Nucleolus as an oxidative stress sensor in the yeast *Saccharomyces cerevisiae*

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In mammals, the nucleolus is thought to be a stress sensor; upon cellular stress conditions, a release of nucleolar proteins and down-regulation of rDNA transcription occurs. Since yeast Rrn3p is a homolog of the mammalian RNA polymerase I (Pol I)-specific transcription factor TIF-IA, we decided to investigate the role of Rrn3p in oxidant-induced nucleolar stress in yeast. We show that, after oxidant treatment, the level of Rrn3p is unaffected but Rrn3p is translocated from the nucleolus into the cytoplasm and a point mutation in the *RRN3* gene leads to hypersensitivity of the yeast to oxidants. This hypersensitivity can be abolished by re-introduction of the active *RRN3* gene, antioxidant supplementation and anoxic atmosphere. Additionally, we employed the PRINS technique to monitor oxidant-mediated changes in the nucleolar structure. Taken together, our results suggest the role of the yeast nucleolus in the response to oxidative stress signals.

Keywords: yeast, *Saccharomyces cerevisiae*, Rrn3p, nucleolus, oxidative stress, antioxidants

Introduction

The nucleolus, in addition to its essential role in ribosome biogenesis, may also be involved in the sequestration of proteins acting in cell-cycle regulation, assembly of signal recognition particles, cellular aging, small RNA processing, mRNA export and degradation, modulation of telomerase activity and may be considered as a stress sensor.^{1–11}

Mammalian cells can immediately sense and respond to oxidative and ribotoxic stress signals by down-regulation of the rRNA synthesis by c-Jun N-terminal kinase 2 (JNK2)-mediated inactivation of an essential transcription factor TIF-IA modulating the activity of RNA polymerase I (Pol I).⁸ Genetic inactivation of TIF-IA results in nucleolar disruption, cell cycle arrest and p53-mediated induction of

programmed cell death.¹² Heat shock, UV irradiation, hypoxia, DNA damaging agents and ROS can induce nucleolar stress resulting in augmentation of the p53 level. Under these conditions, proteins like ARF, L5, L11, L23 or B23/nucleophosmin are released from the nucleolus to the nucleoplasm and can bind to MDM2 (E3 ubiquitin ligase) inhibiting its activity and, in turn, stabilizing p53 which leads to cell cycle arrest and/or apoptosis.^{6,10,13,14} Additionally, TIF-IA localization is affected by stress: after hydrogen peroxide and rapamycin exposure, TIF-IA moves from the nucleolus into the nucleoplasm and cytoplasm, respectively.^{7,8,15} Such redistribution is connected with modifications leading to inhibition of TIF-IA: oxidative stress causes phosphorylation of TIF-IA by JNK at threonine 200, while mTOR inhibitor rapamycin leads to hyperphosphorylation of TIF-IA at serine 199.^{8,15}

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Abbreviations: CumOOH, cumene hydroperoxide; DAPI, 4',6'-diamino-2-phenylindole; DTT, dithiothreitol; GFP, green fluorescent protein; GSH, glutathione; NAC, N-acetylcysteine; PRINS, primed *in situ* DNA synthesis; ROS, reactive oxygen species; *tert*-BOOH, *tert*-butyl hydroperoxide

Rrn3p is the yeast homolog of the mammalian TIF-IA.^{16,17} It was shown that TIF-IA was able to rescue the lethal deletion of *RRN3* gene when expressed in the yeast which may suggest that the regulation of eukaryotic RNA polymerase I transcription is functionally conserved in evolution.¹⁶ Similar to TIF-IA, Rrn3p can form a complex with Pol I rendering the enzyme transcriptionally active and enabling initiation of rRNA transcription.^{18–21}

The aim of this study was to investigate the role of Rrn3p in oxidant-mediated nucleolar stress in the yeast by: (i) monitoring cellular localization of Rrn3p under stress conditions; and (ii) estimating the level of Rrn3p during oxidant exposure; and (iii) examining the impact of oxidants on the growth rate of a temperature sensitive (ts) yeast strain harboring the point mutation in the *RRN3* gene leading to substitution of serine to proline at position 213 (S213P). Additionally, protective effects of anoxic atmosphere and antioxidants against oxidant-mediated toxicity were investigated.

Materials and methods

Chemicals

Cumene hydroperoxide was from Fluka (Buchs, Switzerland) and mounting medium with DAPI II Counterstain was from Abbott Molecular (Illinois, USA). All other reagents, if not stated otherwise, were purchased from Sigma (Poznan, Poland) and were of analytical grade.

Yeast strains and growth conditions

The following yeast strains were used: a *RRN3*-GFP clone containing the GFP and *HIS3* marker gene tagged ORF at its chromosomal location in parental ATCC 201388 *MATa his3 leu2 met15 ura3* background²² purchased from Invitrogen (Carlsbad, USA); wild-type W303-1A *MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his 3-11,15 can1-100*, its isogenic *rrn3* mutant strain harboring point mutation in the *RRN3* gene leading to substitution of serine to proline at position 213 (S213P) NOY1075, a *RRN3* disruptant NOY1170 *MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his 3-11,15 can1-100* containing a pNOY452 carrying *RRN3* tagged with (HA)₇ at the N terminus,²³ which were kindly provided by Professor Masayasu Nomura and Dr Loan Vu (University of California, Irvine, CA, USA). A cross between NOY1075 and NOY1170 strains was obtained by standard micromanipulation technique.²⁴ Yeast was grown either on liquid YPD

medium (1% Difco Yeast Extract, 1% Difco Yeast Bacto-Peptone, 2% glucose) or on minimal (YNB; 0.67% Yeast Nitrogen Base, 0.5% glucose) medium, or on solid YPD or YNB medium with appropriate additives, containing 2% agar, at 28°C.

RRN3-GFP, NOY1170 and NOY1075 strains were checked on YNB plate +Ura +Met +Leu –His; on YNB plate +Ade +Ura +Trp +Leu –His and on YPD Petri dish kept at 37°C, respectively.

Yeast cell fixation

The *RRN3*-GFP clone was grown overnight in YPD medium, then the cells were sedimented by centrifugation (2200 g, 3 min), washed twice in phosphate-buffered saline (PBS; Gibco, Invitrogen Corporation, Grand Island, NY, USA), diluted to 10⁷ cells/ml in 20 ml of YNB fresh medium lacking histidine ± oxidants tested and grown for 5, 15, 30, 60 and 120 min. After addition of 37% formaldehyde (1:9, v/v) and 1-h incubation, the cells were washed twice with PBS and resuspended in 500 µl of the spheroplast buffer (1.2 M sorbitol in sterile PBS). A 3.2 µl aliquot of 1.42 M β-mercaptoethanol and 5 µl of 5 mg/ml Zymolyase 100T (USBiological, Massachusetts, USA) were added to 200 µl of the cell suspension which was then incubated with shaking at 30°C for 1 h. After sedimentation, the cells were washed, suspended in 100 µl of the spheroplast buffer, spread onto slides, incubated with ice-cold methanol for 5 min and with ice-cold acetone for 30 s and left to air-dry.

Immunofluorescence

We used goat polyclonal antibody against GFP (biotin) (Abcam, Cambridge, UK), monoclonal anti-avidin conjugate clone WC19.10 (Sigma, St Louis, MO USA) mouse monoclonal against Nop2p (nucleolar marker; Abcam) and goat polyclonal against mouse IgG (Chromeo™ 546; Abcam).

Immunostaining was performed by the standard protocol according to the manufacturer's instructions with minor modifications. Unspecific signals were blocked by incubation the slides with 3% BSA in PBST (PBS supplemented with 0.25% Triton X-100) under a coverslip in a humidified chamber at 37°C for 30 min. The slides were incubated in a mixture of two primary antibodies: against GFP (biotin; diluted 1:1000 in 3% BSA in PBST) and against the nucleolar marker Nop2p (diluted 1:500 in 3% BSA in PBST) under a coverslip in a humidified chamber overnight at 4°C. After three 5-min washes with PBST in the dark, the slides were incubated with a secondary antibody against mouse IgG (Chromeo™ 546; diluted 1:1000 in 3% BSA in PBST), then with FITC-

conjugated avidin (diluted 1: 200 in 3% BSA in PBST; Sigma), and with monoclonal anti-avidin conjugate clone WC19.10 (diluted 1:200 in 3% BSA in PBST) and again with FITC-conjugated avidin. Each incubation was performed under a coverslip in a humidified chamber at 37°C for 45 min and separated by three 5-min washes in PBST in the dark and a final wash in PBS in the dark for 5 min. After the last wash, the slides were counterstained with a drop of mounting medium with 4',6'-diamino-2-phenylindole DAPI II Counterstain and analyzed in a Axiophot Zeiss-Opton fluorescence microscope (Carl Zeiss, Germany) equipped with a Cohu High Performance CCD camera and a LUCIA computer image analysis system. For searching Rrn3p-GFP and/or Nop2p positive cells (nucleolar signals), a total of 600 cells (from three independent experiments for each oxidant concentration) were analyzed.

Primed in situ DNA synthesis (PRINS) with oligonucleotides complementary to 18S rDNA

Oxidant exposed and fixed *RRN3*-GFP cells were treated with 100 µg/ml RNase in 2× saline sodium citrate (SSC) buffer (Sigma) in a humidified chamber at 37°C for 1 h for better results. Next, the slides were washed three times with 2× SSC buffer and washed once with PBS with 50 mM MgCl₂ and passed through a set of ethanol solutions (70%, 80% and 95%).

The specific primers for the yeast 18S rDNA (*RDNI8-2* gene) were used as previously described.²⁵ The PRINS reaction mixture in a total volume of 25 µl per slide contained: 0.5 mM dATP, dCTP and dGTP; 0.025 mM dTTP; 1 nM Cy3-dUTP; 2 U *Taq* polymerase; 1× *Taq* polymerase buffer; 2.5 mM MgCl₂, 1 nM each of Fwd primers and 2 nM Rev primer; 5% glycerol and sterile distilled water (milliQ). The PRINS reaction mixture was applied on the slide with a coverslip, sealed with fixogum to prevent evaporation and was performed on a preheated block at 94°C for 5 min and next at 62°C for 30 min.

The PRINS reaction was stopped by transferring the slides to a stop buffer containing 50 mM NaCl and 50 mM EDTA (pH 8.0) at 62°C for 5 min and next to a stop buffer (50 mM NaCl and 50 mM EDTA, pH 8.0) at room temperature for 10 min. Next, the slides were washed twice with 2× SSC at room temperature for 5 min. Before detection, the slides were dehydrated through a set of ethanol solutions (70%, 80% and 95%). As soon as the slides were air-dried in the dark, 10 µl of an antifade solution containing DAPI II Counterstain was applied and the slides were covered

with a coverslip. The slides were analyzed in a Axiophot Zeiss-Opton fluorescence microscope (Carl Zeiss) equipped with a Cohu High Performance CCD camera and a LUCIA computer image analysis system.

Oxidant treatment and antioxidant growth restoration assay

Liquid yeast cultures (total volume of 150 µl of initial cell density of 5×10^6 cells/ml) containing various concentrations of oxidants were run in a Heidolph Inkubator 1000 at 1200 rpm at 28°C and their growth was monitored turbidimetrically at 600 nm in an Anthos 2010 type 17550 microplate reader after 12 h. For spotting experiments, several dilutions (1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 cells/ml) of yeast exponential phase culture in a volume of 5 µl were inoculated on YPD agar containing the tested agents ± antioxidants, incubated at 28°C and inspected after 48 h. Stock solutions of antioxidants were added to sterile media which were cooled to just above the solidification point (temperature of ~50°C). All stock solutions of antioxidants were freshly prepared before adding to the media.

Anoxic atmosphere was achieved by placing Petri dishes in a desiccator flushed with oxygen-free nitrogen (Linde Gaz, Cracow, Poland).

Flow cytometry analysis

The *RRN3*-GFP construct was grown overnight in YPD medium, cells were washed off, diluted to OD₆₀₀ 0.4 and pre-incubated in YNB medium lacking histidine for 30 min, then freshly prepared oxidants were added to the cultures. The median fluorescence intensity of 5×10^4 cells at 0, 15, 30, 45, 60, 90, 105 and 120 min after addition of the agents tested was measured in a Becton Dickinson LSRII flow cytometer (San Jose, CA, USA), equipped with a 488 nm argon laser.

Statistical analysis

The results represent mean ± SD from at least three independent experiments. Statistical significance of differences in (i) the growth rate between oxidant-treated cells (wild-type versus cells harboring the point mutation in the *RRN3* gene) (ii) fluorescence of the Rrn3p-GFP construct under control conditions (cells incubated in the His-free YNB medium) and after treatment with oxidants was evaluated using one-way analysis of variance (ANOVA) with *post-hoc* testing using a Dunnett's multiple comparison test. *P*-values of less than 0.05 were considered significant. Statistical analysis of the data was performed using a StatSoft, Inc. (2005), STATISTICA, v 7.0, <www.statsoft.com>.

Figure 1 Kinetics of oxidant-induced redistribution of Rrn3p. *RRN3*-GFP clone was subjected to oxidative stress conditions for 5, 15 and 30 min, and then fixed. *RRN3*-GFP cells were immunostained against GFP (biotin; green) and against the nucleolar marker Nop2p (red). The slides were stained with a drop of DAPI II counterstain (blue) and analyzed in a fluorescence microscope equipped with a CCD camera and Lucia software. For searching Rrn3p-GFP and/or Nop2p positive cells (nucleolar signals), a total of 600 cells were analyzed. The percentage reflects the amount of cells which are Rrn3p-GFP and/or Nop2p nucleolar positive. The micrographs shown are representative for at least three independent experiments involving all oxidants used

Results

Oxidant-induced redistribution of nucleolar *Rrn3p*

Since, in mammals, exogenous stress-mediated nucleolar disruption involves the release of nucleolar

proteins like ARF, L5, L11, L23, B23/nucleophosmin or TIF-IA into the nucleoplasm, we decided to check the impact of oxidative stress on the cellular localization of Rrn3p, the yeast homolog of TIF-IA. Under standard growth conditions, Rrn3p was found

Figure 2 Long-term oxidant treatment causes the leakage of the nucleolar proteins. *RRN3*-GFP clone was subjected to oxidative stress conditions for 1 h and 2 h, and then fixed. *RRN3*-GFP cells were immunostained against GFP (biotin; green) and against the nucleolar marker Nop2p (red). The slides were stained with a drop of DAPI II counterstain (blue) and analyzed in a fluorescence microscope equipped with a CCD camera and Lucia software. For searching Rrn3p-GFP and/or Nop2p positive cells (nucleolar signals), a total of 600 cells were analyzed. The percentage reflects the amount of cells which are Rrn3p-GFP and/or Nop2p nucleolar positive. The micrographs shown are representative for at least three independent experiments involving all oxidants used

Figure 3 Scheme showing the effects of short- and long-term oxidant treatment on yeast cell, especially on the structure of nucleolus

in the nucleolus while after stimulation with oxidants (up to 30 min) it moved into the cytoplasm (Fig. 1).

We observed that both hydroperoxides (H_2O_2 , *tert*-BOOH, CumOOH) and menadione, a superoxide generating agent, can induce the translocation of Rrn3p. After 30 min oxidant incubation, 15–30% of Rrn3p has translocated from the nucleolus to the cytoplasm while Nop2p, the nucleolar marker was present in the nucleolus almost in the control amount (Fig. 1). After prolonged treatment (1–2 h), we observed the structural damage of nucleolus and loss of signals of both nucleolar proteins (Fig. 2) which indicates that the long-term oxidant treatment induced disruption of nucleolus integrity and leakage of the nucleolar proteins.

A schematic pattern of events following yeast short- and long-term exposure to oxidative agents is shown in Figure 3.

Additionally, we used the PRINS reaction with oligonucleotides complementary to 18S rDNA to monitor changes in the nucleolus structure during oxidative stress. After short-term oxidant treatment, the nucleolus was unchanged while long-term oxidant treatment caused structural damage of the nucleolus (Fig. 4). At this late state of incubation, rDNA is rather degraded. In our opinion, it reflects the general cell state at this end-point (the last step of nucleolus disintegration linked with cell death). These results are consistent with the data obtained by immunostaining.

The rrn3 (S213P) mutant is hypersensitive to oxidants

To study the role of Rrn3p in the oxidant-induced nucleolar disruption, we used cells with the point mutation in the *RRN3* gene leading to substitution of serine to proline at position 213 (S213P).²³ Such amino acid alteration in the Rrn3p was found to confer the oxidant-sensitive phenotype. After 12-h treatment with 0.5 and 1 mM hydrogen peroxide, the growth rate of the *rrn3* (S213P) mutant was about 2- and 4-fold, respectively, lower compared to parental wild-type strain (Fig. 5A).

The effect of organic hydroperoxides was even more pronounced. After 12-h exposure to 0.4 and 0.5 mM *tert*-BOOH and to 0.1 mM CumOOH the growth of the cells lacking active *RRN3* gene was 4-, 6- and 8-fold slower in comparison with the wild-type cells, respectively (Figs 5B and 5C). The *rrn3* (S213P) mutant was also sensitive to menadione, a superoxide generating compound, as estimated by the spotting assay (Fig. 7). The re-introduction of the active *RRN3* gene in the *rrn3* null background (NOY1170 strain) and a crossing

Figure 4 Long-term oxidant treatment promotes nucleolus disintegration. *RRN3*-GFP clone was incubated $\pm$ oxidants for 2 h and fixed. The nucleoli were visualized by PRINS technique (red). The nuclear DNA was stained with DAPI II counterstain (blue). The slides were analyzed in a fluorescence microscope equipped with a CCD camera and Lucia software. The micrographs shown are representative for at least three independent experiments involving all oxidants used

between NOY1075 and NOY1170 strains abolished the hypersensitivity to oxidants (Figs 5 and 6).

Anoxia and antioxidants suppress the oxidant-sensitive phenotype of the *rrn3* (S213P) mutant

We have found that reduction of the oxygen tension alleviates the hypersensitivity of the *rrn3* (S213P) mutant to oxidants. Anoxic atmosphere abolished the

growth restriction caused by oxidative agents (Fig. 7).

Anoxic conditions restored the growth of the *rrn3* (S213P) mutant completely and partially in the case of hydroperoxides and menadione treatment, respectively (Fig. 7). The slightly higher sensitivity of the *rrn3* (S213P) mutant to menadione visible also under anoxic conditions of growth may be the effect of initial handling under air, before establishing anoxic atmosphere.

Additionally, we tested the ability of exogenously added antioxidants to modify growth inhibition of the *rrn3* (S213P) mutant caused by oxidants (Fig. 7). Ascorbate at concentrations ranging from 1–30 mM was able to restore the growth rate of the *rrn3* (S213P) mutant to the level observed in the absence of hydroperoxides. In the case of menadione, it completely recovered the colony forming ability of the cells lacking functional *RRN3* gene at concentrations from 5–30 mM (data not shown). Thiol antioxidants like GSH, NAC, cysteine and DTT were also found protective against the oxidant-induced growth inhibition. Their positive effects were seen at relatively lower concentrations than for ascorbate (data not shown).

Oxidants have no effect on *Rrn3p* expression

To study yeast *Rrn3p* expression, we used the *RRN3*-GFP strain and measured the cell fluorescence intensities by flow cytometry. We were unable to detect any significant induction of *Rrn3p* expression level after both hydroperoxides and menadione treatment when compared with control (Fig. 8).

Discussion

Exposure of mammalian cells to endo- or exogenous stress of various kinds (aberrant ribosome biogenesis, DNA damage, hypoxia, heat shock, augmentation in ROS level) leads to nucleolar stress and, subsequently, to an enhancement in p53 level and cell cycle arrest or apoptosis;^{7,10,26} hence, the nucleolus is thought to be a sensor for cellular stress signals.

The nucleolus, apart from its role in the regulation of cellular stress responses by p53 stabilization, may also supervise rRNA transcription. It has been demonstrated that, after ribotoxic and oxidative stress, c-Jun

Figure 5 Hydroperoxides toxicity to the *rrn3* (S213P) mutant assessed in complete liquid medium. Cells: wild-type, the NOY1075 strain which is the *rrn3* (S213P) mutant (strain harboring point mutation in the *RRN3* gene leading to substitution of serine to proline at position 213) and the NOY1170 strain, from the logarithmic phase at a concentration of 5×10^6 cells/ml were grown in liquid YPD medium containing various concentrations of (A) H_2O_2 – 1, no stress, 2, 0.2 mM H_2O_2 , 3, 0.5 mM H_2O_2 , 4, 0.7 mM H_2O_2 , 5, 1 mM H_2O_2 , 6, 1.5 mM H_2O_2 , 7, 2 mM H_2O_2 ; (B) *tert*-BOOH – 1, no stress, 2, 0.05 mM *tert*-BOOH, 3, 0.1 mM *tert*-BOOH, 4, 0.2 mM *tert*-BOOH, 5, 0.3 mM *tert*-BOOH, 6, 0.4 mM *tert*-BOOH, 7, 0.5 mM *tert*-BOOH; (C) CumOOH: 1, no stress, 2, 0.05 mM CumOOH, 3, 0.1 mM CumOOH, 4, 0.2 mM CumOOH and their growth was monitored turbidimetrically at 600 nm after 12 h. Bars indicate SD, $n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with oxidant-treated wild-type cells (one-way ANOVA and Dunnett's multiple comparison test)

Figure 6 Crossing *rrn3* (S213P) mutant with *RRN3* disruptant containing a pNOY452 carrying *RRN3* gene restores oxidant-resistant wild-type phenotype. The cells were grown on solid YPD medium $\pm$ oxidants and inspected after 48 h. Additionally, we provided analogical data concerning well-established temperature-sensitive phenotype of the NOY1075 strain

Figure 7 Anoxic atmosphere and antioxidants reduce the hypersensitivity of the *rrn3* (S213P) mutant to oxidants. The cells were grown on solid YPD medium $\pm$ H_2O_2 , *tert*-BOOH, CumOOH and menadione, under aerobic or anoxic atmospheres or $\pm$ antioxidants and inspected after 48 h

N-terminal kinase 2 (JNK2) can inactivate the transcription factor TIF-IA and down-regulate rRNA synthesis.⁸ Under stress conditions, TIF-IA is phosphorylated by JNK2, which leads to its inability to bind to RNA polymerase I (Pol I) and to inhibition of rRNA transcription, and translocation from the nucleolus into the nucleoplasm.⁸ Since Rrn3p is the yeast homolog of mammalian TIF-IA and the regulation of eukaryotic Pol I transcription is functionally conserved,^{16,17} we decided to examine the role of Rrn3p in oxidant-mediated nucleolar stress in the yeast. After oxidant exposure, Rrn3p expression was unaltered when compared to standard growth conditions (Fig. 8), which may imply that co-ordination of yeast rRNA synthesis involves different regulation than at the Rrn3p transcriptional/translational level.

We observed a clear-cut effect of oxidative stress on cellular Rrn3p localization. After hydroperoxides and menadione treatment, Rrn3p migrated from the nucleolus into the cytosol as seen by the fluorescence of Rrn3p-GFP while the level of nucleolar marker protein Nop2 in the nucleolus was unchanged (Fig. 1). This result is in agreement with previous findings on mammalian cells concerning hydrogen peroxide- and rapamycin-dependent redistribution of TIF-IA from the nucleolus into the nucleoplasm and cytoplasm, respectively.^{7,8,15} Divergent migration of TIF-IA was preceded with its phosphorylation at different residues.^{8,15} Furthermore, under stress conditions, when nucleolar functions are impaired, other mammalian proteins like ARF, L5, L11, L23 or B23/nucleophosmin are also released from the

Figure 8 Effects of oxidants on the Rrn3p level. The *RRN3*-GFP clone was incubated in a histidine-free YNB medium $\pm$ the agents tested: H_2O_2 , *tert*-BOOH, CumOOH and menadione at 28°C for 2 h. GFP intensity was estimated by flow cytometric analysis of 5×10^4 cells every 15 min during a 2-h incubation. Bars indicate SD, $n = 3$

nucleolus. Such relocation allows them to bind to MDM2 which, in turn, leads to an increase in p53 level, cell cycle arrest and/or apoptosis.^{6,10,13,14} Moreover, in our hands, prolonged oxidant treatment (1–2 h) caused the nucleolus disruption which was shown with PRINS technique (Figs 2 and 4).

Subsequently, we studied the importance of Rrn3p in the oxidant-induced disturbance to nucleolus functions. In the yeast, *RRN3* is an essential gene and a null $\Delta rrn3$ mutant is non-viable; thus, we used the cells with the point mutation in the *RRN3* gene leading to substitution of serine to proline at position 213 (S213P)²³ for growth sensitivity assays. We found that the *rrn3* (S213P) mutant was hypersensitive to agents inducing peroxide and superoxide stress when compared to its parental wild-type (Figs 5 and 7). Moreover, the oxidant-sensitive phenotype was abolished after the re-introduction of active *RRN3* gene in the *rrn3* background and after a crossing between NOY1075 and 1170 strains (Figs 5, 6 and 7). These data suggest the significance of nucleolar Rrn3p in the regulation of yeast oxidative stress signals.

Here, we also showed that modifications of yeast growth environment may contribute to improvement of Rrn3p function under oxidative stress. Anoxic atmosphere and antioxidants were able to restore growth deficiency of the *rrn3* (S213P) mutant subjected to oxidants challenge (Fig. 7). Under such conditions affecting cell redox homeostasis, production of ROS is limited which abolishes the

hypersensitivity of the *rrn3* (S213P) mutant to oxidants. Protection of ascorbate and glutathione against oxidant-induced growth restrictions of yeast cells lacking antioxidant proteins was previously reported by our laboratory.^{27,28}

Here, we showed for the first time the importance of yeast nucleolus in stress sensing, especially in oxidative stress sensing which seems to be a crucial cellular function of the nucleolus. Additionally, a range of oxidants was discovered to induce such response (hydrogen peroxide, organic peroxides and menadione) which could shed some light on the yet little-known subject of cell biology and stress responses.

It has been demonstrated that the *rrn3* (S213P) mutant exhibits a temperature sensitive phenotype.²³ When the temperature was shifted from 25°C to 37°C, the cells lacking active *RRN3* gene grew evidently slower than the wild-type strain. The 95% inhibition of Pol I activity observed in the *rrn3* (S213P) mutant may account for this temperature-sensitive phenotype.²³ It is well known that oxidative stress is implicated in heat-induced cell death in yeast, since mutations in antioxidant genes sensitize the yeast to heat shock, overexpression of antioxidant genes protects the cells against heat-induced lethality, while anoxia abolishes the effects of heat shock. Augmentation in ROS production and an increase in GSH content are induced by heat shock.^{29–32} Collectively, all these data suggest that under various stress conditions Rrn3p is required for proper Pol I

function. However, further experiments are needed to confirm such assumption and to describe in detail yeast nucleolar stress response.

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References

- Shou W, Seol JH, Shevchenko A *et al.* Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. *Cell* 1999; **97**: 233–244.
- Jacobson MR, Pederson T. Localization of signal recognition particle RNA in the nucleolus of mammalian cells. *Proc Natl Acad Sci USA* 1998; **95**: 7981–7986.
- Zhang Y, Xiong Y. Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. *Mol Cell* 1999; **3**: 579–591.
- Straight AF, Shou W, Dowd GJ *et al.* Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell* 1999; **97**: 245–256.
- Pederson T. The plurifunctional nucleolus. *Nucleic Acids Res* 1998; **26**: 3871–3876.
- Olson MO. Sensing cellular stress: another new function for the nucleolus? *Sci STKE* 2004; **2004**: pe10.
- Mayer C, Grummt I. Cellular stress and nucleolar function. *Cell Cycle* 2005; **4**: 1036–1038.
- Mayer C, Bierhoff H, Grummt I. The nucleolus as a stress sensor: JNK2 inactivates the transcription factor TIF-IA and down-regulates rRNA synthesis. *Genes Dev* 2005; **19**: 933–941.
- Boisvert FM, van Koningsbruggen S, Navascues J, Lamond AI. The multifunctional nucleolus. *Nat Rev Mol Cell Biol* 2007; **8**: 574–585.
- Rubbi CP, Milner J. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J* 2003; **22**: 6068–6077.
- Sirri V, Urcuqui-Inchima S, Roussel P, Hernandez-Verdun D. Nucleolus: the fascinating nuclear body. *Histochem Cell Biol* 2008; **129**: 13–31.
- Yuan X, Zhou Y, Casanova E *et al.* Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. *Mol Cell* 2005; **19**: 77–87.
- Rubbi CP, Milner J. Non-activated p53 co-localizes with sites of transcription within both the nucleoplasm and the nucleolus. *Oncogene* 2000; **19**: 85–96.
- Gallagher SJ, Kefford RF, Rizos H. The ARF tumour suppressor. *Int J Biochem Cell Biol* 2006; **38**: 1637–1641.
- Mayer C, Zhao J, Yuan X, Grummt I. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev* 2004; **18**: 423–434.
- Moorefield B, Greene EA, Reeder RH. RNA polymerase I transcription factor Rrn3 is functionally conserved between yeast and human. *Proc Natl Acad Sci USA* 2000; **97**: 4724–4729.
- Bodem J, Dobrev G, Hoffmann-Rohrer U *et al.* TIF-IA, the factor mediating growth-dependent control of ribosomal RNA synthesis, is the mammalian homolog of yeast Rrn3p. *EMBO Rep* 2000; **1**: 171–175.
- Yamamoto RT, Nogi Y, Dodd JA, Nomura M. *RRN3* gene of *Saccharomyces cerevisiae* encodes an essential RNA polymerase I transcription factor which interacts with the polymerase independently of DNA template. *EMBO J* 1996; **15**: 3964–3973.
- Milkereit P, Tschochner H. A specialized form of RNA polymerase I, essential for initiation and growth-dependent regulation of rRNA synthesis, is disrupted during transcription. *EMBO J* 1998; **17**: 3692–3703.
- Keener J, Josaitis CA, Dodd JA, Nomura M. Reconstitution of yeast RNA polymerase I transcription *in vitro* from purified components. TATA-binding protein is not required for basal transcription. *J Biol Chem* 1998; **273**: 33795–33802.
- Fath S, Milkereit P, Peyroche G, Riva M, Carles C, Tschochner H. Differential roles of phosphorylation in the formation of transcriptional active RNA polymerase I. *Proc Natl Acad Sci USA* 2001; **98**: 14334–14339.
- Huh WK, Falvo JV, Gerke LC *et al.* Global analysis of protein localization in budding yeast. *Nature* 2003; **425**: 686–691.
- Claypool JA, French SL, Johzuka K *et al.* Tor pathway regulates Rrn3p-dependent recruitment of yeast RNA polymerase I to the promoter but does not participate in alteration of the number of active genes. *Mol Biol Cell* 2004; **15**: 946–956.
- Sherman F. Getting started with yeast. *Methods Enzymol* 2002; **350**: 3–41.
- Wnuk M, Lewinska A, Bugno M, Bartosz G, Slota E. Rapid detection of yeast rRNA genes with primed *in situ* (PRINS) labeling. *FEMS Yeast Res* 2009; **9**: 634–640.
- Pestov DG, Strezoska Z, Lau LF. Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition. *Mol Cell Biol* 2001; **21**: 4246–4255.
- Lewinska A, Bilinski T, Bartosz G. Limited effectiveness of antioxidants in the protection of yeast defective in antioxidant proteins. *Free Radic Res* 2004; **38**: 1159–1165.
- Lewinska A, Bartosz G. Protection of yeast lacking the Ure2 protein against the toxicity of heavy metals and hydroperoxides by antioxidants. *Free Radic Res* 2007; **41**: 580–590.
- Davidson JF, Whyte B, Bissinger PH, Schiestl RH. Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 1996; **93**: 5116–5121.
- Lee SM, Park JW. Thermosensitive phenotype of yeast mutant lacking thioredoxin peroxidase. *Arch Biochem Biophys* 1998; **359**: 99–106.
- Sugiyama K, Izawa S, Inoue Y. The Yap1p-dependent induction of glutathione synthesis in heat shock response of *Saccharomyces cerevisiae*. *J Biol Chem* 2000; **275**: 15535–15540.
- Sugiyama K, Kawamura A, Izawa S, Inoue Y. Role of glutathione in heat-shock-induced cell death of *Saccharomyces cerevisiae*. *Biochem J* 2000; **352**: 71–78.