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The Conditional Mutation *cdc6-1* Affects Chromosome Segregation in *Saccharomyces cerevisiae*

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Summary

CDC6 is an essential gene of *Saccharomyces cerevisiae* involved in the initiation of DNA replication. Interacting with ORC complex of proteins, Cdc6 protein has a pivotal role in loading Mcm proteins on origins of replication. Although much evidence about its function in S phase of the cell cycle is available, only a few data indicate a regulatory function of this protein in G2/M transition phase of the cell cycle. By synchronisation with a microtubule-destabilising drug nocodazole and Fluorescence Activating Cell Sorting (FACS) analysis it was possible to provide evidence that *CDC6* is responsible for a proper sequence of genetic events and accurate chromosome segregation during mitosis.

Key words: *cdc6-1*, chromosome segregation, cell cycle control, nocodazole

Introduction

Regulation of the cell division cycle is one of the most challenging issues of modern molecular biology. Although eukariotic organisms are very different regarding DNA content, chromosome number and several metabolic functions, basic principle and key molecules remain the same. Several ortologues of yeast *CDC6* gene were already found in different eukariotc organisms evolutionary so distal as yeast and humans *(1–4)* and function of the gene was extensively studied during last years. A *cdc6-1* allele of this gene was described first as temperature sensitive mutation in a collection of cell division cycle mutants *(5)*, and it was reported that this mutant remained sensitive to hydroxyurea after incubation at the restrictive temperature *(6)*. Similarly to other *cdc* mutations, *cdc6-1* exhibits very high frequency of segregation of heterozygous markers and chromosome loss after exposure to the non-permissive temperature of 36 °C *(7)*. The most studied function of the *CDC6* gene in the yeast *S. cerevisiae* is connected with the initiation of DNA replication. Several reports in literature indicated that Cdc6 physically interacts with ORC as well as with Mcm proteins and by this determines the competence of origins for DNA replication *(8–11)*.

Early indications *(5)*, inferred by double-shift experiments with hydroxyurea, suggested the possibility that *CDC6* could have another function in the cell cycle, besides replication which was described for the first time in the paper on the role of *CDC6* gene in chromosome segregation *(12)*. It was found out that the deregulation of *CDC6* gene expression causes delay in mitosis in *S. cerevisiae* as well as in *Schizosaccharomyces pombe*. The second indication about the function of *CDC6* gene in G_2-M transition came from the results of sequence similarity with the *cdc18+* gene from *S. pombe (1)*. The *cdc18-K46* mutant cells arrest at non-permissive temperature with 2C DNA content, deletion of the gene results in 1C DNA content and, in some cells, premature chromosome segregation, results in less than 1C DNA.

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By fluorescence *in situ* hybridisation (FISH) techniques, it was demonstrated that cells deprived of Cdc6 protein underwent reductional anaphase with intact chromosomes *(13)* and that the termosensitive mutation *cdc6-1* induced chromosome rearrangements *(14)*.

In this paper it is shown that after release from G_2 arrest caused by nocodazole, *cdc6-1* mutant cells not only omit replication at restrictive temperature but also almost at the same time when the control population replicates their DNA, mutant cells segregate, indicating that *CDC6* is also a cell cycle regulator.

Materials and Methods

Genotypes of *Saccharomyces cerevisiae* strains:

N349–3B *a, cdc6, his4, ura1, MAL2, gal1*;
A364A *a, ade1, ade2, ura1, tyr1, his7, lys2, gal1*.

Growth conditions: yeast cells were grown aerobically in standard YPD medium (2 % glucose, 2 % Bacto peptone, 1% Bacto yeast extract) at 23 °C as the permissive temperature and at 36 °C as the restrictive temperature. Nocodazole was added to the liquid medium containing 1 % DMSO to the final concentration of 15 μg mL^{-1}.

FACS analysis

Cells for FACS analysis were prepared following previously described procedure (*15*). Samples were taken from liquid medium every 30 min, centrifuged and fixed at least overnight in cold 70 % ethanol. Prior to FACS analysis, cells were washed with TBS (50 mM TRIS/HCl pH = 7.5, 150 mM NaCl), briefly sonicated and incubated in RNAse A (0.5 mg mL^{-1} in TBS) for 2 h at 36 °C. Cells were diluted to 10^6 mL^{-1} and stained with propidium iodide (100 μg mL^{-1}) for 20 min in the dark. Fifteen thousand cells per sample were analysed with a Becton Dickinson Cell Sorter.

Results

To determine the period in the cell cycle when *CDC6* function is vital, the cells were synchronised in G_2 phase by the microtubule-destabilising drug nocodazole. Over 80 % of synchronisation was regularly obtained after 3 h of incubation at 23 °C in YPD. After the drug was removed by centrifugation, cells were resuspended in pre-warmed YPD at 23 °C and 36 °C, respectively.

Nocodazole-synchronised cells were tested for viability by plating them on YPD. Several repetitions of the experiment were done and the results were obtained by normalising the number of colonies of each plate with the number of colonies on plate at the time zero.

The difference between active and inactive cdc6 gene product became obvious in samples withdrawn 60 min after release from nocodazole block. Rapid decrease of the mutant strain viability was observed at non-permissive temperature, while the wild type strain at the same temperature and the mutant strain at permissive temperature did not show substantial decrease in viability (Fig. 1).

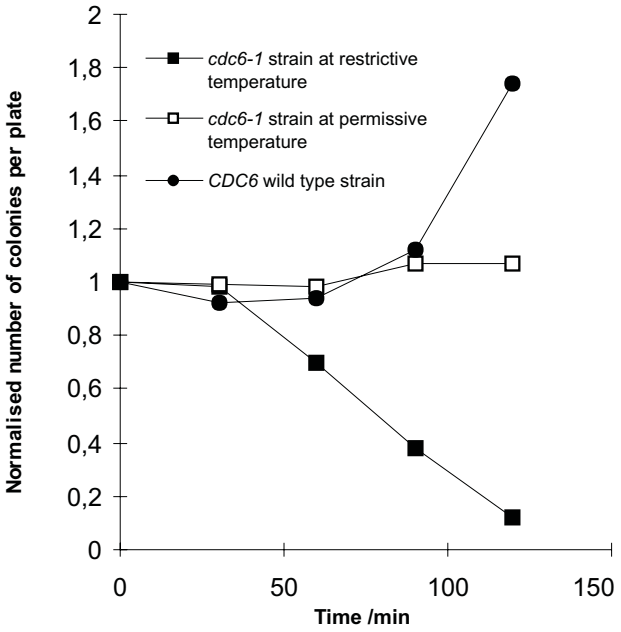

Fig. 1. Viability of nocodazole synchronised cell cultures. After nocodazole synchronisation, cells were resuspended in YPD pre-warmed to 23 °C and 36 °C. Every 30 min, samples were taken and diluted in sterile H_2O by 10^3. Each sample was plated onto 3 plates and the average number of colonies per plate was normalised by an average number of colonies at the time 0 after nocodazole block. Several experiments were pooled by this method and the average values of these experiments are presented

Yeast cells were also monitored by microscope. Nocodazole-arrested cells have a dumbbell phenotype of large budded cells. In the population of cells released from nocodazole at 23 °C, the first changes in cell type distribution appeared after 60 min of incubation, and after 90 min 15 % of the population formed buds. The budding phenotype, consisting of large budded cells with 1 or 2 additional buds and small budded cells, became predominant after 120 min of incubation (Fig. 2A).

Nocodazole-synchronised cells released to the restrictive temperature segregated immediately and after 60 min about 35 % of the population formed small buds. After 120 min *cdc6–1* dumbbell shape terminal phenotype represented the majority of the mutant cell population and continued to accumulate with time (Fig. 2B).

The DNA content of mutant cells was monitored by FACS (Fig. 3). At the permissive temperature, the population of cells released from nocodazole increased the DNA content after 60 min of incubation corresponding to the time of the first changes in cell type distribution and started the segregation process after 120 min (Fig. 3B). At the restrictive temperature, the population of cells released from nocodazole, showed an opposite behaviour (Fig. 3A). Approximately at the same time, when the control population at the permissive temperature increased its DNA content, the population at non-permissive temperature segregated its DNA in a faulty manner causing a rapid decrease of viability.

The FACS data are the opposite of the data of cell type distribution since the population of small budded

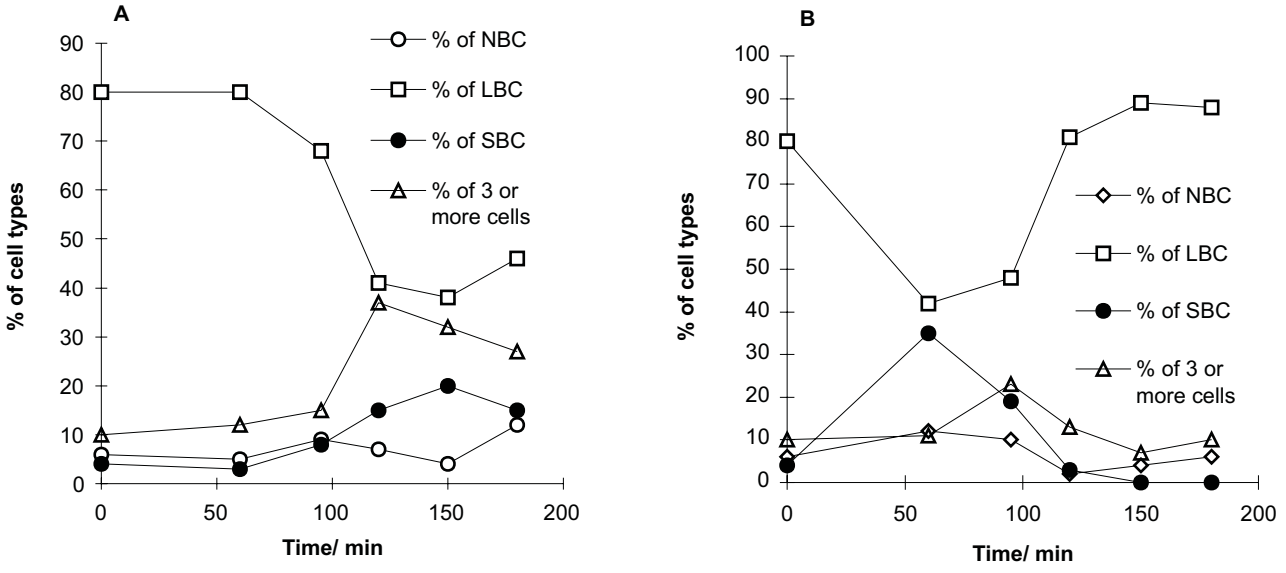

Fig. 2. Distribution of cell phenotypes after nocodazole synchronisation at permissive and restrictive temperature. Cells were synchronised in liquid YPD containing nocodazole. After centrifugation, cells were resuspended in YPD pre-warmed to 23 °C and 36 °C. Every 30 min samples were withdrawn, fixed with ice-cold NaN_3 and cells were counted by heamocytometer. **A)** Culture of nocodazole synchronised cells released to C 23 °C. **B)** Culture of nocodazole synchronised cells released to 36 °C. Legend: SBC – small budded cells, LBC – large budded cells, NBC – non-budded cells

cell takes place of large budded cells after 60 min of incubation at restrictive temperature. From phenotypic point of view, increasing DNA content would be expected. Since *cdc6-1* mutant at restrictive temperature cannot make DNA, any changes in DNA content would be expected regarding the control population at permissive temperature. Besides the loss of viability at the time of DNA replication, the loss of feedback control is also demonstrated since no cell cycle arrest at the phenotype level could be observed.

Discussion

The key mechanism of the cell cycle control is the cyclin dependent kinase activity that oscillates throughout the cell cycle (*16*). Several authors showed that Ccd6 protein is the target of Cdc28 kinase complex (*17–19*). Transcription of the gene starts already in M phase of the cell cycle but high kinase activity at this period direct the protein to be destructed by anaphase promoting complex (*20*). When kinase activity is low, Cdc6 protein is stabilised and thus pre-replicative complex can be formed. Since thermosensitivity of *cdc6-1* mutation prevents the protein from forming pre-replicative complex the replication is blocked (Fig. 3), but other functions such as budding (Fig. 2) and even segregation (Fig. 3) are not restrained. In nocodazole arrested, G_2 phase cells the kinase activity is high (*13*) and *CDC6* gene is transcribed (*21*). When cells are shifted to 23 °C, DNA replication occurred within an hour indicating that kinase activity dropped, pre-replicative complex was formed and replication was initiated. At 36 °C, inactive Cdc6 was synthesised but pre-replicative complex was not formed and cells segregated without DNA replication. Although the DNA content is 2C, the segregation is faulty leading to extensive cell death (Fig. 1). To find

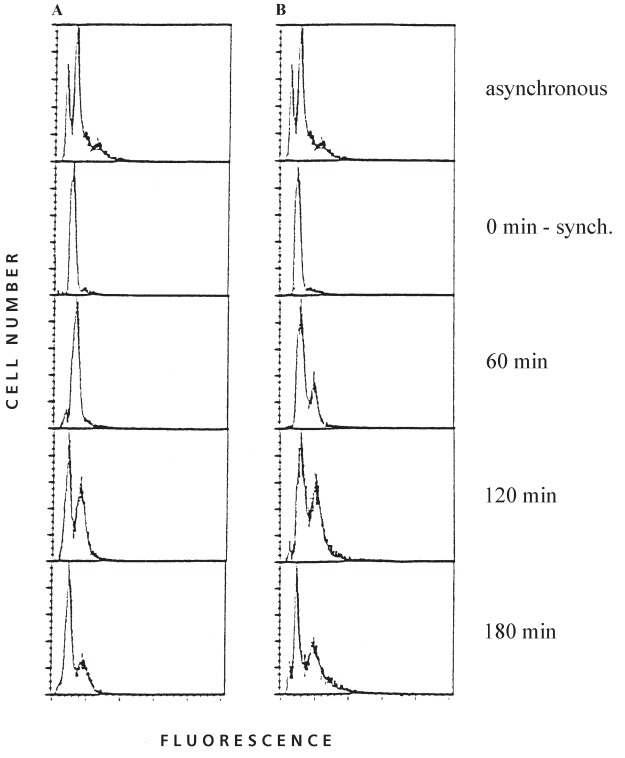

Fig. 3. FACS analysis of DNA content. After nocodazole synchronisation, cells were resuspended in YPD pre-warmed to 23 °C and 36 °C. Every 30 min, samples were centrifuged and fixed overnight in cold ethanol. Cells were stained with propidium iodide and analysed with a Becton Dickinson Cell Sorter
A) Culture of s nocodazole synchronised cells released to C 23 °C.
B) Culture of nocodazole synchronised cells released to 36 °C

proteins involved in chromosome segregation interacting with Cdc6, the protein was used as bait in two-hybrid reaction (*20*,*22*). No such protein was found so far which could also be due to the fact that Cdc6 protein is highly unstable.

Loss of replication ability also skips the function of checkpoint genes, which are triggered by the appearance of free DNA ends (*23*). Budding of cells at non-permissive temperature indicates that phosphorylation and dephosphorylation of other cyclin/kinase dependent targets is not affected. It seems that cells perceive no mistake in the system so they proceed the cell cycle, as DNA would be replicated.

At the end, it can be concluded that abolishing Cdc6 function uncouples DNA replication from other cell cycle functions and that although extensively degraded in M/G2 phase of the cell cycle, Cdc6 protein is involved in proper chromosome segregation.

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Uvjetna mutacija *cdc6-1* utječe na segregaciju kromosoma u *Saccharomyces cerevisiae*

Sažetak

CDC6 jedan je od bitnih gena kvasca *Saccharomyces cerevisiae* koji sudjeluje u procesu započinjanja replikacije DNA. U interakciji s ORC proteinskim kompleksom, Cdc6 protein ima bitnu ulogu u gomilanju Mcm proteina na ishodišta replikacije. Iako ima mnogo podataka o njegovoj funkciji u S-fazi staničnog ciklusa, samo neki podaci upućuju na regulacijsku funkciju tog proteina u G2/M tranzicijskoj fazi staničnoga ciklusa. Istodobnom primjenom nocodazola, lijeka koji destabilizira mikrotubule, te analizom razvrstavanja fluorescentno aktiviranih stanica (FACS) bilo je moguće dokazati da je *CDC6* odgovoran za ispravan slijed genetičkih procesa i za besprijekornu segregaciju kromosoma tijekom mitoze.