cultured first in YPRaff for 2 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium for 2 h at 37°C. Cells were washed and released into the fresh YPGal and cultured at 37°C for indicated periods. (A) Experimental outline and timing of sampling. (B) The chromatin loading of Mcm2p and Orc5p monitored by immunoblotting, as described in the legend of Fig. 1. For loading control (bottom panel), gels were stained with silver.

Fig. 8C, Mcm2p released from chromatin more rapidly in *orc5* mutants than in wild-type cells, however, the difference was not so clear. This may be due to that the degradation of Orc5p in *orc5* mutants was not so clear in α-factor-blocked cells as in the nocodazole-blocked cells, as shown in Fig. 8D and E.

DISCUSSION

In yeast, the heat-inducible degron mutant system is very useful to reveal the exact roles of proteins essential for cell growth. This system has helped us to study the functions of various DNA replication-related proteins *in vivo* (*21*, *23*, *24*, *42*), but its application to ORC has not yet been reported. Biochemical studies of ORC have revealed its functions *in vitro*, but, as described in the INTRODUCTION section, genetic studies using temperature-sensitive mutants have so far failed to reveal ORC's precise role *in vivo*. In this study, we constructed a heat-inducible degron mutant for every ORC subunit. All mutants showed temperature-sensitive growth phenotype, confirming that ORC is essential for budding-yeast cell growth. Using these mutants, we examined the role and function of ORC *in vivo*.

In *orc* degron mutants, under the non-permissive conditions, both the targeted ORC subunit, and other subunits, were degraded. Due to technical problems, we

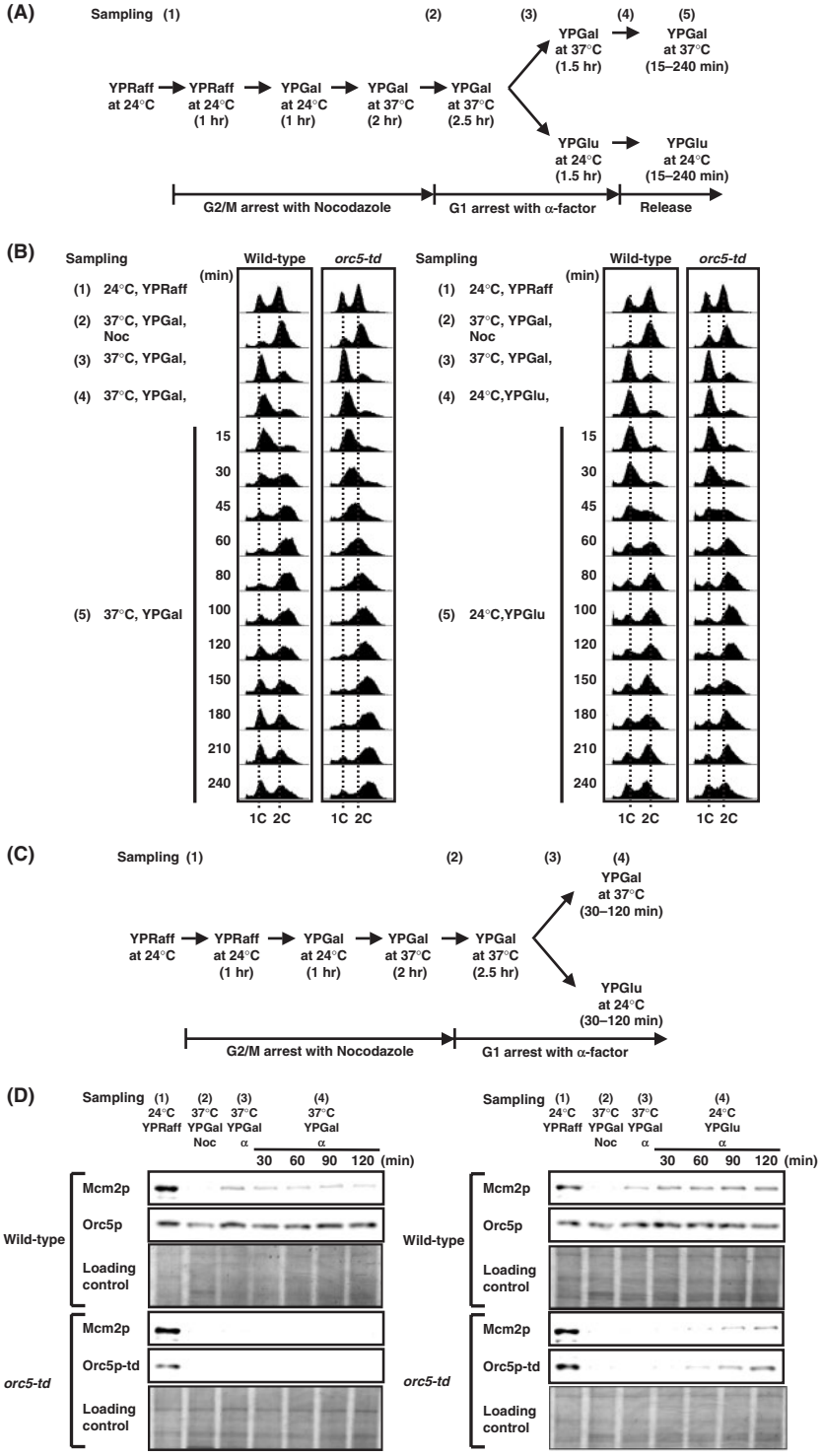

Fig. 7. **The presence of ORC at late G1 rather than early G1 and G2/M phases is important for cell cycle progression and the pre-RC formation.** YYM114 (wild-type) and YYM115 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were cultured first in YPRaff for 1 h at 24°C then in YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium for 2 h at 37°C. Cells were washed and released into the fresh YPGal containing α-factor and cultured for 2.5 h at 37°C. At this point, cell cultures were divided and incubated at 37°C in YPGal with α-factor, or at 24°C in YP medium with 2% glucose (YPGlu) plus α-factor, for 1.5 h (B) or indicated periods (D). Finally, cells were released into fresh YPGal or YPGlu and cultured for indicated periods (B). FACS analysis and chromatin-binding assay was performed as described in the legends of Figs 1 and 3 (B and D). For loading control, gels were stained with silver (D). (A) Experimental outline and timing of sampling for Fig. 7B. (C) Outline and sampling for Fig. 7D.

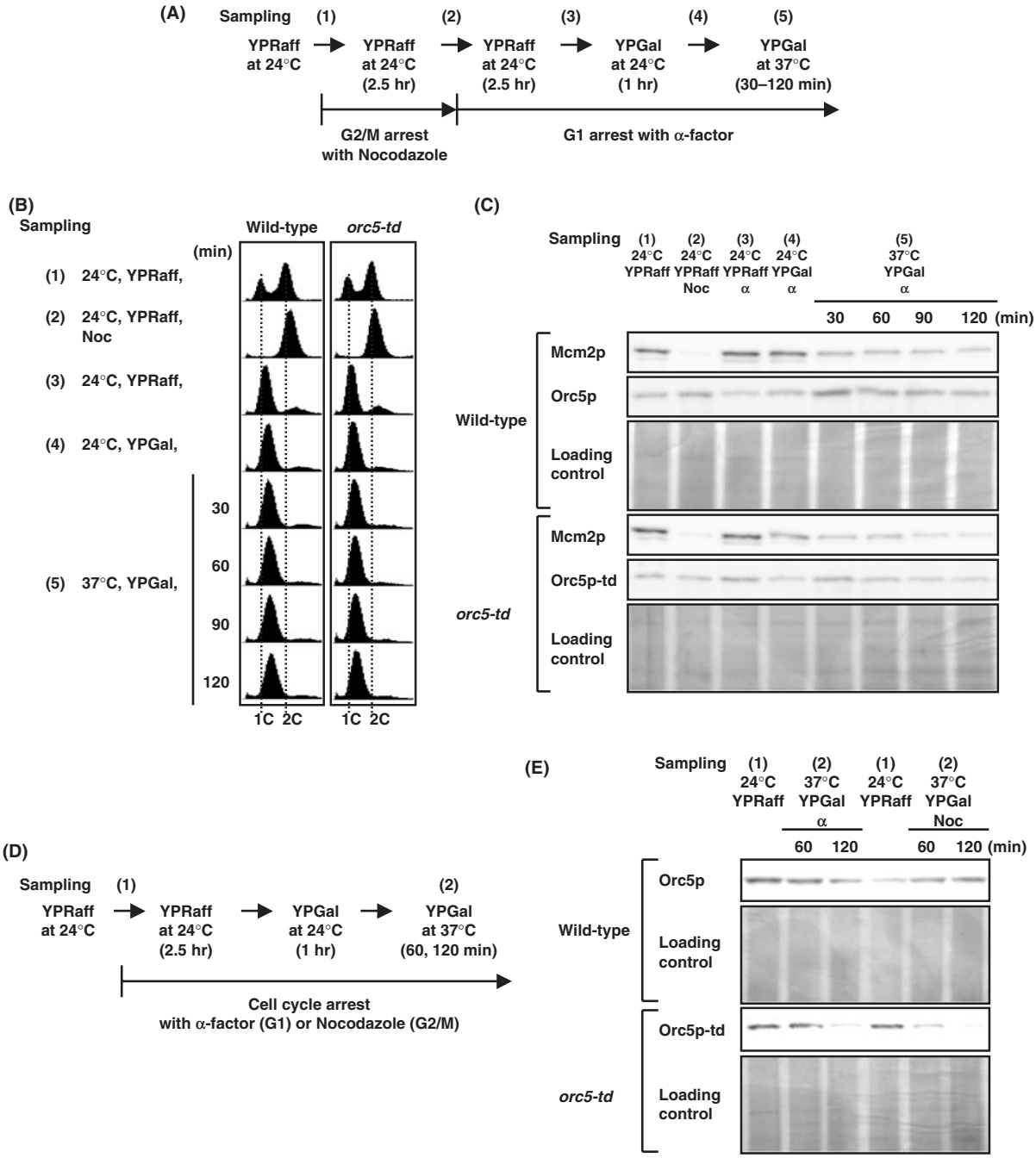

Fig. 8. **Stability of pre-RC after degradation of ORC.** YYM114 (wild-type) and YYM115 (*orc5-td*) cells were cultured in YP medium with 2% raffinose (YPRaff) to logarithmic phase at 24°C and synchronized at G2/M phase by incubation with nocodazole. Cells were washed and released into the fresh YPRaff containing α-factor and cultured for 2.5 h at 24°C, then YP medium with 2% galactose (YPGal) for 1 h at 24°C and finally in the same medium at 37°C for indicated periods. (A) Experimental outline and timing of sampling. (B) FACS analysis. (C) The chromatin loading of Mcm2p and Orc5p monitored by immunoblotting, as described in the legend of Fig. 1. For loading control (bottom panel), gels were stained with silver. YYM114 (wild-type) and YYM115 (*orc5-td*) cells were cultured in YPRaff to logarithmic phase at 24°C and synchronized at G1 phase or G2/M phase by incubation with α-factor or nocodazole for 2.5 h at 24°C, then YPGal for 1 h at 24°C and finally in the same medium at 37°C for indicated periods. (D) Experimental outline and timing of sampling. (E) The Orc5p on chromatin was monitored by immunoblotting, as described in the legend of Fig. 1. For loading control (bottom panel), gels were stained with silver.

could not verify the degradation of all ORC subunits. However, it is reasonable to speculate that under the non-permissive conditions, all subunits of ORC would have been degraded in each of the *orc* degron mutants. Thus, results suggest that ORC probably becomes unstable when any one of its subunits is degraded. We previously reported that in the temperature-sensitive *orc5-A* mutant (a strain expressing ORC with

Orc5pK43E, a mutation in the ATP binding domain of Orc5p), not only Orc5p but also all other subunits were rapidly degraded at non-permissive temperatures by ubiquitin-proteasome system (*15*, *43*). Similar degradation was observed with other temperature-sensitive *orc* mutants (*16*, *44*). Therefore, in yeast, when any one of the ORC subunits is degraded, other subunits may be also degraded by ubiquitin-proteasome system and we speculate that subunits of ORC other than targeted one, are degraded by this system in *orc* degron mutants. But since the same system degrades the targeted subunit, we could not study the degradation of targeted and non-targeted subunits separately.

Block and release experiments suggested that ORC is required for G1/S transition but not for S and G2/M phase progression. Degradation of Orc5p (ORC) at S or G2/M phases did not inhibit the S or G2/M phase progressions, but elimination of Orc5p (ORC) from G2/M to late G1 phase, inhibited the subsequent G1/S transition. This conclusion is consistent with results in many previous studies using temperature-sensitive *orc* mutants, and genetic shut-off systems for Orc6p (*13*, *16–19*, *34*, *45*). As well as various temperature-sensitive *orc* mutants, incubation of asynchronously growing *orc* degron mutant cells under non-permissive conditions, caused accumulation of cells with nearly 2C DNA content. There are two possible explanations. First, as reported for other DNA replication-related mutants (*41*, *46*), inefficient loading of MCM onto chromatin caused by the low level of ORC in *orc* degron mutant cells is responsible for slow S phase progression and accumulation of cells with nearly 2C DNA. Second, the low level of ORC induces DNA damage and the DNA replication checkpoint systems that block cell cycle progression before mitosis. Supporting this idea, we showed that incubation of *orc5* degron mutant cells under non-permissive conditions caused phosphorylation of Rad53p. Furthermore, in other temperature-sensitive *orc* mutants, non-permissive temperatures induce not only DNA damage and DNA replication checkpoint systems, but also spindle assembly checkpoint systems (*14*, *34*). Both possibilities imply that ORC functions primarily as the initiator of DNA replication. The accumulation of cells with nearly 2C DNA content, seen in various temperature-sensitive *orc* mutants, may be due to inefficient MCM loading and/or induction of checkpoint systems.

For cell cycle progression, Orc5p (ORC) is required at late G1 phase rather than early G1 and G2/M phases. Even after elimination of Orc5p (ORC) from G2/M to late G1 (the point of α-factor block), α-factor-blocked *orc5* mutants could enter into S phase normally, after the release, as long as Orc5p (ORC) had been restored before the release. The heat-inducible degron system did not work well in cells blocked with α-factor (i.e. blocked after pre-RC formation). Therefore, we could not test the effect of eliminating Orc5p (ORC) in late G1 on the following G1/S transition. Thus, we do not know whether ORC is required at late G1 for the subsequent G1/S transition. However, recent studies using genetic shut-off systems for Orc6p, showed that the elimination of Orc6p in cells blocked with α-factor did inhibit the subsequent G1/S transition (*45*), suggesting that ORC is required at late G1. Furthermore, we showed that pre-RC can form in cells in late G1 phase provided that Orc5p (ORC) is restored. This is interesting because previous studies suggested that pre-RC is formed in late M or early G1 phase (*29*, *35*). It seems that the pre-RC can be formed in relatively wide window in the cell cycle, from late G2/M (after the decrease in Cdc28p kinase activity) to late G1 (the point of α-factor block). This wide window may help yeast cells to maintain cell cycle progression under stress, which might otherwise inhibit pre-RC formation.

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