

An essential role for Orc6 in DNA replication through maintenance of pre-replicative complexes

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The heterohexameric origin recognition complex (ORC) acts as a scaffold for the G₁ phase assembly of pre-replicative complexes (pre-RC). Only the Orc1-5 subunits appear to be required for origin binding in budding yeast, yet Orc6 is an essential protein for cell proliferation. Imaging of Orc6-YFP in live cells revealed a punctate pattern consistent with the organization of replication origins into subnuclear foci. Orc6 was not detected at the site of division between mother and daughter cells, in contrast to observations for metazoans, and is not required for mitosis or cytokinesis. An essential role for Orc6 in DNA replication was identified by depleting it at specific cell cycle stages. Interestingly, Orc6 was required for entry into S phase after pre-RC formation, in contrast to previous models suggesting ORC is dispensable at this point in the cell cycle. When Orc6 was depleted in late G₁, Mcm2 and Mcm10 were displaced from chromatin, cells failed to progress through S phase, and DNA combing analysis following bromodeoxyuridine incorporation revealed that the efficiency of replication origin firing was severely compromised.

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Introduction

The origin recognition complex (ORC) plays an essential role in the initiation of DNA replication by binding to origin sequences throughout the cell cycle and acting as a scaffold for the association of additional protein factors in G₁ phase (reviewed in Bell, 2002). Originally isolated and characterized in the budding yeast *Saccharomyces cerevisiae* (Bell and Stillman, 1992), ORC is composed of six distinct subunits, and orthologs of each have now been found in a wide range of eukaryotic species (reviewed in DePamphilis, 2005). In early

G₁ phase, ORC promotes the origin-association of the clamp loading protein Cdc6 in an ATP-dependent manner (Speck *et al.*, 2005). Another factor, Cdt1 (Devault *et al.*, 2002; Tanaka and Diffley, 2002) directs the nuclear import of the MCM (minichromosome maintenance) family of proteins, Mcm2–7, which act as replication fork helicases (reviewed in Bell and Dutta, 2002). Once ORC and Cdc6 are present at origins, Cdt1–Mcm2–7 can also associate with origin DNA. Hydrolysis of ATP by Cdc6 is then thought to result in Cdt1 dissociation and a stronger Mcm2–7 binding at origins. Subsequent ATP hydrolysis by ORC catalyzes the loading of additional Mcm2–7 complexes (Kawasaki *et al.*, 2006; Randell *et al.*, 2006). Once tightly bound, the continued association of at least Mcm2 with chromatin in G₁ phase requires the presence of another ORC-associated protein, Mcm10 (Homesley *et al.*, 2000). Collectively, this assemblage of proteins is known as the pre-replicative complex (pre-RC). In addition to pre-RC formation, the initiation of DNA replication requires the activation of two kinase complexes, Clb5/Cdc28 and Dbf4/Cdc7, which promote the origin-association of Cdc45 (Nougarede *et al.*, 2000; Zou and Stillman, 2000). Cdc45 in turn recruits DNA polymerases to origins (Mimura and Takisawa, 1998; Aparicio *et al.*, 1999; Zou and Stillman, 2000).

Curiously, only five of the six ORC subunits are required for origin recognition and binding *in vitro* (Lee and Bell, 1997). Even though Orc6 is an essential protein in budding yeast (Li and Herskowitz, 1993), it appears to be dispensable for these functions and its role in cell cycle progression has yet to be determined. Clearly, Orc6 association with the other budding yeast ORC subunits suggests a function in DNA replication. Li and Herskowitz disrupted one copy of *ORC6* in a diploid yeast strain and, following sporulation, were able to observe up to two of rounds of cell division from spores inheriting the *ORC6* knockout. Arrested cells had a large budded phenotype often observed for DNA replication mutants, but the stage of cell cycle arrest could not be determined by FACS analysis due to an insufficient number of cells. Studies involving the replication of *Xenopus* sperm DNA in *Drosophila* egg extracts indicate that Orc6 can promote DNA replication in this *in vitro* system (Chesnokov *et al.*, 2001). With human cancer cells, depletion of Orc6 by transfection with siRNA duplexes resulted in a significant reduction in the number of positive cells in BrdU incorporation assays, consistent with a replicative function (Prasanth *et al.*, 2002). As well, research with both human and fruit fly cells point to mitotic and/or cytokinetic functions in addition to a role for Orc6 in DNA replication (Prasanth *et al.*, 2002; Chesnokov *et al.*, 2003).

Here, we demonstrate that Orc6 is required for the initiation of DNA replication in budding yeast cells and is dispensable for progression through mitosis and cytokinesis. We further show that Orc6 is required for the maintenance of MCM protein association with chromatin, and that depletion of Orc6 after pre-RC formation inhibits replication origin firing.

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Results

Orc6 localizes to subnuclear foci in living yeast cells

As a first step in characterizing the essential role of Orc6, we examined its cellular localization. This was accomplished by tagging genomic *ORC6* with a sequence encoding the GFP variant eYFP in a haploid yeast strain (DY-1), resulting in expression of full-length Orc6 with a C-terminal eYFP tag, under the control of the natural *ORC6* promoter (DY-41). Studies with human cancer cells indicate that, in addition to the expected nuclear localization during interphase, cytoplasmic pools of Orc6 exist with localization observed at both the cell periphery during mitosis and at the midbody during cytokinesis (Prasanth *et al*, 2002). Similar results have been reported for *Drosophila* cells (Chesnokov *et al*, 2003). When we followed single cells as they progressed through the cell cycle, only nuclear Orc6 localization was observed (Figure 1A). To confirm that *S. cerevisiae* Orc6 is confined to the nucleus, we constructed a double-tagged haploid strain expressing Orc6 with a C-terminal eCFP fusion as well as the nuclear pore protein Nup49 with a C-terminal eYFP fusion (DY-65). Irrespective of cell cycle stage, Orc6 was consistently bounded by the nuclear membrane, as designated by the signal for Nup49 (Figure 1C). Although we cannot rule out additional pools of Orc6 below our threshold of detection, we conclude that there was no significant localization of budding yeast Orc6 at either the cell periphery or at the mother-bud neck.

To further investigate the subnuclear localization of Orc6 in live cells, 20 images taken in rapid succession were stacked to increase the signal as described in the Materials and methods. The resultant higher resolution images revealed a pattern of punctate Orc6 foci (Figure 1B), similar to what has been previously observed in fixed cells for other presumed replication factory constituents such as Orc2 (Pasero *et al*, 1999; Lengronne *et al*, 2001). Chromatin immunoprecipitation (ChIP) analysis further indicated that Orc6 specifically binds to replication origin sequence (Supplementary Figure 1).

Depletion of Orc6 leads to an S-phase arrest

Given that the cellular localization of Orc6 was indicative of a role in DNA replication, and provided no evidence for additional cellular function(s), we wanted to determine the point in the cell cycle at which cells arrest when depleted of Orc6. A haploid yeast strain (DY-36) was generated replacing the natural *ORC6* promoter with a glucose-repressible *GAL1* promoter. As part of this strain construction, a sequence encoding three copies of the HA epitope was fused to the start of the *ORC6* ORF, to facilitate the monitoring of Orc6 levels. Cultures of DY-36 and isogenic wild-type (wt) strain DY-26, were grown on 2% galactose/1% raffinose (YPG/R) medium overnight, washed in ddH₂O, and resuspended in 2% glucose (YPD) medium. Following 4 h of growth in YPD, the amount of Orc6 in the *GAL1-ORC6* cells had fallen below normal endogenous levels as judged by immunodetection of whole-cell extracts (Figure 2A), and these cells clearly showed growth defects at subsequent time points, relative to the wt controls (Figure 2B). We further compared *GAL1-ORC6* and wt strain growth in YPD by removing aliquots at 3-h intervals for FACS analysis. At all time points following the shift to YPD, the wt strain exhibited prominent 1C and 2C peaks characteristic of asynchronous cultures (Figure 2C). In

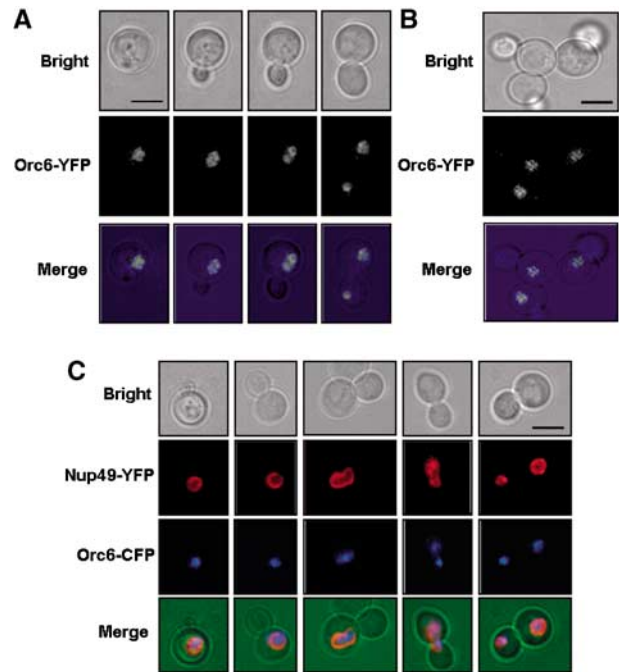


Figure 1 Orc6 localizes to subnuclear foci throughout the cell cycle. Orc6-eYFP, Orc6-eCFP and Nup49-eYFP cell preparation and live imaging was performed as described in Materials and methods. (A) A single cell was imaged over a 3 h time period. Both a bright field and a single fluorescent image were taken every 10 min; representative images of different cell cycle stages are shown. Multiple fluorescent images could not be taken at each time point to reduce background as in (B), since increased exposure to the light source results in cell bleaching over the 3 h time course. (B) A representative field of cells is shown. Since expression of Orc6-eYFP is low under its endogenous promoter, a stronger signal with reduced background was obtained by taking 20 fluorescent images under low gain in rapid succession and stacking them using ImageJ 1.30v. (C) Co-localization of Orc6 and a nuclear membrane protein (Nup49) in a series of Orc6-eCFP/Nup49-eYFP cells shows that Orc6 is found exclusively within the nucleus at all stages of the cell cycle observed, as judged by bud morphology. All scale bars correspond to 5 μ m.

contrast, by 3 h the *GAL1-ORC6* cells were already showing defects in S phase progression, with an accumulation of cells with a DNA content between 1C and 2C. No significant accumulation of cells with 2C or greater DNA content was observed, as would be expected for mitotic or cytokinetic defects. Indeed, by 6 h, the size of the 2C peak was markedly reduced compared to earlier time points, and the wt control.

Orc6 is required for entry into S phase

In order to determine whether Orc6 is required for entry into and/or progression through S phase, Orc6 was depleted in *GAL1-ORC6* cells synchronized at G₂/M by initially arresting cells in YPG/R supplemented with nocodazole, washing the culture and resuspending the cells in YPD/nocodazole. Since origins are rendered competent for DNA replication during G₁ phase, if Orc6 is essential in this process one would expect to see the formation of a 1C peak as Orc6-depleted cells fail to efficiently fire origins following release from the block. Such a 1C peak was indeed observed with the *GAL1-ORC6* strain following Orc6 depletion (Figure 3A).

A central function of the ORC complex in promoting DNA replication is to facilitate the loading of the MCM proteins at

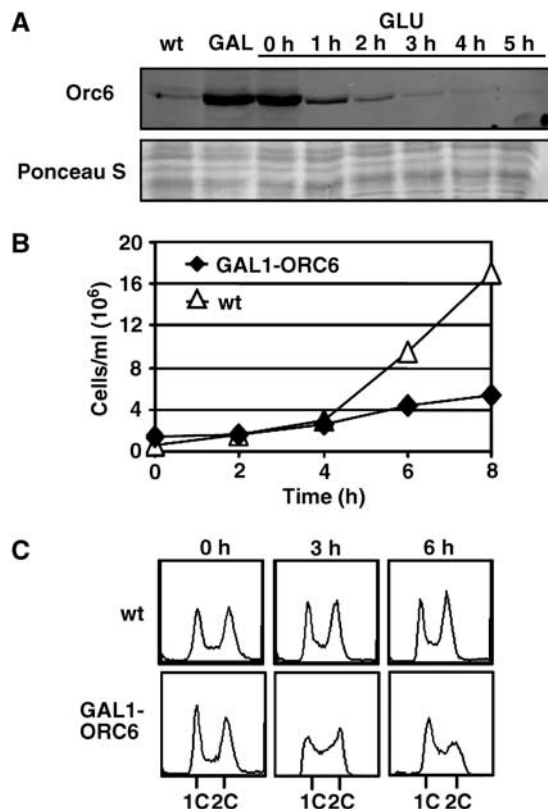


Figure 2 Depletion of Orc6 results in an accumulation of S phase cells. (A) An asynchronous *GAL1-3HA-ORC6* (DY-36) culture was grown in YPG/R (2% galactose, 1% raffinose) medium to 10^6 cells/ml, washed and resuspended in YPD (2% glucose) medium. Whole-cell extracts were prepared from culture aliquots taken prior to (GAL) and at the indicated time points following resuspension in YPD. A whole-cell extract was also prepared from an asynchronous exponential culture of an isogenic strain (DY-93) in which Orc6 is tagged with the same number of HA epitopes, but remains under the control of its endogenous promoter (wt). Eighty micrograms of each extract was used for immunoblot analysis. HA-tagged Orc6 was detected using an anti-HA antibody (Roche) and fluorescent secondary antibody (Invitrogen). Ponceau S staining of the region detected by the blot to verify equal loading of whole-cell extracts is also shown. (B) Asynchronous *GAL1-3HA-ORC6* (DY-36) and isogenic wt (DY-26) cultures were grown as described in (A). Cell counts of both cultures were taken every 2 h for 8 h following transfer to YPD. Growth defects were observed in the *GAL1-3HA-ORC6* strain after 4 h, compared to what was seen for wt cells. (C) FACS analysis of culture aliquots removed at indicated time points after transfer to YPD.

origins (reviewed in Bell and Dutta, 2002). To further explore the role of Orc6 in rendering origins competent, we monitored Mcm2 loading by means of a chromatin fractionation assay (Donovan *et al*, 1997; Liang and Stillman, 1997) using *GAL1-ORC6* cells that had been depleted of Orc6 during a G_2/M arrest. Mcm2 association with the chromatin pellet was reduced, in Orc6-depleted cells relative to wt cells, following release into a G_1 block with α -factor (Figure 3B). To confirm that the decline of Mcm2 loading following Orc6 depletion in G_2/M was not the result of ORC destabilization, we also monitored the level of Orc2 to examine the overall state of the ORC complex. *GAL1-ORC6* cells depleted of Orc6 in G_2/M and then released into the G_1 block had levels of chromatin-bound Orc2 comparable to wt cells (Figure 3B), consistent with previous *in vitro* studies indicating Orc6 is not required for ORC to bind replication origins (Lee and Bell, 1997). It

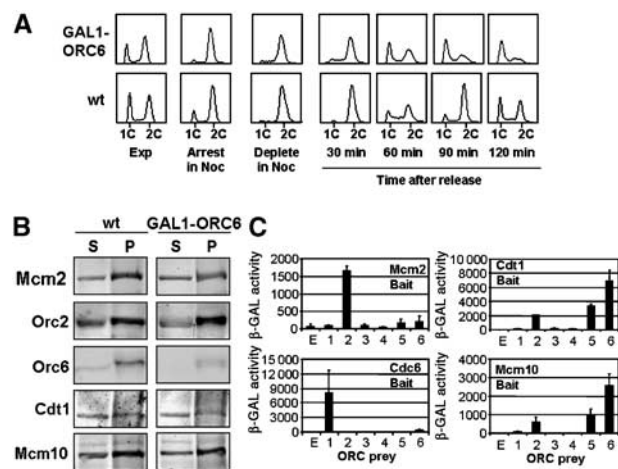


Figure 3 Orc6 is required for S-phase progression. (A) Asynchronous cultures of *GAL1-3HA-ORC6* cells (DY-36) and its wt parental strain (DY-26; labeled wt) were grown to 5×10^6 cells/ml (Exp) and then arrested at G_2/M with nocodazole (15 μ g/ml) for 3 h in YPG/R. Once blocked, the cells were washed and transferred to YPD again with nocodazole (12 μ g/ml) for 4 h to maintain the cell cycle arrest. The cells were then washed and released into fresh YPD without any arrest agents and samples were removed at the indicated intervals for FACS analysis. (B) *GAL1-3HA-ORC6* and wt cells were arrested in YPG/R with nocodazole and then transferred to YPD/nocodazole for 4 h as described in (A). Cultures were subsequently released into YPD supplemented with α -factor to arrest cells in late G_1 phase. Samples of the culture were taken after 2 h in α -factor for chromatin binding assays. Thirty microliters of the chromatin-associated pellet (P) and 15 μ l of the supernatant (S) were analyzed by immunoblot. HA-tagged Orc6 was detected using an anti-HA antibody (Roche); anti-Mcm2 (1:500; goat polyclonal, Santa Cruz) and anti-Orc2 (1:1000; rabbit polyclonal) antibodies were also used to analyze pellet and supernatant fractions for isogenic strains DY-36 (*GAL1-3HA-ORC6*) and DY-93 (*ORC6-3HA*). Cdt1 was detected using (anti-Myc; Sigma) for isogenic strains DY-83 (*CDT1-13Myc*) and DY-91 (*GAL1-3HA-ORC6*, *CDT1-13Myc*), while Mcm10 was detected with the same antibodies for strains DY-84 (*MCM10-13Myc*) and DY-92 (*GAL1-3HA-ORC6*, *MCM10-13Myc*). (C) Liquid two-hybrid assays were performed using pEG-Mcm2, pEG-Cdc6, pEG-Cdt1 and pEG-Mcm10 as baits in combination with each ORC subunit (pJG-ORC1-6) or empty pJG4-6 vector (E) as preys. All two-hybrid results are representative of two or more assays and error bars represent standard deviation.

therefore appears that the reduction in Mcm2 loading was specific to the absence of Orc6.

Orc6 interacts with members of the pre-RC involved in MCM loading and maintenance

To determine whether Orc6 physically interacts with Mcm2 or factors that play a role in MCM chromatin association, the full-length *MCM2*, *CDC6*, *CDT1* and *MCM10* coding sequences were cloned into the two-hybrid bait vector pEG-202 (Ausubel *et al*, 1995). Each of these constructs was separately transformed into the wt yeast strain DY-1, already harboring the lacZ reporter-plasmid pSH18-34, and one of six prey plasmids expressing Orc1-6. When Mcm2 was tested as bait, the strongest β -galactosidase signal was obtained with Orc2, indicating that this ORC subunit and not Orc6, is most likely to associate directly with Mcm2 (Figure 3C). We next evaluated ORC subunit interactions with Cdc6 and Cdt1, which are required for MCM loading onto chromatin (reviewed in Bell and Dutta, 2002). The strongest signal for Cdc6 was obtained with the Orc1 subunit, consistent with the previously reported Orc1-Cdc6 interaction in both yeast

(Wang *et al*, 1999) and human cells (Saha *et al*, 1998). However, Orc2, Orc5 and Orc6 preys produced positive two-hybrid signals when co-expressed with the Cdt1 bait, with Orc6 the highest of the three. Finally, we tested the ORC subunits against Mcm10, which is required to preserve the interaction of Mcm2 with chromatin in G₁ phase (Homesley *et al*, 2000). Here, we noticed a very similar pattern to that observed for Cdt1, with Orc6 again exhibiting the highest two-hybrid signal. Since both Cdt1 and Mcm10 interacted with Orc6, we evaluated the effect of G₂/M Orc6 depletion on their chromatin association in G₁. There was no appreciable effect on the degree chromatin binding of either protein (Figure 3B).

Orc6 is required to maintain MCM chromatin association following pre-RC formation

Studies with *Xenopus* egg extracts indicate that once the MCM proteins are assembled onto chromatin, ORC and Cdc6 can be removed without affecting their putative DNA helicase function (Hua and Newport, 1998; Rowles *et al*, 1999). Therefore, we were interested to determine whether Orc6 is still required for the efficient initiation of DNA replication after MCM proteins have been loaded onto chromatin. This was carried out by synchronizing cultures of *GAL1-ORC6* and its parental strain growing in YPG/R in late G₁ phase by adding α -factor. Following the initial arrest, cells were transferred to YPD again with α -factor to deplete Orc6 in the *GAL1-ORC6* strain. After release from the block, an accumulation of 1C cells was observed for the Orc6-depleted *GAL1-ORC6* culture by 30 min, while the isogenic wt strain displayed a prominent 2C peak at the same time point, consistent with a role for Orc6 in S phase progression (Figure 4A). As an additional control, a *GAL1-ORC4* strain was similarly assessed. Orc4 depletion in this strain occurs with similar kinetics to what is observed for Orc6 depletion (Supplementary Figure 2), but in this case cells were able to replicate their DNA, albeit at a slower rate than the wt control (Figure 4A). Therefore, it appears that Orc6, as opposed to the entire ORC complex, is required in late G₁ phase for subsequent DNA replication. Since the most likely explanation for a lack of DNA replication following Orc6 depletion in late G₁ phase was again a destabilization of the MCM complex at origins, we monitored the chromatin association of Mcm2 prior to and following Orc6 depletion. While equivalent chromatin pellet to supernatant Mcm2 ratios were observed during the initial α -factor arrest between the wt and *GAL1-ORC6* strains (GAL; Figure 4B), there was a clear displacement of Mcm2 from the pellet to the supernatant fraction following Orc6 depletion in the *GAL1-ORC6* strain (GLU; Figure 4B). Densitometry indicated that there was close to a 50% reduction in the Mcm2 pellet:supernatant ratio in the *GAL1-ORC6* strain relative to the isogenic wt strain (Supplementary Figure 3). Interestingly, we also observed a dramatic reduction in the level of chromatin-associated Orc2 following Orc6 depletion. Although we cannot rule out the possibility that an overall destabilization of ORC is responsible for the displacement of Mcm2, the observation that a robust Mcm2 chromatin association is maintained in cells depleted of Orc4 (Figure 4B) argues against this. In the case of Cdt1, little difference was observed between cells with normal Orc6 levels or those that had been depleted, although in each case, as expected, very little Cdt1 was detected in the

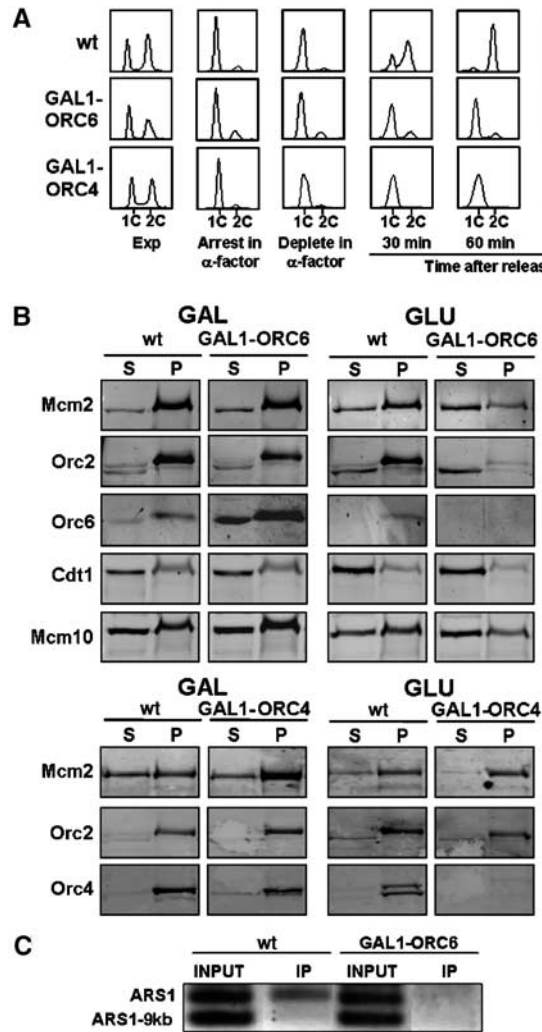


Figure 4 Depletion of Orc6 in late G₁ destabilizes MCM association with chromatin and impedes S phase entry (A) wt (DY-26), *GAL1-3HA-ORC6* (DY-36) and *GAL1-3HA-ORC4* (DY-103) strains were grown to 5 × 10⁶ cells/ml (Exp) and arrested at G₁ with α -factor (50 μ g/ml) for 2–3 h in YPG/R. The cells were then pelleted, washed with ddH₂O and transferred to YPD containing α -factor (50 μ g/ml) for 4 h. Cells were subsequently washed and released in YPD without α -factor. Culture aliquots were taken at the indicated times for FACS analysis. (B) Isogenic wt, *GAL1-3HA-ORC6* and *GAL1-3HA-ORC4* cells were arrested with α -factor in YPG/R, washed and incubated in YPD with α -factor, as described in (A); DY-109 (*ORC4-3HA*) was used as the wt control for the experiments with *GAL1-3HA-ORC4* cells. Chromatin fractionation and immunoblotting was performed as described in Figure 3, with culture aliquots removed following the initial arrest (GAL), and at the end of the YPD/ α -factor incubation (GLU). (C) ChIP was carried out with a mixture of Mcm2 and Mcm5 antibodies as described in Materials and methods, for DY-26 and DY-36 cells cultured as described in (A). PCR using sample aliquots taken after sonication (INPUT) and following purification of co-immunoprecipitated DNA (IP) was carried out using primers specific for ARS1 origin sequence, and a region 9 kb upstream of ARS1 (ARS1–9 kb).

chromatin fraction (Randell *et al*, 2006). In contrast, the high levels of chromatin-bound Mcm10 initially detected were markedly reduced following Orc6 shut-off (Figure 4B and Supplementary Figure 3). These results are consistent with a mechanism whereby Orc6 promotes Mcm10 chromatin association, and Mcm10 in turn stabilizes the MCM complex (Homesley *et al*, 2000). Finally, we confirmed that MCM

proteins are actually displaced from replication origins following late G₁ Orc6 depletion, by carrying out ChIP analysis (Figure 4C), which demonstrated a dramatic drop in the level of ARS1 DNA co-immunoprecipitating with MCMs when Orc6 is absent.

Depletion of Orc6 in late G₁ reduces the efficiency of DNA replication initiation

The inability of cells lacking Orc6 to progress through S phase may have been due to a reduction of origin firing, inhibition of elongation, or a combination of both. To investigate the effect of Orc6 depletion on initiation events, we constructed a TK+ *GAL1-ORC6* strain (DY-79), to allow the incorporation of BrdU into newly synthesized DNA. Cultures of DY-79 and its parental TK+ strain (DY-67) were initially synchronized in late G₁ with α -factor in YPG/R, then shifted to YPD with α -factor, to deplete Orc6 in the DY-79 cells. The cells were subsequently washed and resuspended in YPD supplemented with BrdU (0.4 mg/ml) and the ribonucleotide reductase inhibitor hydroxyurea (HU; 0.2 M), which normally results in a mid-S phase arrest after about half the replication origins have fired. Following 90 min in HU, cells were harvested, genomic DNA was isolated and single molecule DNA combing was carried out, as previously described (Versini *et al*, 2003). Comparison of inter-origin distances from *GAL1-ORC6* and wt cells revealed that approximately half the number of origins fired in cells depleted of Orc6 (Figure 5A), consistent with a role for Orc6 in promoting initiation events. The length of individual BrdU tracks was more than double those observed with the wt controls. This can be explained by the fact that nucleotide pools are greatly reduced when HU is administered, thus cells that have fewer origins firing have more nucleotides available for the migration of each individual replication fork. To examine the possibility that origin firing inhibition was caused by Orc6 depletion triggering a G₁ checkpoint, we monitored the abundance and phosphorylation status of Rad53 and did not detect upregulation or higher mobility forms on immunoblots for samples taken either immediately following Orc6 depletion or an hour after release from the α -factor block (Supplementary Figure 4).

Whether Orc6 might play a role in the elongation stage as well as the initiation stage of DNA replication was also investigated. *GAL1-ORC6* and isogenic wt cells were arrested in YPG/R supplemented with HU, then washed and resuspended in YPD/HU for 4 h. Following release from the HU block in YPD, the two cultures progressed through S phase at comparable rates (Figure 5B), suggesting that Orc6 was not required for efficient replication fork progression. Although we cannot exclude the possibility that residual Orc6 following our shut-off regime may have played a role in replication fork progression, we can rule it out as the cause of the S-phase arrest we see for this length of depletion (Figure 2).

Discussion

The essential cell cycle role of Orc6 is specific to S phase

Since its original biochemical purification from budding yeast (Bell and Stillman, 1992), ORC has been shown to play a central role in the initiation of DNA replication (reviewed in Bell, 2002). ORC lacking Orc6 is fully competent to bind origin sequences, but omission of any other subunit abrogates this capability (Lee and Bell, 1997). Orc6 is nevertheless

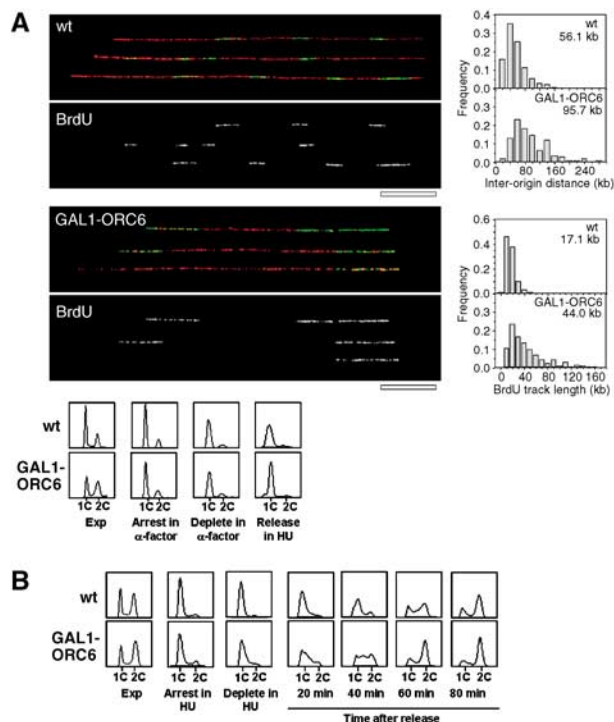


Figure 5 Orc6 is required for the initiation of DNA replication following pre-RC formation. (A) Initiation frequency was monitored in both wt (DY-67) and *GAL1-3HA-ORC6* (DY-79) TK+ cells. Cells were synchronized in G₁ using α -factor and then transferred to YPD medium for 4 h to deplete Orc6 while still arrested with the pheromone. Cells were then released into YPD containing HU (0.2 M) to block elongation, and BrdU to monitor new strand synthesis. DNA plugs were prepared 90 min after the transfer. FACS profiles of each stage of the experiment are presented in the bottom left. DNA combing was performed on silanized coverslips as described in Materials and methods. Representative DNA fibers from both the wt and *GAL1-3HA-ORC6* strains are shown. BrdU incorporation appears green, DNA red. Inter-origin distances as well as BrdU tract lengths following analysis of over 200 origins are shown in the histograms, and in each case the average value is indicated. Scale bar represents 50 kb. The Mann–Whitney rank-sum test was used to show that the differences between wt and *GAL1-ORC6* cells are significant ($P < 0.001$). (B) wt (DY-93) and *GAL1-3HA-ORC6* (DY-36) strains were grown in YPG/R to 5×10^6 cells/ml and arrested at S phase with HU (0.2 M). The cells were washed with ddH₂O and transferred to YPD with HU (0.36 M) for 4 h. Cells were then washed twice with ddH₂O and released into fresh YPD. Aliquots of each culture were taken every 20 min for FACS analysis.

an essential protein for cell proliferation (Li and Herskowitz, 1993), and previous studies suggest that some budding yeast ORC subunits may have important functions at other cell cycle stages (Bell *et al*, 1993; Dillin and Rine, 1998). Recent attention has focused on additional roles for the Orc6 subunit in mitosis and cytokinesis in metazoans (Prasanth *et al*, 2002; Chesnokov *et al*, 2003). It is therefore reasonable to ask whether budding yeast Orc6 is required for DNA replication and/or other cell cycle events. Our localization of Orc6-YFP/CFP to subnuclear foci in living cells provides compelling evidence for a replicative function, since it is similar to the punctate pattern previously shown for immunolocalization of Orc2 in fixed cells (Pasero *et al*, 1999), and with the detection of discrete zones of new DNA synthesis in TK+ yeast cells following a BrdU pulse (Lengronne *et al*, 2001). To our knowledge, the visualization of Orc6 foci represents the first detection of a protein constituent of what are thought

to be replication factories involving clusters of replication forks (reviewed in Laskey and Madine, 2003) in living *S. cerevisiae* cells. Intriguingly, we have also found that among ORC subunits, both Orc5 and Orc6 are able to homodimerize and thus may conceivably contribute to the formation of replication compartments by bridging different origin sequences (unpublished data).

In contrast to what has been seen in both human and fruit fly cells, we observed no accumulation of Orc6 at the mid-plane of division between mother and daughter cells (Figure 1A and C). Given that the nuclear envelope in budding yeast remains intact throughout the cell cycle, it is possible that Orc6 could play a karyokinetic role that is analogous to the cytokinetic function of its metazoan orthologs. Our results argue against this, however, as we do not observe a population of Orc6 at the site of nuclear division in any of the cells we observed (Figure 1A and results not shown). Moreover, FACS analysis following depletion of Orc6 from an asynchronous culture shows no accumulation of cells with $\geq 2C$ DNA content (Figure 2C). The fact that no significant 2C DNA peak was observed even 6 h after Orc6 was depleted suggests that chromosome segregation was not compromised as reported for human cells treated with Orc6 siRNA (Prasanth *et al*, 2002). Indeed, cells depleted for Orc6 during a G₂/M block were fully capable of completing mitosis and proceeding into G₁ phase following release (Figure 3A). Although we cannot rule out that residual levels of Orc6 capable of carrying out mitotic and/or cytokinetic functions remain following our depletion protocol, it is clear from the marked accumulation of cells with DNA content between 1C and 2C following Orc6 depletion in an asynchronous culture, that the cell cycle arrest we observed under these conditions was primarily due to S phase defects.

Why would metazoan roles for Orc6 in cytokinesis and mitosis not be present in budding yeast? An important consideration in answering this question is that *S. cerevisiae* Orc6 shares only low conservation with its *Drosophila* (5% identity, 19% similarity) and human (6% identity, 15% similarity) orthologs. Furthermore, sequence comparison of Orc1–Orc6 from these species suggests that Orc6 evolved more quickly than any of the other ORC subunits (Dhar and Dutta, 2000). Thus, it appears that metazoan Orc6 has conserved its original role in DNA replication, while acquiring cytokinetic and, at least in the case of human Orc6, mitotic functions. It was proposed that Orc6 helps to coordinate the processes of DNA replication and chromosome segregation with cytokinesis in metazoans (Prasanth *et al*, 2002), which might be more critical for multicellular than for unicellular organisms. The fact that these additional functions are not present in *S. cerevisiae* makes it an advantageous system to further characterize the role of Orc6 in DNA replication, without the complication of mitotic and cytokinetic phenotypes.

Orc6 mediates the G₁ phase chromatin association of Mcm2

To our knowledge, the most substantial evidence of an essential role for *S. cerevisiae* Orc6 in DNA replication comes from the original work of Li and Herskowitz (1993) in which they found that following sporulation and tetrad dissection of *ORC6*^{+/-} heterozygotes, haploid *ORC6*–spores undergo up to two rounds of cell division, arresting

with the type of large bud morphology often associated with mutations affecting DNA replication. More recently, the hyperphosphorylation of both Orc6 and Orc2 following START was shown to be part of a series of overlapping mechanisms that prevent DNA re-replication, which also include Cdc6 degradation and export of Mcm2–7 from the nucleus (Nguyen *et al*, 2001). All three processes are Clb/Cdc28 (CDK) mediated, and a direct interaction between Clb5 and Orc6 has been reported in budding yeast (Wilmes *et al*, 2004). Either mutation of both the Orc2 and Orc6 consensus CDK phosphorylation sites or abrogation of the Clb5/Orc6 interaction predisposes cells to re-replication in combination with stabilized Cdc6 and/or constitutively nuclear Mcm2–7 (Nguyen *et al*, 2001; Wilmes *et al*, 2004; Green *et al*, 2006; Tanny *et al*, 2006). However, neither change has any effect on cell cycle progression in the presence of wt Cdc6 and MCM proteins. Thus, prevention of re-replication is not the essential function of Orc6.

In the present report, we provide strong evidence that Orc6 is required for efficient initiation of DNA replication. Cells that were released from a G₂/M block during which Orc6 was depleted, arrested in the next cell cycle with a 1C DNA content (Figure 3A). How is the lack of Orc6 inhibiting DNA replication? One possibility is that the absence of Orc6 prevents proper pre-RC assembly, which normally culminates in the chromatin loading of the MCM complex. There was a modest drop in the level of chromatin-associated Mcm2 after cells depleted of Orc6 in nocodazole were released and subsequently blocked with α -factor (Figure 3B). More striking, however, was the strong displacement of previously loaded Mcm2 by depleting Orc6 during an extended α -factor arrest (Figure 4B and C). This pointed to a role for Orc6 in maintaining the origin association of MCM complexes after they have associated with the pre-RC. Possible mechanisms for how Orc6 facilitates MCM chromatin association were suggested by our observations that Orc6 interacts with both Cdt1 and Mcm10, two factors required for stable association of Mcm2 with chromatin (Homesley *et al*, 2000; Devault *et al*, 2002; Tanaka and Diffley, 2002). While there appeared to be no significant effect on the chromatin levels of either Cdt1 or Mcm10 following the G₂/M Orc6 depletion, the chromatin pellet to supernatant ratio of Mcm10 dropped by approximately 50% when Orc6 expression was shut-off in late G₁ phase (Figure 4B, Supplementary Figure 3), again suggesting a maintenance role. Interestingly, Mcm10 has also been shown to regulate the chromatin association of DNA polymerase- α (Ricke and Bielinsky, 2004), providing another way in which its displacement may inhibit DNA replication. In contrast to what was seen with Orc6 depletion at the earlier time point, Orc2 was displaced from chromatin (Figure 4B). Thus, a general destabilization of ORC may be responsible for the MCM displacement, and subsequent replication defects; however, the fact that Orc4 depletion neither displaces Mcm2 from chromatin (Figure 4B) nor prevents progression through S phase (Figure 4A) argues against this and points to a role for Orc6 that is distinct from other ORC subunits.

Orc6 plays a role in the initiation of DNA replication after pre-RC formation

Previous models of pre-RC assembly and function have proposed that once MCM proteins have been loaded onto chromatin, ORC is dispensable for DNA replication.

Treatment of either budding yeast late G₁ chromatin or pre-RCs assembled on magnetic beads with high salt has been reported to remove ORC, while MCM proteins remain present (Donovan *et al*, 1997; Bowers *et al*, 2004). In each case, however, there was a reduction in the level of associated MCM proteins and the consequences of the ORC removal on DNA replication were not evaluated. Subsequent DNA replication was determined to be unaffected by salt extraction of ORC after pre-RC formation in a *Xenopus* cell-free system, although the successful removal of only the Orc1 and Orc2 subunits was monitored (Rowles *et al*, 1999). In contrast to these results, our data clearly points to a role for Orc6 in promoting initiation events after the pre-RC has formed, since its depletion in late G₁ severely reduced the number of origins that fired (Figure 5A) and clearly inhibited S phase progression (Figure 4A). One explanation for varying observations with budding yeast and *Xenopus* is that there are simply differences between the two species with respect to the cell cycle window during which ORC is required to ensure origin firing. Another possibility is that different ORC subunits are required to varying degrees for initiation after pre-RC formation. We favor this idea, since we observed that depletion of Orc4 in late G₁ does not result in Mcm2 chromatin displacement or prevent cells from traversing S phase, in contrast to what is seen with Orc6 turnover (Figure 4A and B). A recent study (Gibson *et al*, 2006) reported that *orc1-161*, *orc2-1* and *orc5-1* temperature-sensitive mutants each exhibited slower progression through S phase than a wt control, after a shift to restrictive temperature in α -factor, and subsequent release at the same temperature. However, these effects were considerably less severe than we have observed following Orc6 depletion. A potential late G₁ requirement for Orc2 has been examined in a number of additional studies, with varying outcomes. Strong inhibition of S phase progression was observed when an *orc2-1* strain was shifted to non-permissive temperature in late G₁ (Zhang *et al*, 2002). Late G₁ Orc2 depletion at permissive temperature using a *GAL1-orc2-1* strain had little effect on S phase progression in one report (Shimada *et al*, 2002), while another study using the same strain found that Orc2 depletion resulted in less efficient DNA replication (Weinberger *et al*, 2005). A possible explanation for the differing outcomes of the two depletion studies is that Orc2 removal and/or pre-RC destabilization is more efficient under one set of conditions used (2 h, 23°C) than the other (1 h, 30°C); however, this remains to be tested experimentally.

Clearly, a better understanding of the functions of individual ORC components is required to fully characterize the multiple roles of this complex in cell cycle progression. For the first time, we have demonstrated an essential function for the Orc6 subunit in promoting DNA replication by mediating the stability of pre-RCs after their formation. It will now be of considerable interest to determine whether this function is conserved among eukaryotes, and to investigate the precise cell cycle roles of other ORC subunits.

Materials and methods

Yeast strains

The genotypes of the strains used in this study are listed in Table I. Unless otherwise indicated, DY-1 was used as our standard wt strain.

Plasmid construction

Genomic tagging of ORFs was performed by homologous recombination with linear PCR fragments amplified using plasmid templates. To create an eYFP tagging vector, the DNA sequence encoding the GST tag was removed from pFA6a-GST-KanMX6 (Longtine *et al*, 1998) through digestion with *PacI* and *AscI*. The gene encoding eYFP was amplified from pEYFP (Clontech) using a High Fidelity PCR Kit (Roche), with forward and reverse primers incorporating 5' *PacI* and *AscI* sites, respectively. Following digestion, *EYFP* was ligated into the pFA6a-KanMX6 vector to create pFA6a-eYFP-KanMX6. Similarly, *ECFP* was amplified from pECFP (Clontech) and cloned as above, creating pFA6a-eCFP-TRP1. All fluorescent strains were created by integration of a PCR-based cassette amplified from the plasmids created above at the 3'-end of the genes being tagged (Longtine *et al*, 1998).

All bait and prey constructs for two-hybrid analysis were developed by amplifying the entire coding sequence of each gene using a High Fidelity PCR Kit (Roche), and cloning the purified products into each vector in frame with the fusion cassette.

Two-hybrid assays

Two-hybrid assays were performed as described previously (Ausubel *et al*, 1995; Duncker *et al*, 2002). LacZ-reporter (pSH18-34), bait (derived from pEG202; Ausubel *et al*, 1995) and prey (derived from pJG4-6, a variant of pJG4-5 with a more extensive polylinker; Gyuris *et al*, 1993) plasmids were serially transformed into yeast strain DY-1. Exponential cultures were grown in SC (2% glucose) medium lacking uracil, histidine and tryptophan to a concentration of 5×10^6 cells/ml, washed with ddH₂O and then resuspended in SC (2% galactose/1% raffinose) lacking the same three components for 6 h to induce prey expression. Following induction, 5×10^6 cells were harvested and permeabilized. The relative strength of interaction was quantified through a β -galactosidase assay utilizing the substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG) (Burke *et al*, 2000) and the formula: β -galactosidase activity = $1000 \times A_{420\text{ nm}} / (t \times v \times A_{600\text{ nm}})$ where *t* is the time of reaction (min) and *v* is the volume of culture used in the assay (ml).

Microscopy

All images were taken with live cells. Prior to imaging cells were grown to $\sim 5 \times 10^6$ cells/ml in SC medium (2% glucose, 0.02% adenine), washed in ddH₂O and resuspended in fresh medium. Cultures were diluted with fresh medium to $\sim 1 \times 10^6$ cells/ml, with 500 μ l added to growth chambers for imaging. Chambers were created using 0.5" ID glass tubing cut to 0.5" and glued to a standard glass (22 \times 66 mm) coverslip with medical grade silicon adhesive. Cells were imaged on a Zeiss Axiovert 100 with a $\times 63$, 1.4 N/A objective lens. To image eCFP and eYFP we used filter sets consisting of exciter D436/ $\times 20$; dichroic 455DCLP; emitter D480/40 m and exciter HQ500/ $\times 20$; dichroic Q515LP; emitter HQ520LP respectively (Chroma Technology, Rockingham, VT). Images were collected with a Sony SX700 CCD (1024 \times 768) and processed in ImageJ 1.30v (NIH, Bethesda). The maximum exposure time with this digital camera is only 2 s and as a result imaging of some FP-tagged proteins under their endogenous promoters resulted in rather weak signals. To obtain a higher signal, 20 fluorescent images were taken in succession and subsequently summed using ImageJ software. Camera gain was adjusted to maximize signal-to-noise ratios in individual frames.

Chromatin binding assay

Approximately 2.5×10^7 cells were harvested at 1000 g and spheroplasted as performed previously (Pasero *et al*, 1999), with modifications. Cells were washed once with ddH₂O and incubated at 30°C for 10 min with gentle mixing in 10 ml/g pre-spheroplasting buffer (100 mM EDTA-KOH (pH 8), 10 mM DTT), followed by incubation in 10 ml/g spheroplasting buffer (0.5XYPD, 1.1 M sorbitol) containing 0.5 mg/ml Zymolyase 20T (Seikagaku Corp., Japan) at 30°C for 10–15 min with gentle mixing. Cells were washed once with 20 ml spheroplasting buffer containing 0.5 mM PMSF followed by resuspension in 1 ml ice-cold wash buffer (5 mM Tris-HCl (pH 7.4), 20 mM KCl, 2 mM EDTA-KOH (pH 7.4), 1 M sorbitol, 1% thiodiglycol, 125 μ M spermidine, 50 μ M spermine) and protease inhibitors (0.1 mM benzamidine HCl, 1 μ g/ml pepstatin, 2 μ g/ml antipain, 2 μ g/ml leupeptin, 0.5 mM PMSF). Cells were pelleted at 400 g for 2 min in a microcentrifuge at 4°C, washed twice with 1 ml

Table 1 Yeast strains used in this study

Strain	Genotype	Source
DY-1	MATa, <i>ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2</i>	S. Gasser Formerly (GA1020)
DY-26	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0</i>	ATCC (BY4733)
DY-36	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::Pgal1-3HA-ORC6/TRP1</i>	This study
DY-39	MATa, <i>ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-13Myc/TRP1</i>	This study
DY-41	MATa, <i>ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-EYFP/kanMX6</i>	This study
DY-65	MATa, <i>ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-ECFP/TRP, nup49::NUP49-EYFP/kanMX6</i>	This study
DY-67	MATa, <i>ura3::URA3[GPD-TK(7x)] ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL psi +</i>	E Schwob Formerly (E1000)
DY-79	MATa <i>ura3::URA3[GPD-TK(7x)] ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL psi +, orc6::Pgal1-3HA-ORC6/TRP1</i>	This study
DY-81	MATa, <i>ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc2::ORC2-13Myc/TRP1</i>	This study
DY-83	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdt1::CDT1-13Myc/HIS3</i>	This study
DY-84	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, mcm10::MCM10-13Myc/HIS3</i>	This study
DY-91	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::Pgal1-3HA-ORC6/TRP1, cdt1::CDT1-13Myc/HIS3</i>	This study
DY-92	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::Pgal1-3HA-ORC6/TRP1, mcm10::MCM10-13Myc/HIS3</i>	This study
DY-93	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::ORC6-3HA/TRP1</i>	This study
DY-103	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc4::Pgal1-3HA-ORC4/TRP1</i>	This study
DY-109	MATa, <i>his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc4::ORC4-3HA/TRP1</i>	This study

ice-cold wash buffer, and resuspended in 0.4 ml ice-cold breakage buffer (5 mM Tris-HCl (pH 7.4), 20 mM KCl, 2 mM EDTA-KOH (pH 7.4), 0.4 M sorbitol, 1% thiodiglycol, 125 μM spermidine, 50 μM spermine) and protease inhibitors as above. Cells were lysed with 0.5 ml ice-cold breakage buffer containing 2% Triton X-100 and incubated on ice for 5 min with occasional mixing. The lysed cells were spun at 16 000 g for 5 min in a microcentrifuge at 4°C. The chromatin pellet was digested on ice for 10 min in 100 μl ice-cold breakage buffer containing 5 mM MgCl₂ and 5 μl DNaseI (1 mg/ml). Digestion was stopped by adding EDTA-KOH (pH 7.4) to 10 mM.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed as previously described (Tanaka *et al*, 1997) with modifications. Cross-linking was carried out with 1% formaldehyde for 20 min at 30°C and the reaction quenched with 125 mM glycine. Cells were harvested, washed with ice-cold PBS and the pellet was snap frozen in liquid nitrogen and stored at -80°C. Cell lysis was performed with glass beads in 500 μl lysis buffer containing protease inhibitors. The soluble fraction of the whole-cell extract was discarded and the insoluble pellet was resuspended in 500 μl lysis buffer containing protease inhibitors. This extract was sonicated five times for 25 s at 5–6 W to achieve an average DNA fragment size of 500 bp using a Microson™ XL 2000 (Misonix). Samples were spun down twice for 2 min at 4500 g to remove cell debris. Immunoprecipitation was carried out with 30 μl anti-goat IgG agarose beads (Sigma, A9294), which were preincubated with 2 μg each of anti-Mcm2 (Santa Cruz, sc-6680) and anti-Mcm5 (Santa Cruz, sc-6686) antibodies. Samples were incubated overnight at 4°C on a rotator.

DNA combing

DNA combing was performed as in Versini *et al* (2003). In brief, wt and *GAL1-ORC6* cultures were released synchronously from G₁ following an initial arrest with α-factor and subsequent 4 h

incubation in YPD with pheromone to deplete Orc6. The cells were released into YPD containing both HU (0.2 M) to stop S phase progression, and BrdU (0.4 mg/ml) to mark nascent DNA synthesis. After 90 min, genomic DNA plugs (800 ng DNA/plug) were made and stored at 4°C until combing. The plugs were initially stained with YOYO-1 (Molecular Probes), and then digested with agarase (Roche) and resuspended at 150 ng/ml in 50 mM MES pH 5.7. Isolated DNA strands were combed on silanized coverslips. Appropriate antibodies were used to detect both the DNA molecules (Argene) and BrdU incorporation (Sera Labs). Images were captured through a Leica DM6000B microscope with a CoolSNAP HQ CCD camera (Roper). Analysis of track lengths was performed using MetaMorph (Universal Imaging Corp.). Adenovirus DNA was used as a standard to convert distances to base pairs.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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