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Noc3p, a bHLH Protein, Plays an Integral Role in the Initiation of DNA Replication in Budding Yeast

Yuexuan Zhang, Zhiling Yu, Xinrong Fu, and Chun Liang[1]
Department of Biochemistry
Hong Kong University of Science and Technology
Clear Water Bay
Kowloon
Hong Kong
China

Summary

Initiation of eukaryotic DNA replication requires many proteins that interact with one another and with replicators. Using a yeast genetic screen, we have identified Noc3p (*n*ucleolar *c*omplex-associated protein) as a novel replication-initiation protein. Noc3p interacts with MCM proteins and ORC and binds to chromatin and replicators throughout the cell cycle. It functions as a critical link between ORC and other initiation proteins to effect chromatin association of Cdc6p and MCM proteins for the establishment and maintenance of prereplication complexes. Noc3p is highly conserved in eukaryotes and is the first identified bHLH (basic helix-loop-helix) protein required for replication initiation. As Noc3p is also required for pre-rRNA processing, Noc3p is a multifunctional protein that plays essential roles in two vital cellular processes.

Introduction

Eukaryotic cells duplicate their genome by initiating DNA replication at multiple sites along each chromosome called origins of DNA replication. Initiation of DNA replication is controlled by the *cis*-acting DNA elements called replicators and the *trans*-acting replication-initiation proteins that interact with the replicators and unwind the DNA double helix at the origins.

In the budding yeast *Saccharomyces cerevisiae*, the replicators and origins of DNA replication have been defined as ARS (autonomously replicating sequence) elements, and many initiation proteins have been identified. Some of the initiation proteins are the initiator ORC (Orc1p–6p; origin recognition complex) (Bell and Stillman, 1992; Bell et al., 1993; Foss et al., 1993; Fox et al., 1995; Liang et al., 1995), Cdc6p (Bueno and Russell, 1992; Hogan and Koshland, 1992; Liang et al., 1995; Cocker et al., 1996), MCM proteins (Mcm2p–7p; minichromosome maintenance) (Hennessy et al., 1991; Yan et al., 1991, 1993; Lei et al., 1996), Cdc45p (Owens et al., 1997; Dalton and Hopwood, 1997; Zou et al., 1997), and Mcm10p (Merchant et al., 1997; Homesley et al., 2000). Based mainly on sequence homology, homologs of these proteins have also been found in other eukaryotes including fission yeast, *Drosophila*, *Xenopus*, mouse, and humans, and they are also required for DNA replication in the respective organisms (Chong et al., 1995; Madine et al., 1995; Muzi-Falconi and Kelly, 1995; Coleman et al., 1996; Jallepalli and Kelly, 1996; Gavin et al., 1997; Thommes et al., 1997; Williams et al., 1997; Saha et al., 1998; Yan et al., 1998). In addition, an initiation protein called Cdt1p was identified in fission yeast (Nishitani et al., 2000; Gopalakrishnan et al., 2001), *Drosophila* (Whittaker et al., 2000), *Xenopus* (Maiorano et al., 2000; Tada et al., 2001), and humans (Wohlschlegel et al., 2000; Nishitani et al., 2001) before it was recently discovered in budding yeast (Tanaka and Diffley, 2002).

Regulation of DNA replication in budding yeast involves interactions of many initiation proteins with one another and with the replicators. According to current models, the initiator ORC binds replicators throughout the cell cycle (Diffley et al., 1994; Aparicio et al., 1997; Liang and Stillman, 1997; Tanaka et al., 1997), serving as the landing pad for other initiation proteins. From the end of mitosis to early G1 phase, Cdc6p interacts with ORC (Liang et al., 1995) and in turn, with the help of Cdt1p (Tanaka and Diffley, 2002), loads MCM proteins onto the replicators (Cocker et al., 1996; Donovan et al., 1997), forming prereplication complexes (pre-RCs). In late G1, Cdc6p is released from, while Cdc45p binds to, the replicators, establishing preinitiation complexes (pre-ICs) or replication complexes (RCs) (Aparicio et al., 1997; Tanaka et al., 1997; Zou and Stillman, 1998; Weinreich et al., 1999, 2001). As cells enter S phase, MCM proteins and Cdc45p sequentially disappear from the replicators, leaving ORC on chromatin forming postreplication complexes (post-RCs). Once the replication origins have been activated, pre-RCs cannot be formed again on the replicators until the end of mitosis. This ordered assembly and disassembly of replication complexes under the control of cyclin-dependent kinases ensure that each replication origin is activated no more than once per cell cycle (Dahmann et al., 1995; Liang and Stillman, 1997; Nguyen et al., 2001).

Identification and characterization of replication-initiation proteins and their regulators have significantly advanced our understanding of eukaryotic DNA replication. However, the detailed mechanism of replication initiation is still elusive, and some replication-initiation proteins may not have been discovered. We have used a yeast genetic screen to identify previously unknown initiation proteins for DNA replication. Here we describe one such protein, previously known as Noc3p. We found that Noc3p interacts with other initiation proteins including ORC and MCM proteins and plays a direct role in the initiation of DNA replication by acting as a corequired factor with ORC at replication origins for the chromatin association of Cdc6p and MCM proteins in the establishment and maintenance of the pre-RCs.

Results

NOC3 Is a Multicopy Suppressor of the *mcm5/cdc46-1* Mutant

To identify proteins that interact with the replication-initiation protein Mcm5p, a subunit of the MCM protein

[1]Correspondence: bccliang@ust.hk

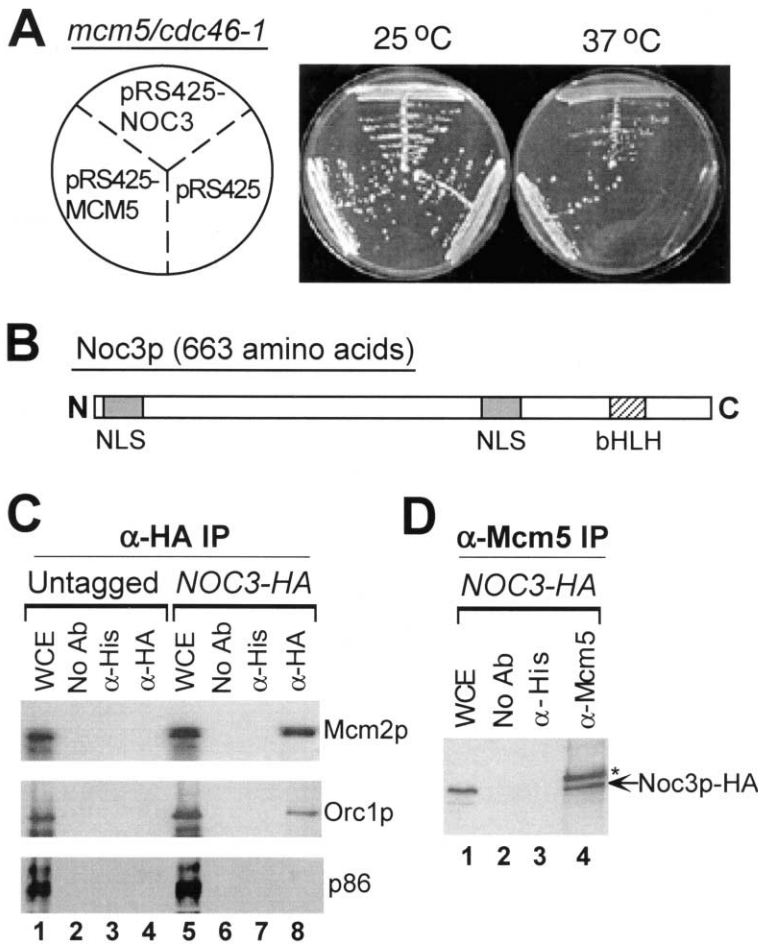


Figure 1. Noc3p Is a Multicopy Suppressor of *mcm5/cdc46-1* and Physically Interacts with MCM Proteins and ORC

(A) The *mcm5/cdc46-1* mutant cells transformed with the multicopy plasmids pRS425-MCM5 (positive control), pRS425-NOC3, or the vector pRS425 were streaked on SCM-Leu (synthetic complete medium lacking leucine) plates, and the plates were incubated at 25°C or 37°C for 3 days.

(B) A diagram of Noc3p, with the bipartite nuclear localization signals (NLS; residues 3–20 and 362–379) and the basic helix-loop-helix (bHLH; residues 541–556) motif noted.

(C) Whole-cell extracts (WCE) from the untagged control and tagged (*NOC3-HA*) cells were precipitated with protein G beads alone as mock IP (lanes 2 and 6), the anti-His control antibody (lanes 3 and 7), or the anti-HA antibody (lanes 4 and 8). Precipitates were immunoblotted with anti-Mcm2, anti-Orc1, or anti-p86 (of Pol α) antibodies.

(D) Whole-cell extracts from *NOC3-HA* cells were precipitated with protein G beads alone (lane 2), the anti-His control antibody (lane 3), or the anti-Mcm5 antibody (lane 4). Precipitates were immunoblotted with the anti-HA antibody. The band marked with an asterisk is a protein crossreacting with the anti-HA antibody after anti-Mcm5 IP.

complex, we have carried out a yeast genetic screen and identified five multicopy suppressors that could rescue the temperature-sensitive lethal phenotype of the *mcm5/cdc46-1* mutant at the restrictive temperature of 37°C. One of the suppressors was identified as YLR002c (Figure 1A), which was recently reported as *NOC3* (*n*ucle*o*lar *c*omplex-associated protein; Milkereit et al., 2001). Database search showed that Noc3p contains a basic helix-loop-helix (bHLH) motif and two bipartite nuclear localization signals (Figure 1B).

The *NOC3* gene was found to be essential for cell viability and for correct processing of pre-rRNA (Milkereit et al., 2001). We also disrupted *NOC3* from the chromosome and found it to be an essential gene in our strain background as well (data not shown).

Noc3p Physically Interacts with MCM Proteins and ORC

Our genetic studies suggested that Noc3p may physically interact with MCM proteins. To test this possibility, a yeast strain in which *NOC3* was tagged at the carboxyl terminus with three copies of the HA epitope (*NOC3-HA*) was constructed. The tagged version of Noc3p is functional, as *NOC3-HA* can replace *NOC3* in haploid cells and allow essentially normal cell growth (data not shown). Noc3p-HA could be detected by using an anti-HA antibody in immunoblotting as a 97 kDa protein in total yeast cell extracts from the HA-tagged but not the untagged strain. To determine if Noc3p-HA and MCM proteins can form a protein complex, we performed reciprocal coimmunoprecipitations (co-IP) between Noc3p-HA and MCM proteins using whole-cell extracts prepared from asynchronous yeast cultures. Mcm2p (Figure 1C, lane 8) and Mcm5p (data not shown) could be precipitated by the anti-HA antibody that recognizes Noc3p-HA. As negative controls, Mcm2p was not precipitated without antibody (Figure 1C, lane 6) or with a control antibody against the $(His)_6$ tag (Figure 1C, lane 7), nor was Mcm2 precipitated by the anti-His or anti-HA antibody from extracts prepared from the untagged control strain (Figure 1C, lanes 1–4). As another negative control, the p86 subunit of the DNA polymerase α was not precipitated by the anti-HA antibody (Figure 1C). These results show that immunoprecipitation of MCM proteins by the anti-HA antibody is dependent on physical interactions (direct or indirect) of MCM proteins with Noc3p-HA. To determine if Noc3p-HA interacts with ORC as well, we immunoblotted the anti-HA immunoprecipitates with an anti-Orc1 antibody and found that Orc1p was also precipitated (Figure 1C). In the reciprocal co-IP between Noc3p-HA and Mcm5p, Noc3p-HA could be precipitated by an anti-Mcm5 antibody (Figure 1D). Taken together with the genetic interaction data, these co-IP results strongly suggest that Noc3p forms

a complex with other initiation proteins including MCM proteins and ORC in vivo. We could not perform co-IP between Noc3p and Cdc6p, as Cdc6p comigrates with IgG heavy chains in SDS-PAGE, and the available anti-Cdc6 antibodies do not efficiently precipitate Cdc6p. As discussed below, however, Noc3p is functionally linked to Cdc6p as well as to ORC and MCM proteins.

Noc3p Is Essential for Minichromosome Maintenance and Initiation of DNA Replication

The genetic and physical interactions of Noc3p with MCM proteins and ORC suggested that Noc3p plays a role in the initiation of DNA replication. To test whether *noc3* mutant cells display defects in the initiation of DNA replication, we first assessed the requirement for Noc3p in the maintenance and initiation of DNA replication of minichromosomes (i.e., ARS-containing plasmids). A strain was constructed in which the *NOC3* locus had been modified such that it only expressed Noc3p fused at its N terminus to a temperature-sensitive degron (td) (Dohmen et al., 1994), effectively creating a temperature-sensitive *noc3-td* mutant strain.

Plasmid loss rates in the *noc3-td* and wild-type control strains were measured using a pair of plasmids, p1ARS and p8ARSs, derived from the previous pDK243 and pDK368-7 (Hogan and Koshland, 1992), respectively, only with the addition of an ampicillin resistance gene. We confirmed that p1ARS and p8ARSs behave in the same way as do pDK243 and pDK368-7 in replication-initiation mutants (Y. Lu, K. Xu, H. Tsang, and C.L., unpublished data). p1ARS (as well as pDK243) contains a single ARS1, and p8ARSs (or pDk368-7) carries seven tandem copies of another ARS in addition to ARS1. It has been shown that, while wild-type cells lose both pDK243 and pDk368-7 at low rates, all previously characterized mutants in replication-initiation proteins including ORC, Cdc6p, MCM, and Cdc45p have high plasmid loss rates with pDK243 and lower loss rates with pDK368-7 (Hogan and Koshland, 1992; Loo et al., 1995; Zou et al., 1997). Correspondingly, all of these mutants have been demonstrated to be defective for the initiation of chromosomal DNA replication (Yan et al., 1993; Fox et al., 1995; Liang et al., 1995; Zou et al., 1997). Therefore, plasmid loss assays with this pair of plasmids can reveal if a mutant is likely defective in the initiation of DNA replication on chromosomes as well as in plasmids.

Plasmid loss rates were measured at the permissive temperature of 25°C and the semipermissive temperatures of 30°C and 32°C (Figure 2; note that plasmid loss assays require colony formation and thus cannot be performed at the restrictive temperature). This analysis revealed that the *noc3-td* mutant cells were severely compromised in their ability to support minichromosome maintenance of p1ARS at 32°C, with a loss rate of 11.0% per generation. In contrast, the plasmid instability was suppressed to a large extent by the addition of multiple ARS elements to the plasmid (p8ARSs; 1.4% per generation). At 30°C, the loss rate per generation was moderately high for p1ARS (5.0%) and decreased to 0.2% for p8ARSs. As these plasmid loss rates are comparable to those reported for other replication-initiation mutants, our results indicate that Noc3p is required for the initiation of DNA replication.

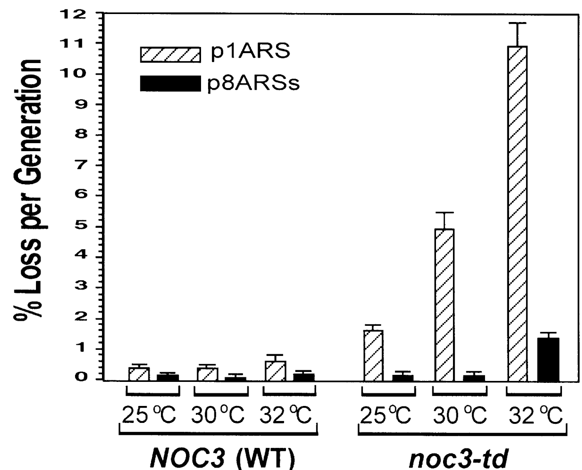


Figure 2. NOC3 Is Essential for Minichromosome Maintenance and Initiation of DNA Replication

Plasmid loss rates were measured for the wild-type and *noc3-td* cells containing either p1ARS or p8ARSs at 25°C, 30°C, and 32°C. Plasmid loss rates are expressed as the averages of percent loss per generation ± SD of three separate experiments.

Mutant *noc3-td* Cells Are Defective for Entry into But Not Progression through S Phase

To further investigate the role of Noc3p in DNA replication, we studied the cell cycle progression of the *noc3-td* cells by analyzing the DNA content of the cells using flow cytometry (also termed FACS, fluorescence-activated cell sorting). The wild-type control and *noc3-td* cells were synchronized in G1 phase by the addition of mating pheromone α factor at 25°C and then released into fresh medium without α factor at the restrictive temperature of 37°C, or at 25°C as the control. Aliquots of cells were analyzed at various time points after release. At both 25°C (Figure 3A) and 37°C (Figure 3B), wild-type cells entered S phase at 30–45 min and finished S phase as a population at 75–90 min after release. In contrast, *noc3-td* cells were defective for entry into S phase. The mutant cells were delayed in entering S phase even at 25°C; they began to enter S phase only after 75 min (Figure 3A). At 37°C, *noc3-td* cells did not enter S phase until after 105–120 min, and by the end of the 180 min experimental time course, approximately 1/3 of the mutant cells in the population still remained with a 1C DNA content (Figure 3B). Approximately 2/3 of the mutant cells did enter S phase at later time points, probably due to the activity of residual Noc3p-td in this somewhat leaky *noc3-td* mutant that could undergo several cell divisions before arrest as large-budded cells at 37°C (data not shown).

In order to ascertain that the mutant cells with a 1C DNA content that appeared at late time points at 37°C were those that had not replicated DNA following the α factor block-and-release, rather than those that had finished DNA replication and undergone mitosis, we examined the effect of adding the microtubule-depolymerizing drug nocodazole on the FACS results. Nocodazole was added at 105 min after release from α factor in order to prevent cells from entering mitosis. The results indicate that a significant population of the mutant cells

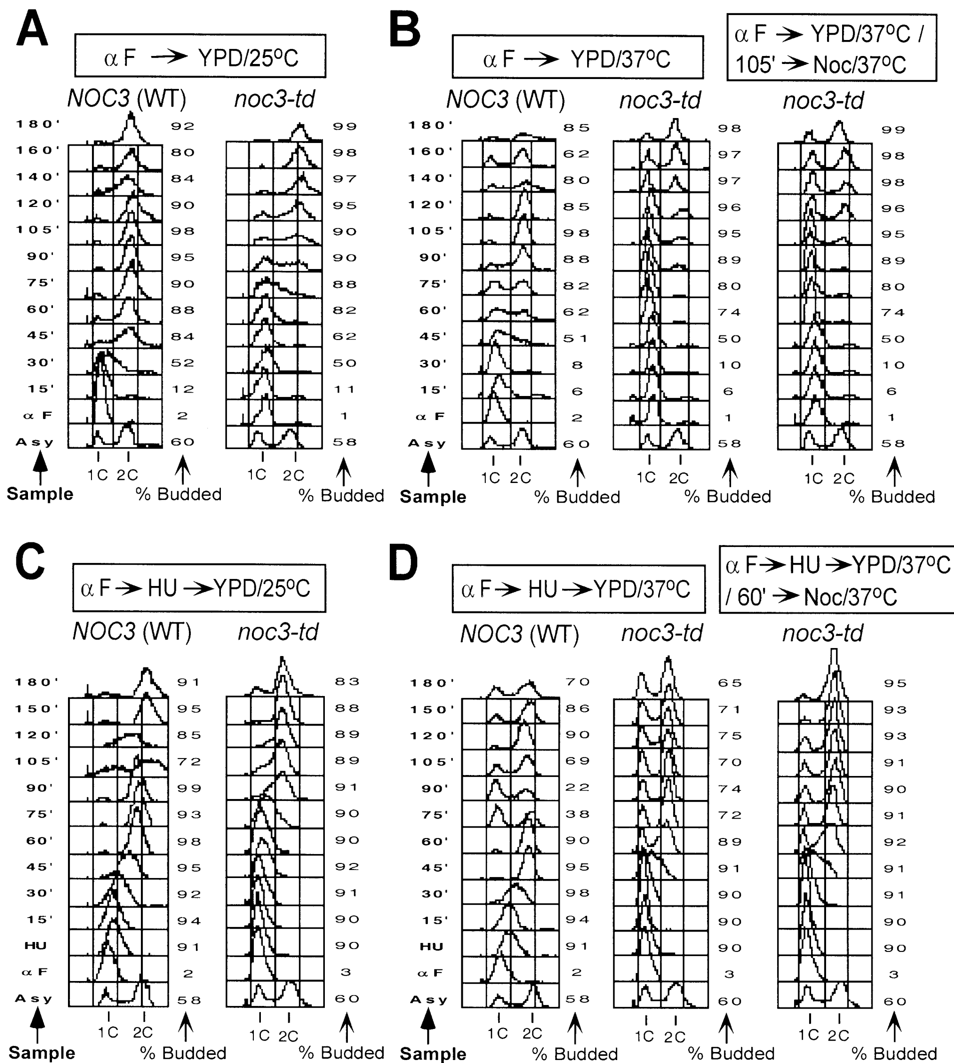


Figure 3. Noc3p Is Essential for Entry into But Not Progression through S Phase

(A and B) Asynchronously (Asy) cycling wild-type (*NOC3* WT) and *noc3-td* cells were arrested in G1 with α factor (α F) at 25°C. Cells were then released into fresh medium at 25°C (A) or 37°C (B). To block mitosis, nocodazole (Noc) was added to an aliquot of the *noc3-td* cells at 37°C at 105 min postrelease from α factor (B). Samples were taken at the time points indicated and analyzed by flow cytometry for DNA contents and by budding index counting (% Budded indicates percent of budded cells in the population).

(C and D) Cycling wild-type and *noc3-td* cells were synchronized in G1 with α factor at 25°C and then released into hydroxyurea (HU)-containing medium for 2 hr at 25°C. The cells were then released from the HU block into fresh medium at 25°C (C) or 37°C (D). Nocodazole was added to an aliquot of the *noc3-td* cells at 37°C at 60 min postrelease from HU (D).

did not enter S phase by 3 hr after being released from G1 at 37°C (Figure 3B). On the other hand, mutant cells underwent budding with similar kinetics to the wild-type cells at both 25°C and 37°C when they were released from the G1 block (Figures 3A and 3B, see "% Budded"). These phenotypes are reminiscent of many previously characterized mutants in replication-initiation proteins.

The results in Figures 3A and 3B show that Noc3p is required for entry into S phase and DNA replication. However, this experiment did not distinguish between an essential role for Noc3p in origin activation and a possible role for Noc3p after initiation, in the elongation phase of DNA replication. To determine if Noc3p was involved in elongation, we asked whether or not Noc3p was required for the completion of DNA replication after the *noc3-td* cells were released from a hydroxyurea (HU) block. HU inhibits ribonucleotide reductase, causing an arrest in early S phase, at a stage where early replication origins would have been activated, so that the cells could complete S phase after release from HU without further activation of replication origins.

Cultures of the wild-type control and *noc3-td* cells were first presynchronized in G1 with α factor at 25°C and released into HU for 2 hr at 25°C to allow activation of early origins. Cultures were then released from HU into fresh medium at 25°C (Figure 3C) or 37°C (Figure 3D). Both the wild-type and *noc3-td* cells could complete S phase at both 25°C and 37°C. At 37°C, the majority of the mutant cells replicated their DNA from 40 to 75 min, and some cells could even enter the next cell cycle

afterwards, as indicated by the appearance of a G1 population with a 1C DNA content and the change of budding index (percent budded cells decreased starting from 75 min; Figure 3D). Furthermore, nocodazole (added at 60 min after release from HU) was used to confirm that DNA replication was completed at 37°C (Figure 3D). These results suggest that Noc3p is dispensable for DNA replication following HU arrest and release. The somewhat slower progression of S phase in the mutant cells compared to the wild-type cells may be attributable to a lower frequency of initiation of DNA replication in the mutant cells during the HU block and subsequent release. Alternatively, Noc3p may play a minor, nonessential role in elongation of DNA replication. The combined results shown in Figures 3A–3D support an essential role of Noc3p for chromosomal DNA replication at the initiation level, consistent with the conclusion from the plasmid loss assays with p1ARS and p8ARSs.

Noc3p Is a Chromatin Binding Protein and Binds to Chromatin Constitutively

Initiation proteins such as ORC, Cdc6p, Cdt1p, MCM proteins, and Cdc45p are chromatin binding proteins. This can be shown by using chromatin binding assays, in which total cellular proteins are separated into soluble and chromatin bound fractions, and specific protein(s) in the two fractions are detected by immunoblotting (Donovan et al., 1997; Liang and Stillman, 1997). In order to confirm that a protein found in a crude chromatin fraction indeed binds to chromatin rather than to the nuclear matrix, the chromatin pellet can be partially digested with micrococcal nuclease (MNase), such that the released polynucleosomes can be pelleted again by ultracentrifugation (Liang and Stillman, 1997).

Cycling *NOC3-HA* cells were analyzed for Noc3p-HA as well as for Orc3p, which served as a control for the assay. A whole-cell extract from the untagged strain was used as the specificity control for Noc3p-HA immunoblotting using the anti-HA antibody (Figure 4A, lane 1). The pattern of Orc3p in different fractions (Figure 4A, lanes 2–8) was the same as reported previously (Liang and Stillman, 1997). Noc3p-HA was found in the crude chromatin pellet (Figure 4A, lane 4) as well as in the whole-cell extract (lane 2) and soluble fraction (lane 3). Furthermore, like Orc3p, Noc3p-HA was also found in the supernatant after MNase digestion (Figure 4A, lane 5) and in the pellet (lane 8) but not the supernatant (lane 7) after ultracentrifugation. These results demonstrate that Noc3p is a chromatin binding protein.

To examine the pattern of chromatin association of Noc3p-HA in the cell cycle, a time course experiment was performed whereby yeast cells were synchronized in G1 with α factor and then released into fresh medium. Aliquots of cells at various time points were processed for flow cytometry to monitor the cell cycle progression (Figure 4C) and for chromatin binding assays for Noc3p-HA, as well as for Orc3p and Mcm2p for comparison (Figure 4B). Orc3p was detected in the chromatin pellet fractions ("Pel") across the cell cycle, as previously shown (Liang and Stillman, 1997; Weinreich et al., 1999). Mcm2p was associated with chromatin periodically from early G1 phase to early S phase, released from chromatin during S phase, and rebound chromatin at the end of mitosis, as published earlier (Liang and Stillman, 1997). Noc3p was found to bind chromatin throughout the cell cycle at a constant level. These data suggest that like ORC, Noc3p is constitutively chromatin bound. The level of Noc3p in the supernatants ("Sup") is also constant in the cell cycle.

Noc3p Binds to ARS But Not Non-ARS Sequences In Vivo

Replication-initiation proteins not only bind to chromatin but also to ARS elements, which are the replicators and replication origins. This can be shown by using chromatin immunoprecipitation (ChIP) assays involving in vivo formaldehyde crosslinking of proteins to DNA as well as proteins to proteins, shearing of DNA, immunoprecipitation of specific proteins, and subsequent PCR detection of the coprecipitated DNA fragments (Aparicio et al., 1997; Tanaka et al., 1997). We modified the ChIP method and used MNase-digested chromatin instead of total cell lysate as the input (see Experimental Procedures). The validity of our method was confirmed by ChIP assay with antibodies against the endogenous ORC subunits. ARS1, but not the control "R2.5" sequence, which does not contain ARS and is 2.5 kb away from ARS1 (Homesley et al., 2000), could be immunoprecipitated by the anti-ORC antibodies (Figure 5A, lane 3). The control anti-HA antibody (the cells were untagged) did not precipitate ARS1 or R2.5 (Figure 5A, lane 2). Both the ARS1 and R2.5 DNA could be detected in the input chromatin (Figure 5A, lane 4), which represented the total genomic DNA before IP.

To determine if Noc3p-HA binds to ARS elements, ChIP assays using an anti-HA antibody were performed with the untagged control and *NOC3-HA*-tagged cells. ARS1 but not the control R2.5 sequence could be detected in the anti-HA immunoprecipitates from asynchronous *Noc3-HA* cells and those synchronized by α factor, hydroxyurea, or nocodazole (Figure 5B, lanes 3–7). Neither ARS1 nor R2.5 DNA was detected in the immunoprecipitate from the untagged control cells (Figure 5B, lane 2), indicating that the anti-HA antibody did not nonspecifically precipitate these DNA fragments. Both the ARS1 and R2.5 DNA could be detected in the input chromatin (Figure 5B, lanes 7–11). Moreover, ARS305, but not the "ARS305+8kb" DNA, which is 8 kb away from ARS305 serving as the non-ARS control (Aparicio et al., 1997), could be found in the anti-HA precipitate from the tagged but not the untagged cells (Figure 5D). These results strongly suggest that, like ORC, Noc3p binds to replication origins in vivo and does so in different phases of the cell cycle, consistent with the idea that Noc3p plays a direct role in the initiation of DNA replication.

Noc3p Is Required for the Chromatin Association of Cdc6p and Mcm2p

After having established that Noc3p is required for the initiation of DNA replication and that it binds specifically to ARS elements, we wanted to understand the mechanism of action of Noc3p in replication initiation. During the M-to-G1 transition, pre-RCs consisting of at least ORC, Cdc6p, Cdt1p, and MCM proteins are assembled

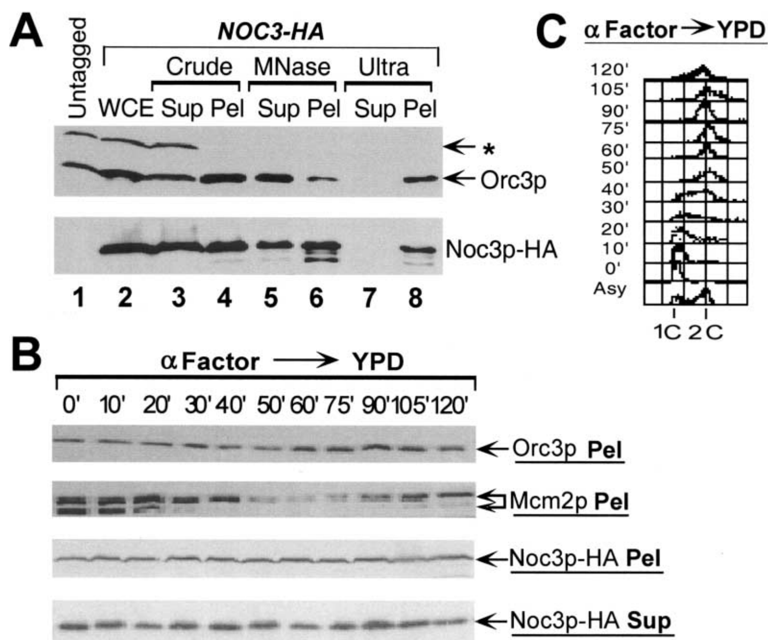


Figure 4. Noc3p Is a Chromatin Binding Protein and Constitutively Binds to Chromatin

(A) Cycling *NOC3-HA* cells were harvested and analyzed with the "7 fraction" chromatin binding assay for Orc3p and Noc3p-HA as indicated. Abbreviations: Untagged, whole-cell extract from untagged cells; WCE, whole-cell extract; Sup, supernatant; Pel, pellet; Crude, crude chromatin fractionation; MNase, micrococcal nuclease limit-digestion of the crude chromatin; Ultra, ultracentrifugation of the MNase Sup. The band marked with an asterisk is a non-chromatin binding protein crossreacting with the anti-Orc3 antibody.

(B) Aliquots of *NOC3-HA* cells were harvested at various time points indicated (at 25°C) after being released from α factor synchronization and were processed for chromatin binding assays. The crude chromatin and supernatant fractions at different time points across the cell cycle were immunoblotted for Orc3p, Mcm2p, and Noc3p-HA as indicated.

(C) The cell cycle progression of the cells in (B) was monitored by FACS.

at replication origins, and in G1 phase, MCM proteins need to be maintained at the pre-RCs so that other initiation proteins such as Cdc45p can be loaded for the subsequent initiation of DNA replication. To investigate the possibility that Noc3p may play a role in the formation and maintenance of the pre-RCs, we used the *noc3-td* mutant to determine if Noc3p is required for the chromatin association of Cdc6p and/or MCM proteins, two components of the pre-RCs.

We first asked if Cdc6p and/or Mcm2p would fail to be loaded onto chromatin during the M-to-G1 transition when Noc3p-td was depleted in *noc3-td* cells (the *noc3-td* locus is also tagged with a single HA to give a 120 kDa protein on SDS-PAGE). The wild-type and *noc3-td* cells were presynchronized in G1 with α factor and released into the cell cycle in fresh medium for 75 min at 25°C (these two steps were to minimize the time of the subsequent incubation of the cells in nocodazole). The cells were then blocked in G2/M with nocodazole at 37°C for 100 min and subsequently released from G2/M into G1 in α factor-containing medium at 37°C. Aliquots of the cells were harvested for chromatin binding assays every 30 min for a total of 2 hr. As a control, the same procedures were carried out, but at 25°C throughout (see diagram in Figure 6, top).

In wild-type cells at 25°C and 37°C, the levels in both the chromatin pellet (Figure 6A) and supernatant (Figure 6B) fractions for Noc3p-HA and Orc3p were constant, and the levels for Cdc6p and Mcm2p were also as expected for the normal cell cycle regulation. Cdc6p and Mcm2p were almost absent from chromatin in nocodazole-blocked cells and were loaded onto chromatin as cells were released into α factor, but then Cdc6p gradually disappeared from chromatin (Figure 6A) and was also mostly degraded (Figure 6B) by 90 min in α factor-containing medium, consistent with previous reports that Cdc6p is released from chromatin in G1 (Weinreich et al., 1999, 2001) and is unstable in α factor-blocked cells (Drury et al., 2000). In *noc3-td* cells at 25°C, the patterns of all four proteins analyzed in both the chromatin (Figure 6D, lanes 1–5) and supernatant (Figure 6E, lanes 1–5) fractions were similar to those in wild-type cells. At 37°C, when Noc3p-td was largely removed from chromatin (Figure 6D, lanes 6–10) and was mostly degraded as anticipated (Figure 6E, lanes 6–10), Orc3p remained bound to chromatin (Figure 6D, lanes 6–10). In contrast, little or no Cdc6p and Mcm2p was found on chromatin (Figure 6D, lanes 6–10), while the level of Mcm2p in the supernatant fractions remained more or less constant and the amount of Cdc6p in the supernatants was little affected by the depletion of Noc3p-td (Figure 6E). This also indicates that the total cellular level of neither Cdc6p nor Mcm2p was controlled by Noc3p (see Discussion). FACS and budding index analyses showed that, by 90 min after release from nocodazole at 25°C or 37°C, the majority of the *noc3-td* as well as wild-type cells completed mitosis and entered the subsequent G1 phase (Figures 6C and 6F). This also suggests that Noc3p is not essential for mitosis. Together, these results indicate that Noc3p is required for loading Cdc6p and MCM proteins onto chromatin as cells pass through mitosis and enter G1 phase.

To provide evidence that Noc3p is a component of the pre-RCs, in addition to being required for recruiting Cdc6p and MCM proteins onto chromatin, we tested the effects of depleting Noc3p-td in α factor-arrested G1 cells in which MCM proteins had been loaded onto, and were associated with, chromatin. The wild-type and *noc3-td* cells were synchronized in G1 with α factor and then shifted to 37°C, or maintained at 25°C as the control, in the continued presence of α factor. Cells were harvested at different time points and processed for chromatin binding assays to analyze Noc3p-HA (or Noc3p-td), Orc3p, and Mcm2p (Cdc6p is not stable in *noc3-td* or wild-type cells in α factor as discussed above, and thus could not be followed in this experiment). For wild-type cells, all three proteins examined remained at more or less constant levels in both the chromatin (Figure 7A)

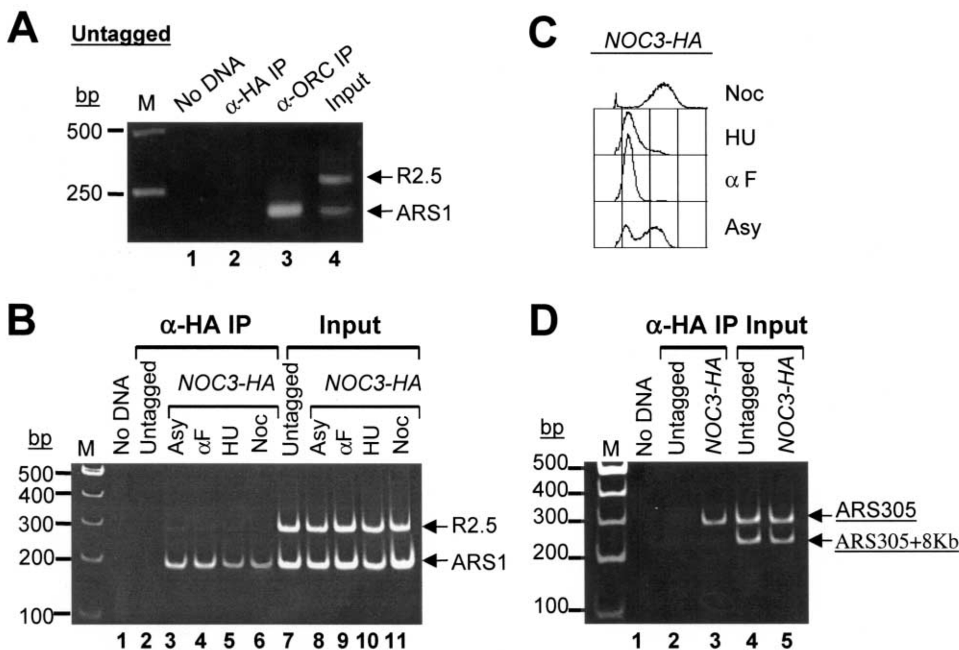


Figure 5. Noc3p Binds to ARS But Not Non-ARS Sequences In Vivo

(A) ChIP assays were performed with asynchronous untagged cells using anti-ORC antibodies or the control anti-HA antibody. Possible PCR products for ARS1 and the control R2.5 fragments amplified from precipitated DNA, and total input DNA were analyzed on an agarose gel and visualized under UV after ethidium bromide staining. Abbreviations: M, 250 bp DNA markers; No DNA, control PCR without DNA template.
(B) ChIP assays were performed using an anti-HA antibody with untagged control cells and *NOC3-HA*-tagged cells that were asynchronous (Asy) and those synchronized with α factor (αF), hydroxyurea (HU), or nocodazole (Noc). Possible PCR products for ARS1 and R2.5 were analyzed on a nondenaturing polyacrylamide gel. M indicates 100 bp DNA markers.
(C) FACS analysis for the *NOC3-HA* cells used in (B).
(D) ChIP assays were performed with asynchronous untagged and *NOC3-HA* cells to detect ARS305 and the control "ARS305+8kb" sequences.

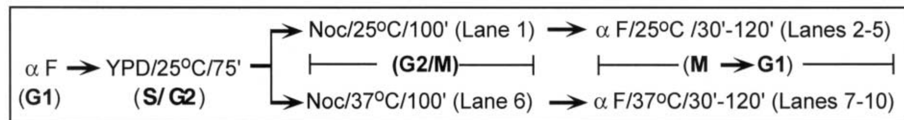
and soluble (Figure 7B) fractions throughout the time course as expected. When *noc3-td* cells were shifted to 37°C, Noc3p-td largely disappeared from chromatin (Figure 7C, lanes 2–6) and was mostly degraded (Figure 7D, lanes 2–6), as anticipated. The removal of Noc3p-td had no effect on the chromatin association or total level of Orc3p (Figures 7C and 7D). However, as Noc3p-td was removed from chromatin, Mcm2p was also released from chromatin (Figure 7C, lanes 2–6), whereas the level of Mcm2p in the supernatant fractions remained constant (Figure 7D, lanes 2–6), consistent with the conclusion from the experiments shown in Figure 6 that the total amount of Mcm2p was not affected by depletion of Noc3p-td (see Discussion). In the control experiment at 25°C, both Noc3p-td and Mcm2p remained associated with chromatin (Figure 7C, lane 7). The combined results from the chromatin binding experiments shown in Figures 6 and 7A–7D demonstrate that, like ORC, Noc3p is essential for both the recruitment of Cdc6p and MCM proteins to, and the maintenance of MCM proteins on, the chromatin, suggesting that, like ORC, Noc3p is a component of the pre-RCs as well as the post-RCs.

ORC Is Required for Stable Chromatin Association of Noc3p

As discussed above, Noc3p is dispensable for chromatin association of ORC. We performed a reciprocal experiment to see if ORC is required for the maintenance of Noc3p on chromatin. An *orc2-1* temperature-sensitive mutant strain, in which *NOC3* (wild-type) was also C-terminally tagged with three copies of HA, was taken through a similar experiment as shown in Figures 7A–7D for the wild-type and *noc3-td* strains. When the *orc2-1* cells in G1 were shifted to 37°C, Orc3p was gradually released from chromatin (Figure 7E, lanes 2–6), as previously reported (Homesley et al., 2000). Mcm2p was rapidly removed from chromatin at 37°C (Figure 7E, lanes 2–6). This is consistent with previous reports that ORC is required for the maintenance of pre-RCs in G1 cells. The level of Noc3p on chromatin decreased gradually in the first 2 hr and then fell to a low level at later time points at 37°C (Figure 7E, lanes 2–6). The levels of all three proteins in the supernatants were little affected (Figure 7F). These data suggest that ORC is required for stable chromatin association of Noc3p. This ORC dependence of chromatin association of Noc3p further supports the conclusion that Noc3p plays a direct role in the initiation of DNA replication together with ORC. The fact that the chromatin association of Noc3p follows ORC more closely than Mcm2p does in *orc2-1* cells (Figure 7E) is consistent with the idea that Noc3p is closely associated with ORC on chromatin and connects ORC to other initiation proteins.

Discussion

We have identified Noc3p as a novel initiation protein for DNA replication. Noc3p forms a complex with ORC and MCM proteins and is required for the initiation of



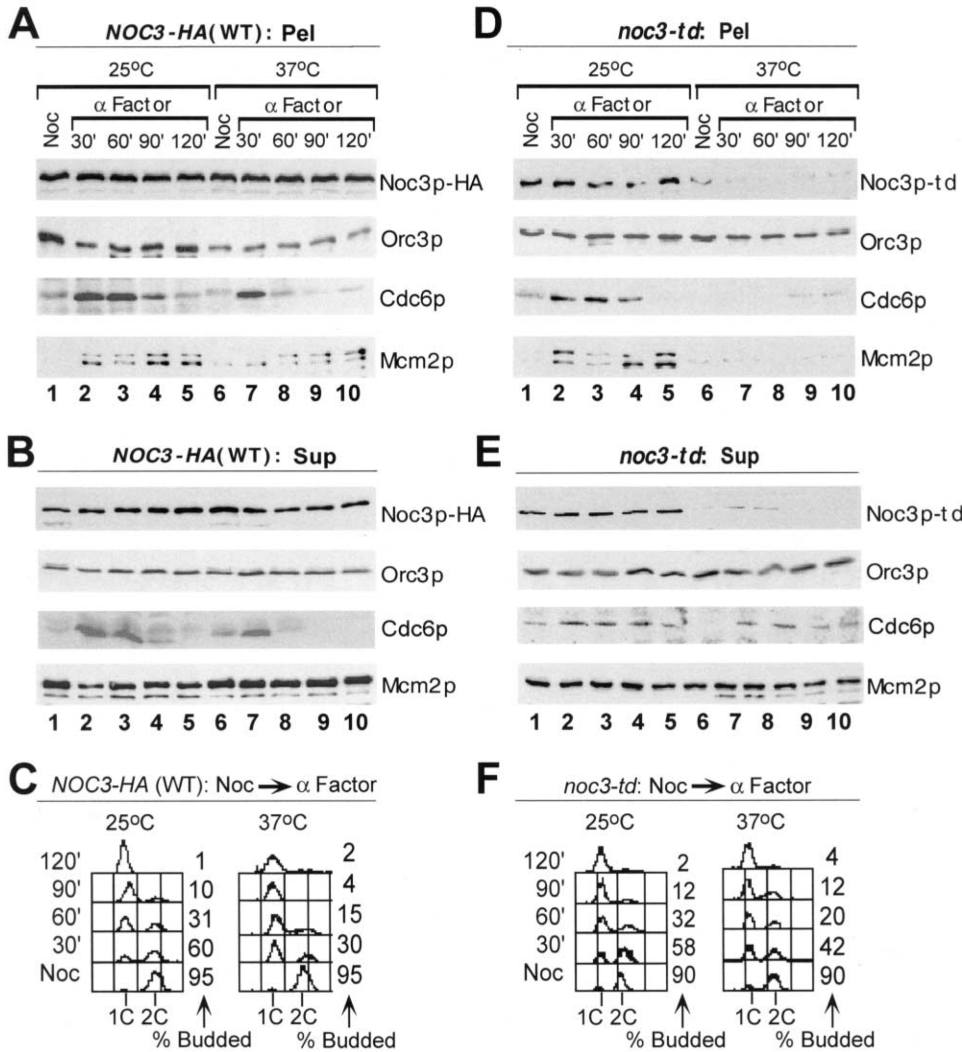


Figure 6. Noc3p Is Essential for Loading Cdc6p and MCM Proteins onto Chromatin during the M-to-G1 Transition

Wild-type control (A–C) and *noc3-td* mutant (D–F) cells were presynchronized in G1 with α factor at 25°C for 3 hr and then released into fresh medium for 75 min at 25°C. Nocodazole was then added, and the cells were shifted to 37°C for 100 min. The cells were then released from nocodazole into α factor-containing medium at 37°C for 120 min and harvested at 30 min intervals. For the control experiment, the cells were taken through the same procedure, except that the cells were kept at 25°C throughout. Chromatin binding assays were performed for the various samples, and the chromatin (A and D) and supernatant (B and E) fractions were immunoblotted for Noc3p-HA/Noc3p-td, Orc3p, Cdc6p, and Mcm2p as indicated.

(C and F) The cell cycle progression of the cells in (A) and (B) and in (D) and (E), respectively, was monitored by FACS.

DNA replication. Like ORC, Noc3p binds chromatin and ARS elements through the cell cycle, and together with ORC, it functions at replication origins to recruit Cdc6p and MCM proteins for the establishment of the pre-RCs and then to maintain MCM proteins on the pre-RCs so that other replication-initiation proteins such as Cdc45p can be loaded during G1. The previously known order of the pre-RC assembly was that ORC recruits Cdc6p and perhaps also Cdt1p, and then ORC-Cdc6p-Cdt1p together recruit MCM proteins. Now we have found another critical link, Noc3p, between the initiator ORC and the other initiation proteins. Without Noc3p, ORC cannot recruit Cdc6p or MCM proteins, and therefore pre-RCs cannot be formed. Without Noc3p, pre-RCs that have been established cannot be maintained at replication origins in G1.

Noc3p was previously found to be required for pre-rRNA processing and pre-ribosome maturation (Milkereit et al., 2001). Therefore, we needed to consider the formal possibility that the replication-initiation pheno-

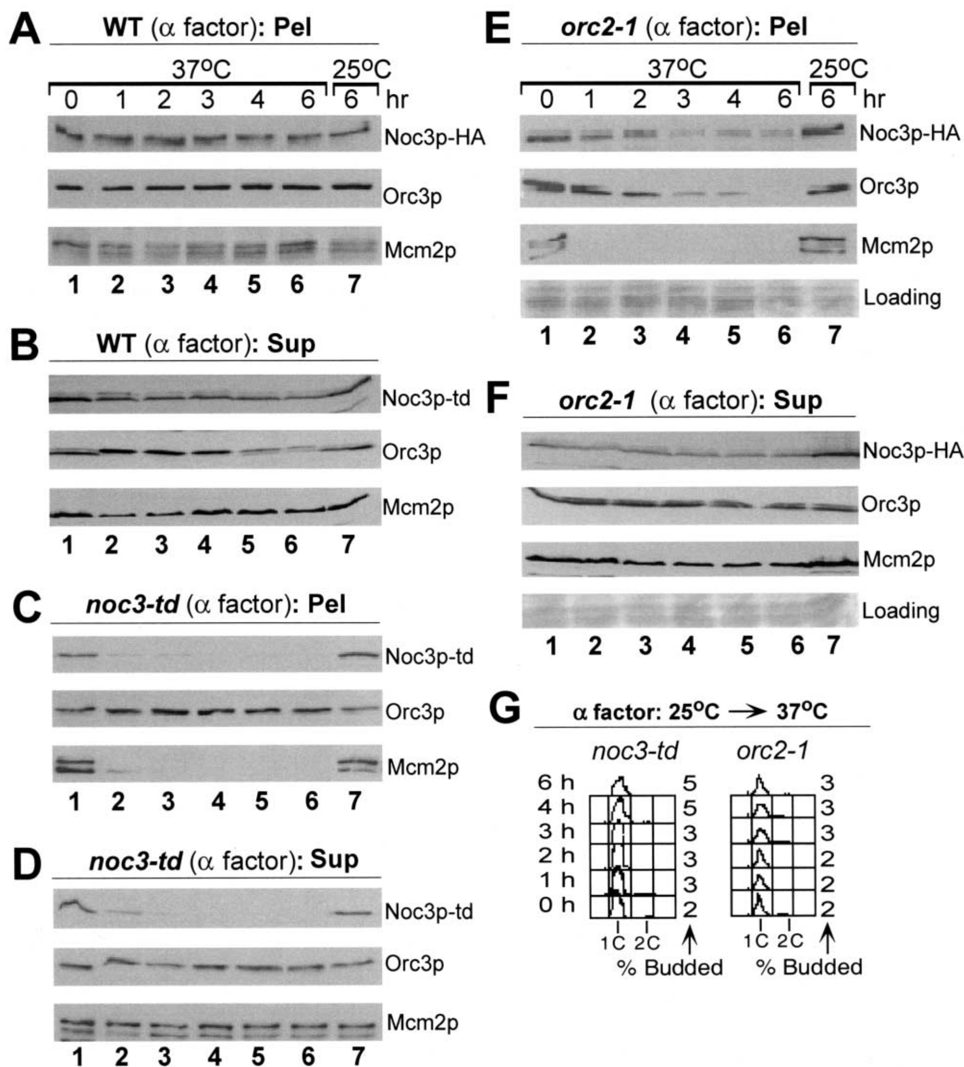


Figure 7. Noc3p Is Essential for the Maintenance of MCM Proteins on Chromatin in G1, and ORC Is Required for Stable Chromatin Association of Noc3p

Wild-type (*NOC3-HA*) (A and B), *noc3-td* (C and D), and *orc2-1* (E and F) cells were synchronized in G1 with α factor at 25°C for 3 hr and then shifted to 37°C for 6 hr or maintained at 25°C in the continued presence of α factor (replenished every 1.5 hr). Chromatin binding assays were performed, and the pellet (A, C, and E) and soluble (B, D, and F) fractions were immunoblotted for Noc3p-HA/Noc3-td, Orc3p, and Mcm2p as indicated.

(G) Aliquots of the cells were analyzed by FACS (not shown for wild-type cells). The experiment in (A) and (B) was carried out with 20 μg/ml cycloheximide (10 or 20 μg/ml was sufficient to block cell growth; data not shown) added to the culture after the first 3 hr synchronization with α factor at 25°C. The same results (not shown) were obtained without cycloheximide. Ponceau S staining was used as the loading control in (E) and (F), which was necessary because Orc3p was not stably bound to chromatin in *orc2-1* cells at 37°C.

types of the *noc3-td* mutant might be the indirect result of defective translation of other replication-initiation proteins due to a possible ribosome deficiency in the mutant cells. In addition, Noc3p belongs to the large family of bHLH proteins with over 240 members that play critical roles in development, cell growth, differentiation, and apoptosis (reviewed in Massari and Murre, 2000; Robinson and Lopes, 2000). Many bHLH proteins are transcriptional regulatory proteins that can activate or repress gene expression. Therefore, we also needed to consider the possible scenario that Noc3p might play only an indirect role in initiation of DNA replication by affecting transcription of other replication-initiation proteins. However, we found that the role of Noc3p in the initiation of DNA replication is direct and not through controlling the gene expression of other initiation proteins at the level of transcription or translation, for the following reasons.

When Noc3p was depleted in the *noc3-td* cells at 37°C, Cdc6p and Mcm2p remained at about the same levels in the cell and only disappeared from chromatin (Figures 6D, 6E, 7C, and 7D). In addition, when *NOC3* was overexpressed from a multicopy plasmid in the *mcm5/cdc46-1* or wild-type cells, the level of Mcm2p or Mcm5p did not change (data not shown). Therefore, the role of Noc3p in replication initiation is not to control

the gene expression of Cdc6p or MCM proteins. Moreover, we confirmed that protein translation is not required for the maintenance of MCM proteins on chromatin in wild-type cells, as the experiment shown in Figure 7A was carried out in the presence of cycloheximide (which inhibits protein synthesis), indicating that MCM proteins can be stably associated with chromatin in the absence of de novo protein synthesis in G1 phase. We can therefore conclude that the failure of the *noc3-td* cells to load Cdc6p and MCM proteins onto chromatin (Figure 6D) and to maintain MCM proteins on chromatin (Figure 7C) did not result from defects in transcription or translation when Noc3p-td was depleted.

Furthermore, our data demonstrate that Noc3p in fact plays a direct role in the initiation of DNA replication. First of all, Noc3p forms a complex with other replication-initiation proteins including MCM proteins and ORC that are known to function directly at replication origins. Second, not only is Noc3p a chromatin binding protein, it also binds specifically to ARS sequences in vivo. Moreover, the experiment with *orc2-1* cells (Figure 7E) suggests that Noc3p is closely associated with ORC on chromatin. The idea that Noc3p might play only an indirect role in DNA replication by controlling the transcription or translation of initiation proteins would be incompatible with the fact that Noc3p physically interacts with other initiation proteins and with replication origins. Finally, Noc3p is required for the maintenance of MCM proteins on chromatin in G1 cells, in addition to its role in recruiting Cdc6p and MCM proteins for the pre-RC formation. The role played by a protein in upholding MCM proteins on chromatin in G1, which does not required protein synthesis, is most likely through direct interactions with the chromatin bound MCM proteins. This role was previously demonstrated only for ORC (Cdc6p disappears from chromatin and is degraded in late G1 as discussed above, and Cdt1p is also cell cycle regulated and is excluded from the nucleus before DNA replication) (Tanaka and Diffley, 2002), and now this role is known to be shared by Noc3p.

Therefore, Noc3p is a multifunctional protein that plays a direct role in the initiation of DNA replication in addition to its previously reported role in pre-rRNA processing. Of note, although protein synthesis was not specifically investigated in this study, we did notice that while the *noc3-td* cells started budding with similar kinetics to wild-type cells when they were released from G1 into S phase at both 25°C and 37°C, the buds did grow more slowly in *noc3-td* than wild-type cells, consistent with Noc3p being required for protein synthesis and cell growth. Interestingly, a multifunctional nature has also been demonstrated for other replication-initiation proteins. For example, ORC, Cdc6p, and MCM are not only required for DNA replication, but also take part in transcriptional silencing (Foss et al., 1993; Loo et al., 1995), mitosis control (Bueno and Russell, 1992; Calzada et al., 2001; Weinreich et al., 2001), and possibly transcription (Yankulov et al., 1999), respectively.

Noc3p is the first bHLH protein found to be an essential initiation protein for DNA replication. How does Noc3p function in the initiation of DNA replication? Association with replication origins should facilitate the action of Noc3p, together with ORC, in recruiting Cdc6p and MCM proteins onto replication origins and in maintaining MCM proteins at the replication origins before initiation. As some bHLH proteins have been implicated in chromatin remodeling (reviewed in Massari and Murre, 2000), it may also be possible that Noc3p modulates chromatin structure at and/or near ARS elements to help the establishment and maintenance of pre-RCs. Finally, it will be interesting to investigate the possibility that Noc3p may play a role(s) in coordinating several cellular pathways by participating in multiple processes as a way to connect cell division with growth. We believe that further characterization of Noc3p and other replication-initiation proteins with which Noc3p interacts should continue to shed new light onto the mechanism and cell cycle control of eukaryotic DNA replication.

Experimental Procedures

Strains and Plasmids

A one-step PCR-based technique (Longtine et al., 1998) was used to create the *NOC3-HA* strain by tagging the chromosomal *NOC3* gene with three copies of the HA epitope at the C terminus in the W303-1A (*Mat***a**, *ade2, leu2, ura3, trp1, his3*) background. The *NOC3* locus in the *orc2-1* mutant in the W03-1A background (Foss et al., 1993; Liang et al., 1995) was also tagged with HA in the same manner as for the *NOC3-HA* strain. The *noc3-td* strain was generated by first inserting a 1.2 kb KpnI-KpnI HA-*NOC3*-containing fragment into the KpnI sites of the plasmid pPW66R (Dohmen et al., 1994) to create the plasmid p-ubi-DHFR^{ts}-HA-NOC3 and then integrating the MscI-linearized plasmid into the *NOC3* locus on the chromosome by homologous recombination in W303-1A. (Multiple attempts to create a *noc3-td* mutant in a *UBR1*-overexpressing strain were not successful.) The *mcm5/cdc46-1* strain is DBY2028 (*Mat***a**, *ade2, ura3, leu2, lys2, cdc46-1*) (Hennessy et al., 1991). p1ARS and p8ARSs (Y. Lu, K. Xu, H. Tsang, and C.L., unpublished data) were constructed by inserting a 1 kb BspHI fragment (filled with the Klenow enzyme) containing the ampicillin resistance gene (for easier selection in *E. coli*, where use of the *LEU2* marker is inconvenient) from pBluescript II into the SmaI sites of pDK243 and pDK368-7, respectively. pRS425-MCM5 was constructed by cloning a 2.6 kb BsaHI-BsaBI fragment containing *MCM5* into the ClaI and SmaI sites of pRS425 (2 μm ARS, *LEU2*), and pRS425-NOC3 was constructed by inserting a 2.8 kb NcoI-NcoI fragment containing *NOC3* into the NcoI sites of pRS425.

Isolation of Multicopy Suppressors of *mcm5/cdc46-1*

The multicopy suppressor screen was conducted essentially as described by Liang et al. (1995). In brief, 5 μg of yeast genomic library DNA with an average insert size of ~6 kb constructed in YEp213 (2 μm ARS, *LEU2*) was transformed into ~10^8 log phase *mcm5/cdc46-1* cells. The transformants were grown on SCM-Leu plates at 25°C for 1 day and then at 37°C for 3–5 days. The transformation efficiency was about 2×10^4 colonies/μg. Total DNA was isolated from strains that could grow at 37°C. Plasmid DNA was then recovered by transformation into *E. coli* and reintroduced into *mcm5/cdc46-1* cells to confirm suppression. The inserts were identified by sequencing the ends followed by database searching. The inserts were subcloned into pRS425 and tested for suppression of *mcm5/cdc46-1*.

Coimmunoprecipitation and Western Blotting

Yeast whole-cell extracts were prepared by bead-beating in Lysis/IP Buffer (20 mM Tris-HCl [pH 7.9], 150 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 5% glycerol, 1 mM EGTA, 0.1% Triton X-100, 10 mM NaF, 0.1 mM $NaVO_3$, and 300 μg/ml EtBr, plus 2 mM $Na_2P_2O_7$ and protease inhibitors added just before use). Extracts were precleared with protein G beads and then precipitated with the anti-HA (12CA5), anti-Mcm5 (Weinreich and Stillman, 1999), or the control anti-His antibody, all preabsorbed to protein G beads. Western blotting was carried out as previously described (Liang and Stillman, 1997; Weinreich et al., 1999).

Plasmid Loss Assay

Plasmid loss assays were performed as described by Yan et al. (1991). Cells containing either p1ARS or p8ARSs were grown to early log phase (2×10^6 cells/ml) in SCM-Leu medium at 25°C. Cells were then diluted to 2×10^5 cells/ml in rich medium and grown at 25°C, 30°C, or 32°C. After 8–10 generations, plasmid loss rates were determined by plating cells on rich and SCM-Leu plates, respectively, in triplicate. Loss rates (percent per generation) were calculated by the equation $[1 - (F/I)^{1/N}] \times 100\%$, where I is the initial percentage of plasmid-containing cells and F is the percentage of plasmid-containing cells after N generations. All loss rates were the average of three separate experiments.

Cell Synchronization, FACS Analysis, and Chromatin Binding Assay

Cell cycle blocks with α factor, HU, or nocodazole, flow cytometry, and chromatin binding assays were as previously described (Liang and Stillman, 1997; Weinreich et al., 1999).

ChIP Assay

The ChIP assay was modified from that in Aparicio et al. (1997). About 1×10^9 cells were harvested, treated with formaldehyde, and subjected to chromatin isolation using the chromatin binding assay (Liang and Stillman, 1997). The crude chromatin pellet was treated with 1 μM unit of micrococcal nuclease (Sigma) in 100 μl of reaction buffer recommended by Sigma for 5 min at 37°C, such that the majority of chromatin was digested into 1–5 nucleosomal unit length. The reaction was stopped by adding EGTA to 2 mM and spun for 10 min at 10,000 rpm in a microcentrifuge at 4°C, and the supernatant was diluted with 400 μl of the Lysis/IP Buffer (Aparicio et al., 1997) and then precipitated with the anti-HA or anti-ORC antibodies preabsorbed to protein G beads. The remaining steps were as described (Aparicio et al., 1997), except that 1 μg of sheared and purified single-stranded salmon sperm DNA instead of glycogen was used as the carrier (added just before the uncrosslinking step) for the purification of immunoprecipitated DNA. The primers for PCR were as described (Aparicio et al., 1997; Homesley et al., 2000). For ARS1 (50 pmol of each primer) and R2.5 (12.5 pmol of each primer), the PCR cycles were 1×(5′/94°C); 35×(30″/94°C, 1′/51°C, 1′/72°C); 1×(4′/72°C). For ARS305 (50 pmol of each primer) and ARS305+8kb (12.5 pmol of each primer), the PCR cycles were 1×(5′/94°C); 35×(30″/94°C, 1′/55°C, 1′/72°C); 1×(4′/72°C). PCR products were separated on 2% agarose or 6% nondenaturing PAGE gels and visualized under UV after EtBr staining.

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