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ORIGINAL PAPER

Giant yeast cells with nonrecyclable ribonucleotide reductase

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Abstract Ribonucleotide reductase (RNR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides and thereby provides the precursors required for DNA synthesis and repair. In an attempt to test cell resistance to a permanent replicational stress, we constructed a mutant *Saccharomyces cerevisiae* strain containing exclusively nonrecyclable catalytic subunits of RNR that become inactivated following the reduction of one ribonucleoside diphosphate. In this *rnr1C883A rnr3Δ* mutant, the synthesis of each deoxyribonucleotide thus requires the production of one Rnr1C883A protein, which means that 26 million Rnr1C883A proteins (half the protein complement of a wild-type cell) have to be produced during each cell cycle. *rnr1C883A rnr3Δ* cells grow under constant replicational stress, as evidenced by the constitutive activation of the checkpoint effector Rad53, and their S phase is considerably extended compared to the wild type. *rnr1C883A rnr3Δ* mutants also display additional abnormalities such as a median cell volume increased by a factor of 8, and the presence of massive inclusion bodies. However, they exhibit a good plating efficiency and can be propagated indefinitely. *rnr1C883A rnr3Δ* cells, which can be used as a protein overexpression system, thus illustrate the robustness of *S. cerevisiae* to multiple physiological parameters.

Keywords Ribonucleotide reductase · *Saccharomyces cerevisiae* · DNA replication · DNA checkpoints · Protein overexpression

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Introduction

S phase is a period of great vulnerability for the genome of eukaryotic cells, due to several factors. First, chromosomes have to be completely unwound and duplicated. This process implies the formation of single-stranded DNA, which is chemically less stable than double-stranded DNA (Lindahl 1993). Second, replication forks can be blocked because of a depletion of deoxyribonucleoside triphosphates (dNTPs) or because of various impediments including DNA lesions, transcription complexes and replication slow zones in *Saccharomyces cerevisiae* (Mirkin and Mirkin 2007). Stalled forks have to be stabilized and restarted to prevent their collapse and the accumulation of DNA structures potentially leading to illegitimate recombination events and chromosome rearrangements (Branzei and Foiani 2007; Tourriere and Pasero 2007). Third, the logic of replication control makes eukaryotic cells especially sensitive to stresses able to erase the memory of cell cycle position during S phase (Nasmyth 1999). Indeed, eukaryotic genomes are replicated from large numbers of replication origins, the activity of which must be tightly coordinated. A two-step mechanism ensures that no origin can fire more than once in a cell cycle: (1) the assembly of

prereplicative complexes (pre-RCs) at origins only occurs when cyclin-dependent kinase (Cdk) activity is low, and (2) origin firing, in contrast, can only occur when Cdks become active (Diffley 2004). Any temporary inactivation of the Cdks in S phase could lead to the assembly of pre-RCs and thereby to a partial, most probably lethal, rereplication of the genome. This could explain the observation that quiescent cells never arrest in the S phase and that the passage through the START or restriction points irreversibly commits the cells to the completion of the cycle up to exit, and entry into a new G_1 phase.

Genomic stresses affecting specifically the S phase can be generated by inhibiting the DNA polymerases with drugs such as aphidicolin or by inducing a dNTP depletion, which causes fork stalling. Cell responses to acute replicational stresses inducing both an increase in fork stalling and an extension of S-phase length are usually observed during a brief period, shorter than a generation time. Here, we have sought to evaluate cell resistance over generations to a constitutive replication stress by mutating two genes encoding the catalytic subunits of ribonucleotide reductase (RNR) in *S. cerevisiae*. Ribonucleotide reductase [for reviews, see (Eklund et al. 2001; Kolberg et al. 2004)] reduces ribonucleoside diphosphates (NDPs) into deoxyribonucleoside diphosphates, thus catalyzing an essential step in the production of dNTPs required for DNA synthesis. In *S. cerevisiae*, RNR is a tetrameric enzyme composed of two dimeric subunits, R1 and R2. The catalytic R1 subunits, which hold the substrate-binding active site, are encoded by two homologous genes, *RNR1* and *RNR3*, but the large majority of R1 subunits are Rnr1/Rnr1 homodimers (Elledge and Davis 1990; Domkin et al. 2002). The R2 subunit is an Rnr2/Rnr4 heterodimer. The reduction of one NDP by Rnr1 or Rnr3 is balanced by the formation of a disulfide bond between two cysteines of the active site (C218 and C443 in Rnr1), which inactivates the enzyme. The catalytic capacity of Rnr1 or Rnr3 is regenerated by the reduction of this disulfide bond by two C-terminal cysteines (C883 and C886 in Rnr1), which are ultimately reduced by thioredoxins or glutaredoxins (Mao et al. 1989, 1992; Camier et al. 2007).

We managed to construct an *rnr1C883A rnr3Δ* mutant strain containing nonrecyclable Rnr1C883A proteins as the only RNR catalytic subunits. *rnr1C883A rnr3Δ* cells grow under constant replicational stress and their S phase is largely extended compared to the wild type. However, they show a good plating efficiency and can be propagated indefinitely, thus demonstrating the tolerance of yeast cells to constitutive replication stress. *rnr1C883A rnr3Δ* mutants also exhibit other abnormalities such as a largely increased cell volume and the production of massive amounts of Rnr1C883A proteins, which accumulate in inclusion bodies. The viability of *rnr1C883A rnr3Δ* cells illustrates the robustness of yeast cells to multiple physiological parameters.

Materials and methods

Plasmids, strains and media

The pBAD54 (2μ, *TRP1*), pRS316/RNR1 (CEN, *URA3*, *pRNR1-HA-RNR1*) and pSC2/RNR1 (2μ, *TRP1*, *pRNR1-HA-RNR1*) plasmids have been described (Camier et al. 2007). The Quick-Change Site-Directed Mutagenesis Kit (Stratagene) was used to give the pSC2/*rnr1C883A* vector. The *pRNR1-HA-rnr1C883A* construct, harbored by the 4.7 kb *Bam*HI-*Xma*I fragment of the pSC2/*rnr1C883A* plasmid, was inserted into the pRS424 GAL1 (2μ, *TRP1*, *pGAL1*) vector (Mumberg et al. 1994) digested with *Bam*HI and *Xma*I to give the pRS424/*rnr1C883A* plasmid (we used the pRS424 GAL1 plasmid with the idea that the *GAL1* promoter could further increase the transcription of the *rnr1C883A* allele if cells were grown on galactose, but this possibility has remained unexplored so far). The pSC2/*rnr1C883A* and pRS424/*rnr1C883A* plasmids gave similar results in all experiments.

To get the pRS424/*rnr1C883A*-green fluorescent protein (GFP) construct, the sequence encoding the *Aequorea victoria* GFP was amplified by PCR using the pFA6a-GFP(S65T)-kanMX6 vector (Wach et al. 1997) as a template and primers that were designed to allow the subsequent recombination of this sequence with the *rnr1C883A* gene, while destroying *rnr1C883A* stop codon. The PCR product was introduced into YPH499 cells along with the *Not*I-digested pRS424/*rnr1C883A* vector (a unique *Not*I site is located just after the stop codon of the *rnr1C883A* gene). pRS424/*rnr1C883A-GFP* constructs resulting from in vivo recombination between the pRS424/*rnr1C883A* plasmid and the GFP sequence were recovered from [Trp^+] transformants. Additional details and primer sequences are available on request.

Strains used in this study are listed in Table 1. With the exception of MCM869, all yeast strains used in this study were isogenic to MCM185, a YPH499 derivative in which the *BAR1* gene had been inactivated by the insertion of a *LEU2* cassette (Sikorski and Hieter 1989). The *RNR1* and *RNR3* genes were disrupted by PCR targeting using the kanMX cassette and the *Schizosaccharomyces pombe his5*$^+$ gene (Wach et al. 1997), respectively, in MCM185 transformants containing pRS316/RNR1, to give MCM720 cells. The *rnr1C883A rnr3Δ* and *rnr1C883A-GFP rnr3Δ* strains (MCM824 and MCM845, respectively) were constructed by first introducing the pSC2/*rnr1C883A* or pRS424/*rnr1C883A-GFP* plasmids into MCM720. Transformants were streaked on 5-fluoroorotic acid (5-FOA)-containing

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
YPH499	*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*	(Sikorski and Hieter 1989)
MCM185	as YPH499 *bar1Δ::LEU2*	(Leroy et al. 2001)
MCM720	as MCM185 *rnr1Δ::kanMX rnr3Δ::his5^{+}*+ pRS316/*RNR1*	This study
MCM824	as MCM185 *rnr1Δ::kanMX rnr3Δ::his5^{+}*+ pSC2/*rnr1C883A*	This study
MCM845	as MCM185 *rnr1Δ::kanMX rnr3Δ::his5^{+}*+ pRS424/*rnr1C883A-GFP*	This study
MCM934	as YPH499 *bar1Δ::hphMX rnr1Δ::kanMX rnr3Δ::his5^{+} leu2-Δ1::p405-BrdU-Inc* + pSC2/*rnr1C883A*	This study
MCM869	*MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 URA3::GPD-TK$_{7x}$ AUR1c::ADH-hENT1 bar1Δ::LEU2 cdc21Δ::kanMX*	This study

plates (5-FOA is toxic to *URA3* cells) to isolate 5-FOA-resistant cells that had lost pRS316/*RNR1*. The MCM934 strain was constructed by using the p405-BrdU-Inc plasmid [a kind gift from Viggiani and Aparicio (2006)] to introduce a BrdU incorporation cassette (encoding the *Herpes simplex* virus thymidine kinase and a human nucleoside transporter). MCM869 is a derivative of the E1000 strain (Lengronne et al. 2001) in which the *CDC21* gene (encoding thymidylate synthase) was disrupted to increase BrdU incorporation (Vernis et al. 2003) and into which a construct coding for an additional nucleoside transporter was inserted.

Unless indicated otherwise, cells were grown in YPD medium (1% yeast extract, 2% bacto-peptone, 2% glucose). Synthetic media SM and SMCA correspond to the synthetic minimal medium described in (Sherman 1991), supplemented with the bases and amino acids corresponding to the strains' auxotrophic markers, and with casamino acids (2%), respectively.

Detection and quantification of BrdU incorporation

Equal amounts of genomic DNA (100 ng) were denatured in 0.4 M NaOH, spotted onto a nitrocellulose membrane and UV-crosslinked. Immunodetection was performed using a monoclonal antibody against BrdU (MD5300, Caltag Laboratories) and a secondary IgG coupled to horseradish peroxidase (W402B, Promega). Signals were detected by electrochemiluminescence (ECL kit, Amersham) and quantified with the ImageJ software. Serial dilutions of BrdU-marked DNA preparation were systematically performed so as to check the linearity of the signal. The fraction of replicated DNA y was deduced from the fraction of incorporated BrdU x (normalized by the maximal amount of incorporated BrdU) using the equation: $y = x/(2 - x)$.

Electron microscopy

Yeast cells were fixed in 1% glutaraldehyde, washed and embedded either in Epon for ultrastructural studies (Fig. 4a–c) or in Unicryl for immunogold labeling (Fig. 4d–e) as described previously (Ni et al. 2006). For immunogold labeling, 90 nm-thick sections were labeled with purified anti-GFP antibodies (Ab290, Abcam) followed by 10-nm protein A-gold, and stained in uranyl-acetate and lead citrate as described (Ni et al. 2006).

Quantification of plasmid copy number

About 30 ng of DNA was digested with *Eco*RV, separated on a 0.8% agarose gel for 15 h at 70 volts and the resulting Southern blot was hybridized with a probe corresponding to the *Eco*RV-*Xcm*I fragment of the *TRP1* gene, which is present both at the *TRP1* chromosomal locus and as a marker in the pSC2/*rnr1C883A* plasmid. The signal corresponding to the pSC2/*rnr1C883A* plasmid was normalized using the signal corresponding to the *TRP1* locus. As a control, we found a normalized copy number close to 1 for the centromeric vector pRS314 (data not shown).

Quantification of *rnr1C883A* ARNm by RT-PCR

Reactions were performed with the Eurogentec kit MESA GREEN qPCR MasterMix Plus for SYBR Assay. For reverse transcription reactions, 200 ng of RNAs was used, and 0.5 ng of cDNAs was used for quantitative PCR. Signals corresponding to the *RNR1* and *rnr1C883A* mRNAs were normalized using signals corresponding to the actin *ACT1* mRNAs.

Determination of cell volume by FACS analysis

FACS analyses were performed with a FACScalibur flow cytometer (Becton–Dickinson) over 1,024 channels by using red laser radiation (635 nm). The relation between the channels and the volume v (in nm) was measured by van Gaal et al. (2010) using a sampling over 256 channels. In our case, the signal was sampled over 1,024 channels

and recorded in a logarithmic scale. Their equation was therefore modified to give:

$$v = 2,048\left(18.39 + 52.36\left(10^{\frac{channel}{256}}\right)^{0.4482}\right).$$

Other techniques

Analysis of Rad53 phosphorylation was performed as described previously (Marsolier et al. 2000). The in situ Rad53 autophosphorylation assay was carried out as described (Pellicioli et al. 1999).

Results

rnr1C883A rnr3Δ cells exhibit an extended S phase

In an attempt to analyze the resistance of *S. cerevisiae* cells to a constitutive replicational stress, we sought to construct a nonrecyclable version of Rnr1 by preventing the reduction of the catalytic cysteines. This was done with the idea that dNTP synthesis in cells containing only nonrecyclable forms of R1 would be strongly slowed down, which would cause a dNTP depletion during the S phase. The carboxyl end of Rnr1 is supposed to act as a flexible shuttle of reducing equivalents from the surface of Rnr1 to the active site, which suggests that the regenerating step of Rnr1 catalytic cycle can be easily disrupted without affecting NDP reduction, by mutating one of the C-terminal cysteines, C883 or C886. We had shown, indeed, that in vivo, a large part of mutant Rnr1C883A proteins (whose cysteine C883 is mutated into alanine) contains a disulfide bond between the two cysteines of the catalytic site (Camier et al. 2007). This result indicates that an Rnr1C883A protein becomes definitively inactivated by the reduction of one NDP, which acts like a suicide substrate.

We constructed a mutant *rnr1C883A rnr3Δ S. cerevisiae* strain by disrupting *RNR1* and *RNR3* chromosomal alleles and introducing the multicopy plasmid pSC2/*rnr1C883A* harboring the *rnr1C883A* allele under the control of its own promoter. In this strain, the nonrecyclable Rnr1C883A proteins are the only catalytic subunits of RNR. The *rnr1C883A rnr3Δ* mutants grew slowly, indicating either low viability or extended generation time.

We first characterized the replicational stress experienced by *rnr1C883A rnr3Δ* cells by analyzing S-phase progression. Maybe due to the heterogeneity of the cell sizes, FACS data for *rnr1C883A rnr3Δ* mutants proved difficult to interpret and did not allow us to extract any quantitative information about S-phase length (see supplementary data). We resorted therefore to the analysis of BrdU incorporation. A BrdU-Inc cassette [which allows efficient cellular uptake and incorporation of thymidine analogs into DNA (Viggiani and Aparicio 2006)] was introduced in *rnr1C883A rnr3Δ* mutants, and DNA replication was monitored after release from α-factor arrest in G_1. Incorporation of BrdU was strongly slowed down in *rnr1C883A rnr3Δ* mutants compared to the control (Fig. 1): the bulk of DNA replication took place between ~120 min and ~400 min after α-factor release in *rnr1C883A rnr3Δ* cells, and between 45 and 120 min after α-factor release in the control. The S phase was thus considerably extended in *rnr1C883A rnr3Δ* mutants, indicating a major stress.

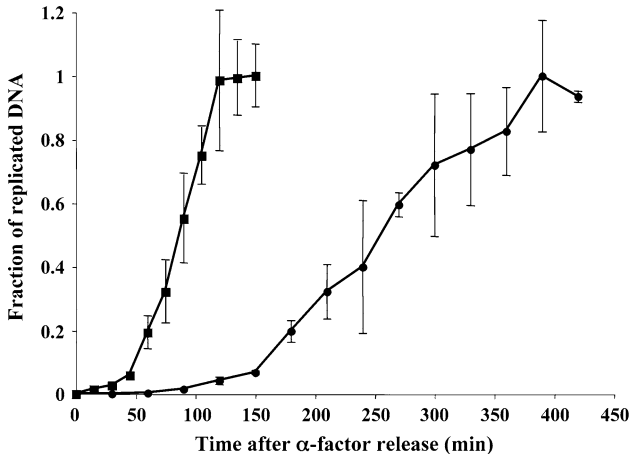

Fig. 1 DNA replication is slowed down in *rnr1C883A rnr3Δ* mutants. Exponentially growing *rnr1C883A rnr3Δ* mutants (MCM934) and control cells (MCM869) were synchronized in G_1 with α-factor (0.5 μM final concentration) for 5.5 h (MCM934) or 2 h (MCM869), washed twice and released into fresh synthetic medium (SMCA) containing 400 μg/ml BrdU. When the majority of the cells had rebudded after release, α-factor was added back to the culture to prevent another replication cycle. Aliquots were collected at various times after release from the α-factor block, and BrdU incorporation in genomic DNA was analyzed by immunodetection. The fraction of replicated DNA is plotted as a function of time for *rnr1C883A rnr3Δ* mutants (MCM934, circles) and for control cells (MCM869, squares). The figure represents the average values obtained using a 3-point Fourier smoothing ± the error corresponding to the deviation of the experimental points

Low, constitutive activation of the replication checkpoint in *rnr1C883A rnr3Δ* mutants

Replication stress can also be assessed via the activation of the S-phase checkpoint, a signal transduction pathway triggered by perturbations of DNA replication resulting from the presence of DNA lesions, defective replication proteins or dNTP depletion (Osborn et al. 2002; Longhese et al. 2003). dNTP depletion induced by treatment with hydroxyurea [HU, a potent inhibitor of RNR that quenches R2 radical (Ehrenberg and Reichard 1972)] has been intensively studied. In the presence of HU, the activation of the S-phase checkpoint, which can be readily monitored by the phosphorylation of Rad53 (one of its key components),

prevents the irreversible dissociation of the replication machinery from DNA, restrains the activity of recombination enzymes at stalled forks, inhibits the elongation of the spindle and delays the firing of replication origins (Branzei and Foiani 2005, 2006).

We first observed that, in contrast to wild-type cells, asynchronously growing *rnr1C883A rnr3Δ* mutants exhibit a slight phosphorylation of Rad53 (data not shown). We analyzed more precisely the activation of the S-phase checkpoint in *rnr1C883A rnr3Δ* mutants in comparison with that induced by a treatment with HU in wild-type cells. *rnr1C883A rnr3Δ* and wild-type cells were synchronized in G_1 with α-factor and released into S phase in the presence (wild-type cells) or absence of 20 mM HU (*rnr1C883A rnr3Δ* mutants). Rad53 appeared slightly phosphorylated (Fig. 2) in *rnr1C883A rnr3Δ* α-factor-arrested cells and exhibited a stronger autophosphorylating activity than in the wild type (Fig. 2). This observation was surprising as *rnr1C883A rnr3Δ* α-factor-arrested cells are supposedly devoid of stalled replication forks and of the DNA damage they can potentially induce. However, given their long generation time and their impaired replication, it cannot be ruled out that a proportion of *rnr1C883A rnr3Δ* cells, even after a 5.5-h treatment with α-factor, could still be blocked in late S phase with compromised replication forks or in G_2 with unrepaired DNA damage. Release of wild-type cells into HU-containing medium induced Rad53 phosphorylation, and Rad53 autophosphorylating activity increased by a factor of 40–60. Rad53 autophosphorylating activity also increased in *rnr1C883A rnr3Δ* cells released from the α-factor block, but only by a factor of ~2.5 (Fig. 2). During the S phase, Rad53 phosphorylation and autophosphorylating activity of HU-treated wild-type cells were higher than those of *rnr1C883A rnr3Δ* cells, although the S phase of *rnr1C883A rnr3Δ* cells was about four times longer than that of the HU-treated wild-type cells (the length of which was not significantly affected compared to untreated wild-type cells).

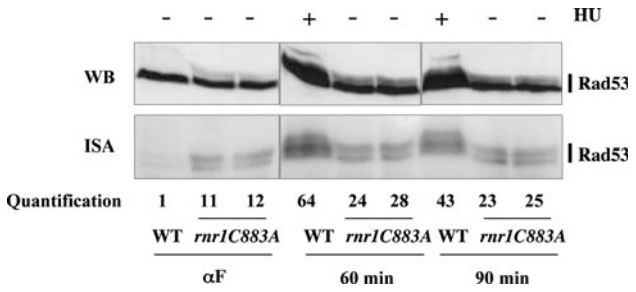

Fig. 2 Analysis of Rad53 phosphorylation state and autophosphorylating activity in *rnr1C883A rnr3Δ* mutants and in HU-treated wild-type cells. Exponentially growing *rnr1C883A rnr3Δ* mutants (*rnr1C883A*, MCM824, in duplicate) and wild-type cells (WT, MCM185) were synchronized in G_1 phase with α-factor (αF, 0.5 μM final concentration) for 5.5 h (*rnr1C883A rnr3Δ*) or for 2 h (wild type), washed twice, released into fresh YPD medium in the presence (+) or absence (−) of HU (20 mM) and collected 60 and 90 min after release. Whole-cell extracts were then analyzed by Western blotting (*WB*), and Rad53 autophosphorylation activity was assessed by an in situ renaturation assay (*ISA*). The relative quantifications indicated below the blots correspond to the amounts of radioactivity (indicative of Rad53 activity) that were quantified using a PhosphorImager system and the ImageQuant software and normalized after background correction by the amount of radioactivity detected for α-factor-blocked wild-type cells

We conclude from these two analyses that *rnr1C883A rnr3Δ* cells are subjected to a strong replication stress, although the activation of Rad53 remains limited. However, in spite of their constitutive replicational stress and extended S phase, *rnr1C883A rnr3Δ* mutants (MCM824) grew robustly in rich medium (YPD), with an apparent generation time (determined in triplicate as the time required for a doubling of the optical density of cell cultures at 660 nm) of 6.2 ± 0.4 h compared to 1.5 ± 0.1 h for the wild-type cells (MCM185). The plating efficiency of *rnr1C883A rnr3Δ* mutants (percentage of cells forming viable colonies, determined in triplicate for three clones) was 74 ± 10 in YPD, 71 ± 8 in synthetic minimum medium supplemented with casaminoacids (SMCA) and 55 ± 7 in synthetic minimum medium supplemented with the amino acids and bases required by the strain auxotrophic markers (SM), compared to 100 for the wild type (MCM185) in all cases. These results illustrate the robustness of *rnr1C883A rnr3Δ* cells to a severe, permanent replication defect, which is not their only anomaly, as detailed below.

The median volume of *rnr1C883A rnr3Δ* cells is increased by a factor of 8 compared to the wild type

With a major defect specific to the S phase, *rnr1C883A rnr3Δ* cells are comparable to *cdc* mutants. These mutants, which contain defective components of the cell division cycle machinery and are blocked at particular phases at restrictive temperature, often die after attaining a large size, due to the decoupling between the cell cycle and cell growth which goes on unabated even when the cells are completely blocked (Hartwell et al. 1973, 1974). However, to our knowledge, no systematic study has so far analyzed the volumes of *cdc* mutants growing under conditions allowing their proliferation for a large number of generations. The systematic search for strains with altered size using either a library of 4,812 haploid deletion strains or a library of 5958 diploid deletion strains (including 1,166 heterozygous strains for essential genes) yielded mutants with at most a 2.4-fold increase in median cell volume compared to the wild type (Jorgensen et al. 2002; Zhang et al. 2002). We found that the median cell volume of *rnr1C883A rnr3Δ* mutants was higher by a factor of 8

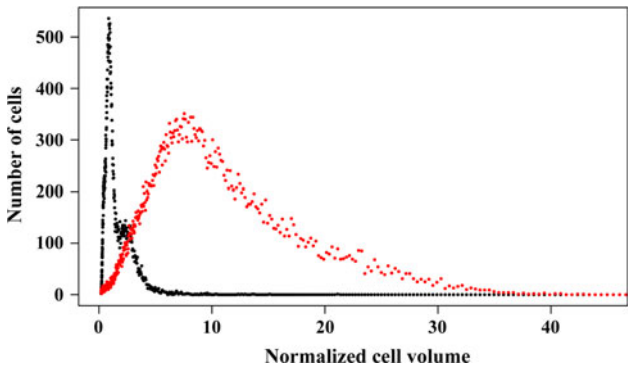

Fig. 3 Distribution of the volumes of wild-type and *rnr1C883A rnr3Δ* cells. The volumes of exponentially growing cells ($N \sim 50{,}000$) were determined by FACS analysis and normalized by the median volume of wild-type cells (42 fL). The *black points* correspond to wild-type cells (MCM185) and the *red points* to the *rnr1C883A rnr3Δ* mutants (MCM824) in the online version. Direct measurements of cell dimensions ($N \sim 200$) made on pictures taken with a Zeiss microscope gave comparable results (data not shown)

compared to the wild type (Fig. 3). We also observed ~5% of giant *rnr1C883A rnr3Δ* cells with volumes 20 times larger than the median cell volume of the wild type (this proportion of giant cells is 2×10^{-5} for the wild type). For microorganisms, cell size is an important physiological parameter which is held constant in a given environment by a tight coordination between cell division and cell growth. We therefore investigated the potential consequences of such a size extension on the cell structure by electron microscopy.

Rnr1C883A proteins accumulate in massive inclusion bodies

Electron microscopy revealed that *rnr1C883A rnr3Δ* cells are similar to the wild type, except for their size and for the presence of agranular, massive bodies, morphologically distinct from the cytoplasm and not bounded by a membrane (Fig. 4a–c). These structures, named inclusion bodies, have been described in eukaryotic and prokaryotic cells overproducing proteins [see (Cousens et al. 1987; Binder et al. 1991) for examples in yeast]. *rnr1C883A rnr3Δ* cells have to synthesize de novo one Rnr1C883A protein for each dNTP produced, which means that they synthesize at least 26 million Rnr1C883A proteins each time they replicate their nuclear DNA. Given that exponentially growing yeast cells contain on average about 50 million proteins (Ghaemmaghami et al. 2003), Rnr1C883A proteins should represent an important part of the proteins synthesized in *rnr1C883A rnr3Δ* mutants. If they are not rapidly degraded, they can accumulate in inclusion bodies. Indeed, using electron microscopy immunogold labeling, we found that the inclusion bodies of *rnr1C883A-GFP rnr3Δ* cells (expressing a GFP-tagged variant of Rnr1C883A) contained Rnr1C883A-GPF proteins (Fig. 4d–e). These observations were corroborated by images of fluorescence microscopy showing that Rnr1C883A-GFP fluorescence was concentrated in massive intracellular patches consistent with inclusion bodies. The accumulation of Rnr1C883A proteins in inclusion bodies probably strongly contributes to the marked increase in cell size of *rnr1C883A rnr3Δ* mutants compared to the wild type (Fig. 4a, b, d vs. Fig. 4c, e and Fig. 4f vs. Fig. 4h). The inclusion bodies were found to fill in large parts of the internal volumes of mother cells, but could not be detected in the buds (Fig. 4g).

Rnr1C883A proteins represent on average half the protein amount of *rnr1C883A rnr3Δ* cells

SDS-PAGE electrophoresis followed by Coomassie staining or Western blotting confirmed that *rnr1C883A rnr3Δ* mutants contained large amounts of Rnr1C883A (Fig. 5). Comparison with serial dilutions of bovine serum albumin (BSA) showed that Rnr1C883A represented approximately half the total amount of proteins in *rnr1C883A rnr3Δ* cells (about 10 μg out of the 20 μg protein separated on the gel). This figure corresponds to an average value, since Rnr1C883A proteins are absent in buds and G_1 cells and also since we can suppose that the amount of Rnr1C883A proteins increases in mother cells after each replication cycle. Finally, we investigated the mechanisms allowing the massive production of Rnr1C883A proteins.

Analysis of the amplification steps in the production of Rnr1C883A proteins

With ~300,000 copies/cell, Rnr1 is one of the 25 most abundant proteins in exponentially growing yeast cells, as determined by Ghaemmaghami et al. (2003). Neglecting Rnr1 degradation, and considering that wild-type cells produce 300,000 Rnr1 proteins per cell cycle, the production of ~26 million Rnr1C883A proteins per cell cycle corresponds to an increase by a factor of ~90. To understand the main steps allowing this increase, we analyzed the copy number of the *rnr1C883A* allele and the corresponding mRNAs.

The copy number of the pSC2/*rnr1C883A* plasmid was determined by Southern blotting. We found that *rnr1Δ rnr3Δ* cells complemented with pSC2/*rnr1C883A* (the *rnr1C883A rnr3Δ* mutants) contained on average 21 ± 3 plasmid copies, whereas *rnr1Δ rnr3Δ* cells complemented with pSC2/*RNR1* (the same vector harboring the wild-type *RNR1* allele) contained only 4 ± 1 plasmid copies. The accumulation of a high number of plasmids containing a defective gene has already been observed for 2 μ-derived vectors harboring the *leu2-d* allele (Erhart and Hollenberg 1983). In that case, the

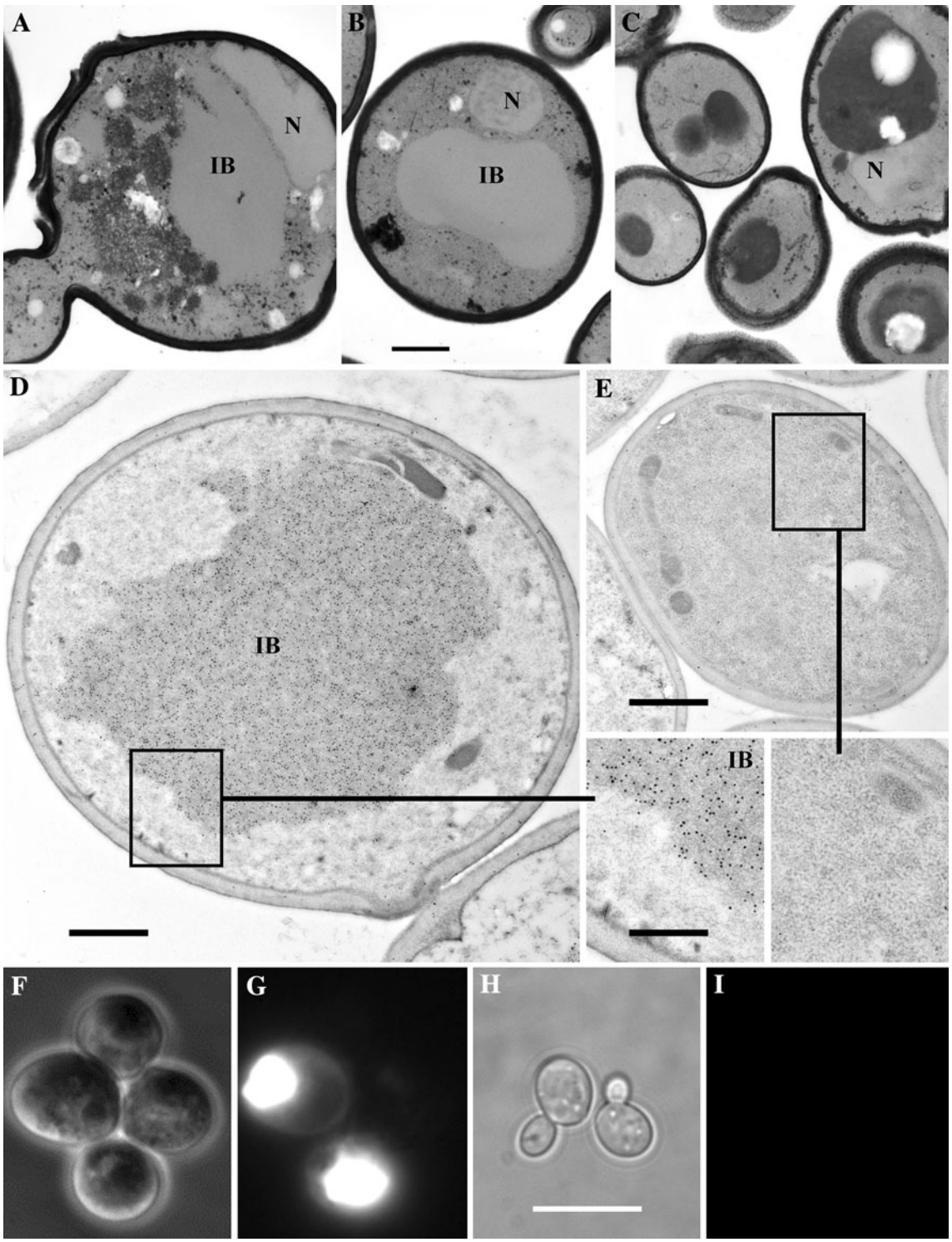

Fig. 4 Rnr1C883A and Rnr1C883A-GFP proteins accumulate in inclusion bodies. *rnr1C883A rnr3Δ* (MCM824) mutants and wild-type (MCM185) cells were grown to exponential phase and analyzed by electron microscopy. Large inclusion bodies (*IB*) were detected in *rnr1C883A rnr3Δ* mutants (**a**, **b**), but were not observed in the wild type (**c**). *N* nucleus. *Bar* = 1 μm in (**a**–**c**). *rnr1C883A-GFP rnr3Δ* (MCM845) mutants (**d**) and wild-type (MCM185) cells (**e**) were grown to exponential phase and analyzed by immunogold labeling using an antibody directed against the GFP moiety of the Rnr1C883A-GFP fusion protein. In *rnr1C883A-GFP rnr3Δ* mutants, IB were packed with gold particles (see **d** inset). Few gold particles were detected outside of IB demonstrating a dramatic accumulation of the Rnr1C883A-GFP protein in these structures. Wild-type cells were devoid of IB and showed only background gold labeling (see **e** inset). *Bar* = 1 μm in (**d**,**e**) and *bar* = 0.5 μm in insets. *rnr1C883A-GFP rnr3Δ* (MCM845) mutants (**f**, **g**) and wild-type (MCM185) cells (**h**, **i**) were examined by phase contrast (**f**, **h**) and by fluorescence microscopy (**g**, **i**). *Bar* = 5 μm in (**f**–**i**)

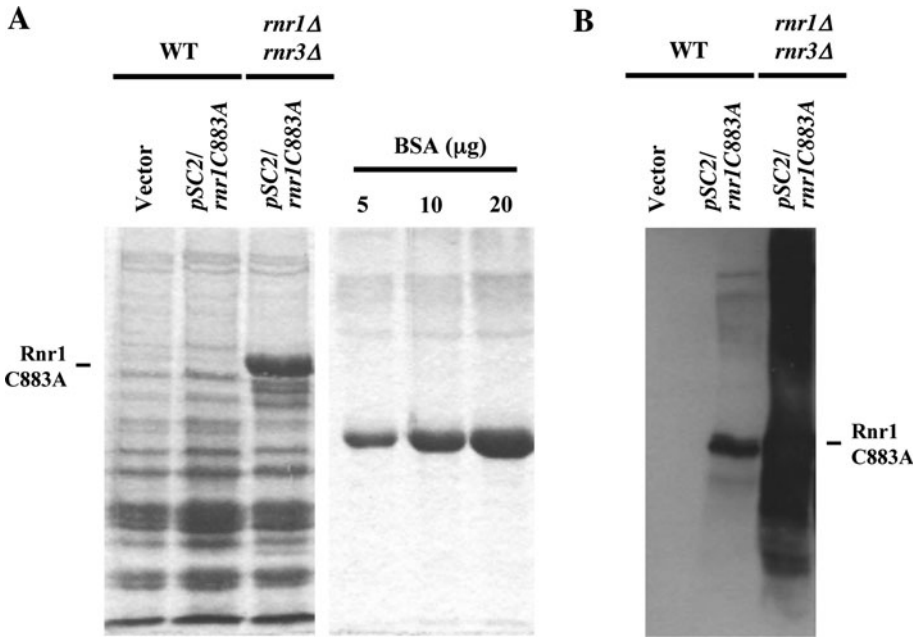

Fig. 5 Analysis of the expression of the *rnr1C883A* allele. Asynchronous cultures of wild-type (WT, MCM185) or *rnr1Δ rnr3Δ* mutant cells (MCM824) harboring either an empty vector (pBAD54) or the plasmid overexpressing the *rnr1C883A* allele (pSC2/*rnr1C883A*) were grown to exponential phase. Total extracts were prepared under nonreducing conditions as described (Camier et al. 2007). Equal amounts of proteins (20 μg) were separated by SDS-PAGE and either stained by Coomassie blue (**a**) or transferred to a nitrocellulose membrane and probed with anti-HA antibodies (12CA5, Abcam) (**b**). Serial dilutions of BSA were also analyzed to provide a standard for the quantification of the Rnr1C883A protein

low level of *leu2-d* expression leads to the predominance of cells with a high copy number of *leu2-d*-containing plasmids under conditions selective for leucine. Similarly, the higher copy number of the pSC2/*rnr1C883A* plasmid compared to pSC2/*RNR1* in *rnr1Δ rnr3Δ* cells can be explained by the low activity of Rnr1C883A and the reduced generation time of cells accumulating pSC2/*rnr1C883A*.

Using quantitative PCR analysis, we found that the abundance of *rnr1C883A* mRNAs in asynchronously growing *rnr1C883A rnr3Δ* cells was equal to 470 ± 80 times the abundance of *RNR1* mRNAs in asynchronously growing wild-type cells. The transcription of *RNR1* is strongly induced in the S phase (Elledge and Davis 1990). Considering that the S phase occupies the largest part of the cell cycle in *rnr1C883A rnr3Δ* mutants, but only about one-third of it in wild-type cells, we can estimate that, during the S phase, the amount of *rnr1C883A* mRNAs in *rnr1C883A rnr3Δ* mutants is about 150 (470/3) times the amount of *RNR1* mRNAs in wild-type cells. The 21-fold increase in copy number of the *rnr1C883A* allele harbored by the pSC2/*rnr1C883A* plasmid compared to the chromosomal *RNR1* allele in wild-type cells cannot account for that difference. This observation could therefore be explained in at least two ways: (1) the transcriptional activity of the *rnr1C883A* allele harbored by the pSC2/*rnr1C883A* plasmid could be higher than that of the chromosomal *RNR1* allele during the S phase (because: the replicational stress experienced by the *rnr1C883A rnr3Δ* mutants leads to an increase in the transcription of *rnr1C883A*; the plasmid contains only part of the *RNR1* promoter; or the plasmidic chromatin context of *rnr1C883A* is different from the chromosomal chromatin context of *RNR1*) or (2) the replicational stress experienced by the *rnr1C883A rnr3Δ* cells could induce an increase in the half-life of *rnr1C883A* mRNAs.

In summary, both a high copy number of the *rnr1C883A* allele and either a higher transcriptional activity of the *rnr1C883A* allele or a higher stability of the *rnr1C883A* transcripts cause a strong increase of *rnr1C883A* mRNAs in *rnr1C883A rnr3Δ* cells compared to *RNR1* mRNAs in wild-type cells. It has to be noted also that the increase of *rnr1C883A* mRNAs, compared to *RNR1* mRNAs, seems higher than the increase of Rnr1C883A proteins, compared to Rnr1 proteins, which suggests that *rnr1C883A* mRNAs could be less efficiently translated than *RNR1* mRNAs.

Discussion

We have constructed a mutant strain containing only nonrecyclable RNR catalytic subunits, in which the production of each dNTP requires the synthesis of one 888 amino acid long Rnr1C883A protein. *rnr1C883A rnr3Δ* cells grow under constant replicational stress and their

S phase is largely extended compared to the wild type. However, we found that *rnr1C883A rnr3Δ* mutants exhibit a good plating efficiency and can be propagated indefinitely, although they grow more slowly than the wild type. Yeast cells thus prove resistant to a constitutive depletion of dNTPs and to a large extension of their S-phase length.

Somewhat paradoxically, the dNTP depletion experienced by *rnr1C883A rnr3Δ* cells, although more severe than the one induced by a 20 mM HU-treatment in wild-type cells (as inferred from their impact on S-phase length), led to a weaker activation of Rad53. This low activation of Rad53 in *rnr1C883A rnr3Δ* cells could be explained by the dependency of Rad53 phosphorylation on the number of active replication forks, as suggested by the fact that mutants partially defective in initiation of DNA replication, such as *clb5Δ*, *cdc7* and *orc2-1* cells, were defective in activating the DNA checkpoint after genotoxic treatments (Shimada et al. 2002; Tercero et al. 2003). When α-factor-arrested wild-type cells are released into HU-containing medium, they start with normal dNTP pools; hence early origins are fired, giving numerous replication forks able to sense the ensuing dNTP depletion [Kitada et al. found that 116 replication origins out of a total of 260 were fired 90 min after α-factor-blocked cells were released in the presence of 0.2 M HU (Yabuki et al. 2002)]. In contrast, *rnr1C883A rnr3Δ* cells probably start their S phase with low dNTP levels. It is conceivable that only few origins can be fired before a signal from the first replication forks activates Rad53 and blocks further origin firing. This would explain that, in *rnr1C883A rnr3Δ* cells, Rad53 activation, triggered by a few replication forks at the beginning of the S phase, is low but sufficient to control further firing. Therefore, at any given time during the S phase, only few replication forks operate simultaneously, maintaining a limited activation of Rad53. Alternative explanations are also plausible, including a decrease in checkpoint activation due to adaptation or to abnormally low amounts of proteins involved in checkpoint signaling caused by the overexpression of Rnr1C883A.

Besides dNTP depletion, *rnr1C883A rnr3Δ* mutants also exhibit a large increase in median cell volume. For a given species in a given environment, cell size is generally held constant by processes coordinating cell growth and division [for recent reviews see (Jorgensen and Tyers 2004; Cook and Tyers 2007)]. However, the physical or physiological selective forces determining cell size are far from clear. Constraints induced by diffusion processes (which affect the import of nutrients and the "mixing times" of intracellular components) probably play a role in favoring small-sized cells, but only a partial one (Young 2006). The *rnr1C883A rnr3Δ* mutants illustrate yeast robustness to size variations.

Another particular aspect of *rnr1C883A rnr3Δ* mutants is the metabolic cost of dNTP production. Using the costs of amino acids and nucleotides, previously calculated by Wagner (2005) for *S. cerevisiae*, and considering growth on glucose under respiratory conditions, we found that the additional cost of one Rnr1C883A protein per dNTP increases the cost of a dNTP in *rnr1C883A rnr3Δ* cells compared to the wild type by a factor of 70 in rich medium (YPD or SMCA, containing all amino acids) and by a factor of 500 in minimal medium (SM, without amino acids). This observation is reminiscent of the large variations in DNA content found in related species of higher eukaryotes, since the metabolic cost of DNA should to a first approximation be proportional to the genome size. Variations of 340-, 240-, 190- and 130-fold have been measured in the genome sizes of flatworms, crustaceans, insects and amphibians, respectively (Gregory 2005). The fact that yeast cells can survive a 500-fold increase in DNA cost suggests that large increases in DNA content can take place in a small number of generations. In contrast with the large genomes of vertebrates and land plants that contain 90-98% of non-coding DNA and up to 120,000 megabases (Mb), the genomes of unicellular eukaryotes, including *S. cerevisiae*, are usually much smaller (3–100 Mb) and more compact as they contain 80–95% of coding DNA (Lynch 2006; Gregory et al. 2007). The fact that yeast cells can accommodate large increases in DNA cost also suggests that there is no stringent metabolic barrier opposing the expansion of DNA content by orders of magnitude in yeast, and that its streamlined genome can rather be attributed to the power of natural selection acting on its large populations to eliminate the metabolic and/or mutational burdens associated with increased DNA content (Lynch 2006).

Finally, another characteristic of *rnr1C883A rnr3Δ* mutants is the production of a quantity of Rnr1C883A proteins equivalent to half the protein complement of a wild-type cell, each time they replicate their DNA, and the accumulation of these proteins. Yeast cells can thus be propagated indefinitely, despite dramatic changes in their transcriptional and translational programs and the formation of massive inclusion bodies. This observation indicates that a protein of interest could be overproduced using fusion constructs including the *rnr1C883A* sequence, as exemplified by the Rnr1C883A-GFP fusion. This strategy could be used for continuous production since the synthesis of the Rnr1C883A-fusion protein is permanently required for cell division. No induction is necessary and cultures can be maintained under stable, optimized conditions.

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