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Yeast growth in raffinose results in resistance to acetic-acid induced programmed cell death mostly due to the activation of the mitochondrial retrograde pathway

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ABSTRACT

In order to investigate whether and how a modification of mitochondrial metabolism can affect yeast sensitivity to programmed cell death (PCD) induced by acetic acid (AA-PCD), yeast cells were grown on raffinose, as a sole carbon source, which, differently from glucose, favours mitochondrial respiration. We found that, differently from glucose-grown cells, raffinose-grown cells were mostly resistant to AA-PCD and that this was due to the activation of mitochondrial retrograde (RTG) response, which increased with time, as revealed by the up-regulation of the peroxisomal isoform of citrate synthase and isocitrate dehydrogenase isoform 1, RTG pathway target genes. Accordingly, the deletion of *RTG2* and *RTG3*, a positive regulator and a transcription factor of the RTG pathway, resulted in AA-PCD, as shown by TUNEL assay. Neither deletion in raffinose-grown cells of *HAP4*, encoding the positive regulatory subunit of the Hap2,3,4,5 complex nor constitutive activation of the RTG pathway in glucose-grown cells due to deletion of *MKS1*, a negative regulator of RTG pathway, had effect on yeast AA-PCD. The RTG pathway was found to be activated in yeast cells containing mitochondria, in which membrane potential was measured, capable to consume oxygen in a manner stimulated by the uncoupler CCCP and inhibited by the respiratory chain inhibitor antimycin A. AA-PCD resistance in raffinose-grown cells occurs with a decrease in both ROS production and cytochrome *c* release as compared to glucose-grown cells *en route* to AA-PCD.

1. Introduction

Cell homeostasis depends on a complex signal transduction network regulating cell capability to adapt or succumb to environmental stress in which different cell components are involved; in this regard besides being cell powerhouse, mitochondria are central organelles in the integration of intracellular signalling pathways and play a pivotal role in the regulation of programmed cell death (PCD) [1–3]. Since signal transduction components and mechanisms are highly conserved among eukaryotes, the unicellular yeast *Saccharomyces cerevisiae* is a suitable model organism to study this issue. In fact, yeast undergoes a PCD process which shares a variety of features with mammalian apoptosis including oxidative stress and the release of pro-apoptotic proteins, including cytochrome *c* (cyt *c*), with attendant late mitochondrial dysfunction [4,5]. With a model system in which PCD of yeast grown on glucose is induced by acetic acid (AA-PCD), the time course of key events which take place *en route* to PCD, including ROS production, cyt *c* release and mitochondrial dysfunction, was described in some detail [5–7]. It was also shown that AA-PCD can also occur in cells lacking cyt *c* and/or the yeast metacaspase *YCA1* via a N-acetyl cysteine (NAC)-insensitive death pathway [8–10]. Although AA-PCD has already been investigated in detail, to date whether and how the cellular energy metabolism can somehow influence the occurrence of either AA-PCD pathways remains to be established.

Glucose is a fermentable carbon source responsible for the down-regulation of respiration [11,12], while raffinose is a poorly fermentable carbon source in which respiration is de-repressed [13]. Here we investigated yeast cell sensitivity to AA-PCD in yeast cells grown either in glucose (GLU-WT cells) or in raffinose (RAF-WT cells). We found that, when raffinose is used as a sole carbon source, differently from what occurs in glucose-grown cells, yeast cells become resistant to AA-PCD in a manner mostly dependent on the retrograde (RTG)-pathway activation. In this regard, that yeast dysfunctional mitochondria can communicate

Abbreviations: AA, acetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cyt *c*, cytochrome *c*; DCF, dichlorophluorescein; GLU, glucose-grown; H_2DCH-DA, 2,7-dihydrodichlorofluorescein diacetate; NAC, N-acetyl cysteine; PCD, programmed cell death; RAF, raffinose-grown; RTG, retrograde; TMRM, tetramethylrhodamine methyl ester; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling; WT, wild type

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with the nucleus via the RTG signalling pathway has already been ascertained in the Butow's laboratory: the expression of a broad array of nuclear target genes was first demonstrated to be largely increased in cells grown on raffinose with impaired mitochondria including those lacking mitochondrial DNA (ρ°) [14,15]. Yeast RTG signalling pathway has been characterized in details [16,17] and its activation was shown to extend replicative life span in yeast [18,19], possibly providing a defense mechanism by which cells can respond to mitochondrial stress leading to mitochondria impairment [20–22]. Surprisingly enough we first found that RTG pathway is active in yeast cells in which mitochondria are still coupled.

2. Materials and methods

2.1. Yeast strains, growth conditions and acetic acid treatment

The *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown at 30 °C in YPD or YPR (1% yeast extract, 2% bactopeptone, and 2% glucose or raffinose, respectively). Acetic acid treatment was carried out as described [23]. Briefly, cells were grown at 26 °C up to exponential phase (OD_{600} = 0.7–0.8) in YPD or YPR, resuspended (10^7 cells/ml) in the same medium adjusted to pH 3.00 with HCl, containing or not containing (control) 80 mM acetic acid and incubated for different times at 26 °C. Cell viability was determined by measuring colony forming units (cfu) after 2 days of growth on YPD plates at 30 °C.

2.2. TUNEL assay and intracellular ROS detection

DNA fragmentation was detected by TUNEL assay. Acetic acid-treated and control cells ($2x10^7$) were harvested at 150 min. Briefly, cells were fixed in 3.7 % formaldehyde solution in PBS, digested with 750 μg/ml zymoliase 20 T and incubated in permeabilization solution (0.1 % Triton-X100, 0.1 % sodium citrate) for 2 min on ice, and then with 30 μl TUNEL reaction mixture (In Situ Cell Death Detection kit, Fluorescein, Roche) for 1 hour at 37 °C. After incubation cells were washed, resuspended in PBS and observed using a Leica TCS SP5 confocal microscope. To detect intracellular H_2O_2 10 μg/ml 2,7-dihydrodichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes) dissolved in ethanol was added to cells either 30 min before or during cell treatment with or without acetic acid. $2x10^7$ acetic acid-treated or control cells were harvested at 15 min and oxidation to the fluorophore dichlorofluorescein (DCF) was detected by confocal fluorescence microscopy analysis.

2.3. Real-time polymerase chain reaction (PCR)

The mRNA levels of peroxisomal citrate synthase and isocitrate dehydrogenase isoform 1 encoding gene (*CIT2* and *IDH1*, respectively) were determined in exponentially growing cells (OD_{600} = 0.7) and in acetic acid-treated or control cells. 20 ml of cell suspension were centrifuged at 3000 ×*g*. Cell pellets were either stored at −80 °C or immediately used to extract total RNA with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) through mechanical disruption of cell walls with glass beads by Tissue Lyser (Qiagen). 1 μg RNA ($OD_{260}/OD_{280} \geq 1.9$) reverse transcription was immediately performed using QuantiTect Reverse Transcription Kit (Qiagen) and cDNA directly used for real-time PCR analysis or stored at −80 °C. Real-time PCR was carried out by QuantiTect SYBR Green PCR Kit (Qiagen) on an ABI Prism 7000 system using the following primer pairs: for *CIT2*: (F) 5′-CGGTTATGGTCATGCTGTGCT-3′ and (R) 5′-GGTCCATGGCAAACTTACGCT-3′; for *IDH1*: (F) TCGACAATGCCTCCATGCA and (R) AAAGCAGCGCCAATGTTGC; for *ACT1*: (F) 5′-CTTTGGCTCCATC TTCCATG-3′ and (R) 5′-CACCAATCCAGACGGAGTACTT-3′. The amount of *CIT2* and *IDH1* mRNA normalized with *ACT1* mRNA was calculated in arbitrary units (a.u.) using the standard curve method.

Table 1
Strains of *Saccharomyces cerevisiae* used in this study.

Strain (name)	Genotype	Reference/source
W303-1B (WT)	MATα ade2 leu2 his3 trp1 ura3 ρ^+	X.J. Chen's lab*
W303-1B (ρ°)	MATα ade2 leu2 his3 trp1 ura3 ρ°	This study
Δrtg2	W303-1B rtg2Δ::LEU2	This study
Δrtg3	W303-1B rtg3Δ::LEU2	This study
Δhap4	W303-1B hap4Δ::kanMX4	This study
Δhap4Δrtg2	W303-1B rtg2Δ::LEU2 hap4Δ::kanMX4	This study
Δmks1	W303-1B mks1Δ::kanMX4	This study

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2.4. Mitochondrial membrane potential and O_2 consumption assay

Mitochondrial membrane potential was monitored essentially as in [24]. Briefly, cells (OD_{600} = 0.6) exponentially growing in YPD or YPR were incubated with 500 nM tetramethylrhodamine methyl ester (TMRM) (λ_{ex} 540/λ_{em}590) (Life Technologies) for 45 min at 26 °C in the dark and then resuspended (10^7 cells/ml) in the same medium adjusted to pH 3.00 with HCl, containing 500 nM TMRM. An aliquot was also incubated with 20 μM CCCP for 15 min to collapse the membrane potential. We confirmed that 20 μM CCCP was not toxic, but can inhibit the growth of WT cells on a non-fermentable carbon source. Cells (about $5x10^5$ cells/ml) were collected and resuspended in phosphate buffer saline (PBS) and analyzed with a FACS Calibur (Becton Dickinson) flowcytometer. Monoparametric detection of fluorescence was performed using FL-2 and data were analyzed using WinMDI 2.9 software. Background fluorescence without dye was analyzed in both glucose and raffinose and subtracted.

O_2 consumption was continuously measured at 25 °C in a thermostatically controlled chamber equipped with a Clark oxygen electrode (Oxygraf, Hansatech Instruments). In a typical experiment cell suspension ($1 x 10^7$ cells/ml) in YPD or YPR medium (final volume equal to 1 ml) was used. The oxygen uptake rate was measured as a tangent at the initial part of the progress curve and expressed as nmol O_2 min^{-1} cell $number^{-1}$. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and antimycin A (Sigma Aldrich) were dissolved in dimethyl sulphoxide and ethanol, respectively; control was made that the solvents have no effect on the electrode sensitivity.

2.5. Protein extraction and cell fractionation

Trichloroacetic acid (TCA) precipitation of total yeast cell proteins was carried out as described by Dilova and Powers [25], with some modifications. 5 ml of cell culture grown to exponential phase (OD_{600} = 0.7) were harvested by centrifugation for 5 min at 3900 rpm (Sepatech omnifuge 2.0 RS, Heraeus). Cells were lysed in 0.225 M NaOH/1 % 2-mercaptoethanol, 10 mM NaF, 1 mM Na_3VO_4 and 2 mM phenylmethylsulfonyl fluoride and proteins were precipitated with 6.1 % TCA. The protein pellet was then washed with 1 M Tris-HCl (pH 6.8) and resuspended in SDS PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 100 mM DTT, 0.002% bromophenol blue). Cytosolic and mitochondrial fractions were isolated from 200 to 400 ml of acetic acid-treated or control cell culture as described in [26]. Protein concentration was determined using the Bradford assay [27].

2.6. Immunoblotting

To detect Rtg3p phosphorylation, equivalent amounts of total cellular protein extracts were loaded on 7.5 % SDS-PAGE gels and transferred to polyvinylidenefluoride (PVDF) membranes (Millipore, Immobilon-P 0.4 μm) by semidry transfer units TE 70 (Amersham Biosciences). Membranes were probed using polyclonal anti-Rtg3p antibody and monoclonal anti-phosphoglycerate kinase (anti-Pgk1p) antibody (Molecular Probes). Immunodetection was performed with

horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using chemiluminescence Western blotting reagents (Amersham ECL Western Blotting Detection Reagent, GE Healthcare Life Sciences). To analyze cyt *c* release from mitochondria, either cytosolic or mitochondrial fractions (10 μg of proteins) were loaded onto a 12% SDS-polyacrylamide gel, separated and transferred to a PVDF membrane which was probed with different antibodies: polyclonal anti-cyt *c*, kindly provided by Fred Sherman (University of Rochester Medical Center, Rochester, NY-USA), anti-Pgk1p and polyclonal anti-acetohydroxyacidreductoisomerase (Ilv5p). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies using enhanced chemiluminescence Western blotting reagents. Densitometric values for immunoreactive bands were quantified using the Electrophoresis Documentation and Analysis System 120 (Kodak). Protein levels were calculated as a percentage of untreated cells taken as 100 in arbitrary units after normalization based on the amount of Pgk1p or Ilv5p, for cytosolic and mitochondrial fraction in each lane on the same filter.

3. Results

3.1. Yeast cells grown on raffinose are resistant to AA-PCD induction and show RTG pathway activation

In order to gain some insights into the role of mitochondrial metabolism on yeast cell sensitivity to AA-PCD we used either GLU-WT cells, already used in AA-PCD investigations [23] or RAF-WT cells, in which whether AA-PCD occurs has not been investigated to date. In the former case a glycolytic energy metabolism occurs, in the latter energy production results mostly from the mitochondrial respiration [28,29]. Thus cell viability was measured as a function of time after AA-treatment in either GLU- or RAF-WT cells (Fig. 1). As in [23] GLU-WT cell viability decreased along time and was zero 200 min after AA addition; this occurred via AA-PCD as shown by 90% positive cells of TUNEL which is a marker of PCD occurrence (Fig. 1A and B). Contrarily, the viability of RAF-WT cells remained mostly constant with 80% viability at 200 min and with only about 10 % of DNA fragmentation at 150 min (Fig. 1A and B).

To find out how AA-PCD resistance occurs, in both cases we investigated whether the RTG pathway was activated. Notice that it has been reported previously that in glucose-grown cells, the RTG pathway is repressed in respiratory-deficient (ρ°) cells [19,30], whereas in raffinose-grown cells it is activated in response to mitochondrial dysfunction [31]. Therefore we generated a variety of mutants; one of them was a mutant lacking *RTG2* (RAF-Δrtg2) encoding a positive regulator of the RTG pathway which is known to regulate *RTG3*, encoding Rtg3p, a transcription factor of the RTG pathway [32]. Since Rtg2p can also play a role in a manner independent of Rtg3p, cells lacking *RTG3* [32] grown in raffinose (RAF-Δrtg3) were also used.

Accordingly, both RAF-Δrtg2 and RAF-Δrtg3 cells were compared to GLU-WT cells and, more importantly to RAF-WT cells with respect to both cell viability and DNA fragmentation upon AA-treatment. In contrast to RAF-WT, both RAF-Δrtg2 and RAF-Δrtg3 cells showed a progressive decline in cell viability, with viability lower than that of WT cells already at 90 min, but 40 % viability was found at 200 min of AA-treatment in both cases (Fig. 1A). The AA-PCD occurrence was shown by a 6-fold increase of TUNEL positive cells (60 %) observed with respect to RAF-WT cells (Fig. 1B). Comparison made of the time course rates showed a lower death rate for RAF-Δrtg2 and RAF-Δrtg3 cells with respect to that of GLU-WT cells ($0.0020 \pm 0.0005\ min^{-1}$ and $0.0020 \pm 0.0001\ min^{-1}$, respectively, versus $0.015 \pm 0.002\ min^{-1}$). This shows that, when the RTG pathway was abolished due to *RTG2*/*RTG3* deletion, differently from WT, yeast cells even if grown on raffinose can undergo AA-PCD. In contrast, the route to AA-PCD of GLU-WT and GLU-Δrtg2/Δrtg3 cells did not differ from one another (data not shown).

Since the change of the carbon source from glucose to raffinose results in the activation of the Hap2,3,4,5 transcriptional complex [12,33] which causes de-repression of a variety of mitochondrial proteins, including components of the electron transport chain and citric acid cycle enzymes, either RAF-WT or RAF-Δrtg2 cells lacking *HAP4* were also used. Both Δhap4 and Δrtg2Δhap4 cell viability in response to AA-treatment did not differ along time from that of WT and Δrtg2 cells, respectively

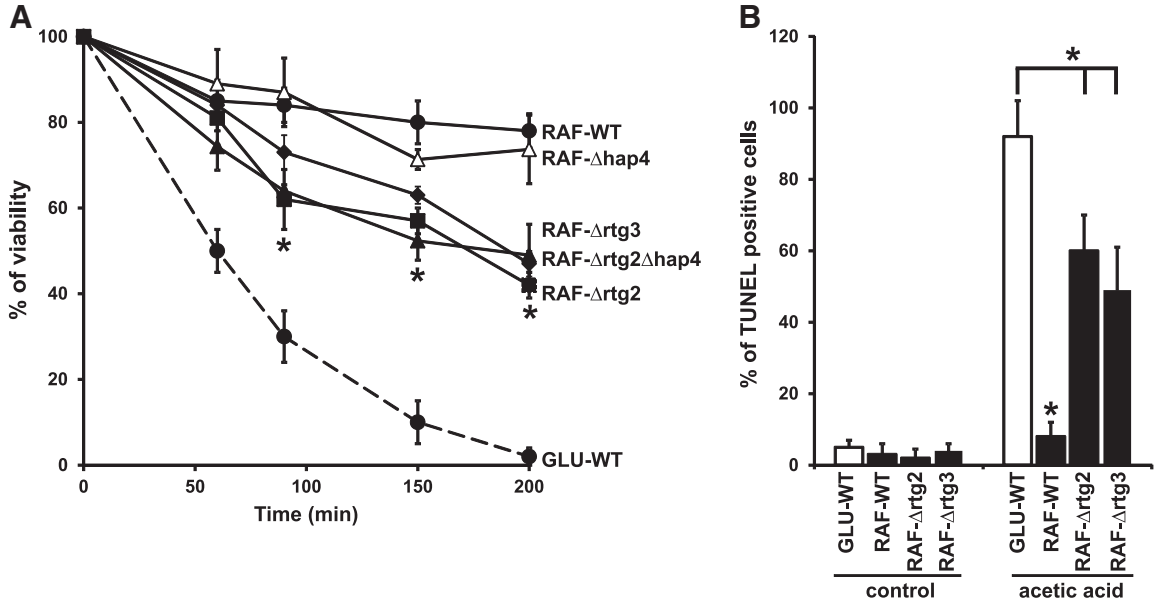

Fig. 1. W303-1B yeast cells are resistant to AA-PCD in an RTG2-dependant manner. (A) WT (●), Δrtg2 (■), Δartg3 (♦) Δhap4 (Δ) and Δrtg2Δhap4 (▲) cells were treated with acetic acid (AA) in the presence of glucose (GLU) (dashed line) or raffinose (RAF) (solid line) as the sole carbon source. Cell viability was analyzed at indicated times by measuring colony-forming units (cfu). Cell survival (100%) corresponds to the cfu at time zero. The means of six independent experiments with standard deviations are reported. Student's t-test: statistically significantly different with (*) $p < 0.005$ when comparing WT *versus* Δrtg2 cells in raffinose. Death rates were calculated as the slope of the linear part of the semi-logarithmic plot of the number of cfu as a function of time (see text). (B) DNA fragmentation was detected by using the TUNEL assay. Percentage of TUNEL positive cells is reported at 150 min. At least 400 cells were analyzed in three samples from each of three independent experiments. Student's t-test: statistically significantly different with (*) $p < 0.0001$ when comparing AA-treated WT cells in glucose (white bars) with AA-treated Δrtg2 and Δrtg3 cells in raffinose (black bars) or AA-treated WT cells in raffinose.

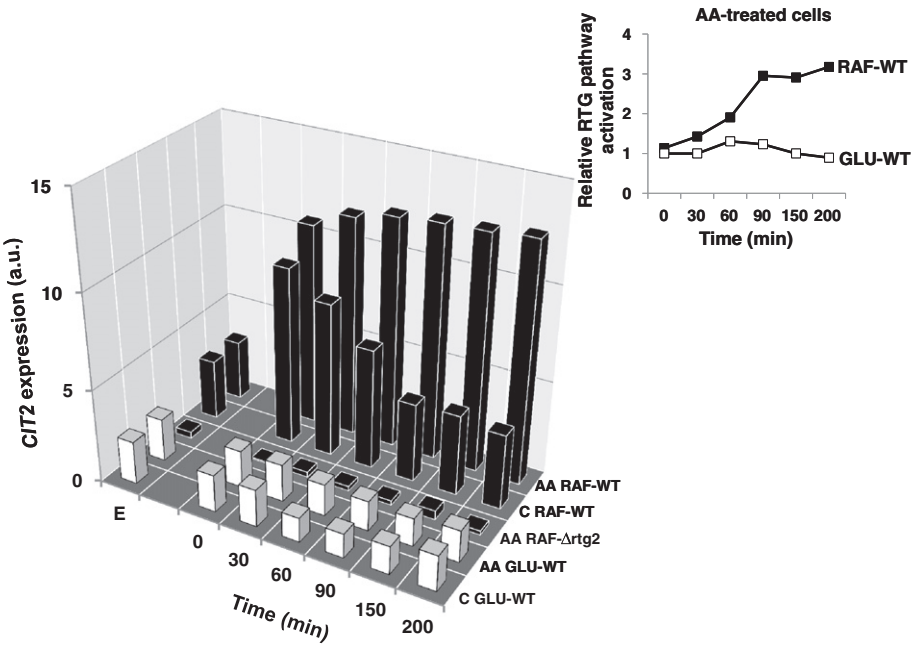

Fig. 2. The RTG pathway activation as a function of time after acid acetic treatment in WT and Δrtg2 cells. *CIT2* mRNA levels were measured by real-time PCR at the indicated times in WT and Δrtg2 cells grown either in glucose (GLU) (white bars) or in raffinose (RAF) (black bars) in the absence of acetic acid (C) or after acetic acid treatment (AA). E refers to cells before starting the AA-treatment (for details see the methods). *CIT2* mRNA levels, normalized to that of *ACT1* mRNA, was reported in arbitrary units (a.u.). In the inset, relative RTG pathway activation, calculated as the ratio between *CIT2* mRNA levels in acetic acid-treated and control RAF-WT cells, is plotted as a function of time.

(Fig. 1A), suggesting that Hap2,3,4,5-dependent transcription is not involved in AA-PCD resistance in RAF-WT cells.

To confirm definitely that the RTG pathway activation is involved in AA-PCD resistance in raffinose-grown cells, we analyzed by real-time PCR *CIT2* mRNA expression, whose up-regulation is a marker of RTG pathway activation [31]. This was made either in RAF-WT or GLU-WT cells *en route* to AA-PCD (Fig. 2). As expected, in GLU-WT cells, no *CIT2* up-regulation was found: *CIT2* mRNA levels were low (≈2 a.u.) and remained constant even after the shift to pH 3.00, and AA addition. In contrast, in RAF-WT cells, the level of *CIT2* mRNA was found to increase up to about 3-fold with respect to the basal level (similar to that in GLU-WT cells) due to pH shift to 3.00 both in the absence and in the presence of AA-treatment, this showing that RTG pathway activation had occurred. However, *CIT2* expression in cells treated or not with acetic acid proved to be significantly different over time: cells shifted to pH 3.00 but without acetic acid addition, showed *CIT2* level decreasing along time up to a constant level in 90–200 min time range, whereas in AA-treated cells *CIT2* mRNA levels remained relatively constant. Notice that the relative RTG pathway activation increased progressively in AA RAF-WT cells which are AA-PCD resistant cells: the ratio of *CIT2* mRNA level between AA-treated and control cells at any times increased along time up to about 2 and 3-fold in 60–200 min interval (inset to Fig. 2). The expression of *IDH1*, another RTG-target gene encoding for isocitrate dehydrogenase isoform 1 [34], was also monitored, showing up-regulation of this gene after AA-treatment in RAF-WT cells (not shown). This result confirms definitely that AA-PCD resistance is found in RAF-WT cells together with a robust RTG pathway activation. Accordingly, *RTG2* deletion resulted in the lack of *CIT2* expression in RAF-Δrtg2 cells (Fig. 2).

3.2. Rtg3p dephosphorylation correlates with AA-PCD resistance in raffinose-grown cells.

Further investigation of the involvement of RTG pathway in AA-PCD resistance was made by monitoring the Rtg3p phosphorylation state. It should be noted that both basal and up-regulated *CIT2* transcription correlates with the nuclear localization of the Rtg3p transcription factor [35]: in the cytosol it is usually in a hyper-phosphorylated state; an Rtg2p-dependent partial dephosphorylation of Rtg3 results in its translocation from the cytoplasm to the nucleus [31] with attendant activation of RTG-target gene expression [25,35]. Thus we investigated whether the Rtg3p phosphorylation state was affected in AA-treated cells grown in raffinose. This was made by immunoblotting with anti-Rtg3p antibody, being the hyper-phosphorylated state shown by the slow mobility of the Rtg3p-immunoreactive band which is smeared likely due to multiple phosphorylations. Equal loading of samples was confirmed by probing membranes with monoclonal anti-Pgk1p antibody, a cytosolic marker protein. RAF-WT cells *en route* to AA-PCD showed increased mobility of the Rtg3p immunoreactive band (Fig. 3, lanes A4–A9) with respect to cells before AA-treatment (Fig. 3, lane A3), i.e. a continuous Rtg3p partial dephosphorylation which correlates with the maximum AA-PCD

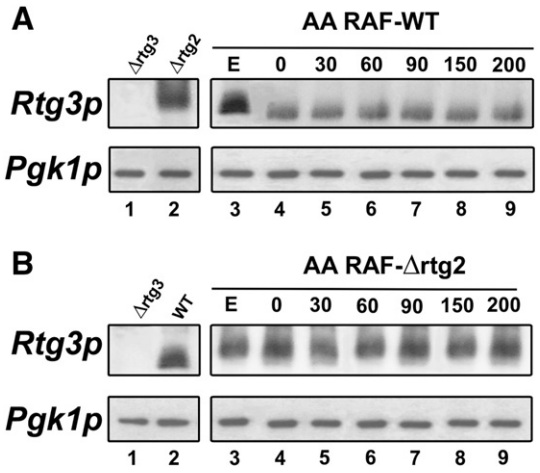

Fig. 3. Rtg3p phosphorylation in WT and Δrtg2 cells treated with acetic acid in raffinose. Cell protein extracts were prepared from (A) RAF-WT and (B) RAF-Δrtg2 cells before (E) and after acetic acid (AA) treatment at the indicated times and analyzed by immunoblot with anti-Rtg3p and anti-Pgk1p antibodies. Cell extracts from Δrtg3 cells have been analyzed as a negative control.

resistance of cells (see Fig. 1); as a control we monitored the maximum Rtg3p hyper-phosphorylated state, as that measured in cells lacking *RTG2* (Fig. 3, lanes A2, B3–B9) which are partially resistant to AA-PCD (see Fig. 1). As expected, we found that in AA RAF-Δrtg2 cells Rtg3p phosphorylation state was constant. Since Rtg3p hyper-phosphorylation correlates with RTG pathway inactivation, as detected by the lack of *CIT2* expression (see Fig. 2), we propose that, based on data in Fig. 3, AA-PCD resistance is not solely dependent on *RTG2* capability to modulate Rtg3p phosphorylation state, but RTG2 itself also play a role in AA-PCD resistance independent of *RTG3*.

A deletion mutation in *MKS1*, encoding a negative regulator of the RTG pathway, results in RTG pathway activation [25,36,37]. After establishing that RTG pathway activation did not occur in GLU-WT cells, we decided to determine whether RTG pathway activation could itself make GLU-cells resistant to AA-PCD. To that end, we investigated *CIT2* expression, cell viability and AA-PCD occurrence in GLU-WT and *MKS1*-knock-out (GLU-Δmks1 cells) cells before and after AA-treatment. In any case, in GLU-Δmks1 cells sustained *CIT2* up-regulation was found with respect to GLU-WT cells either with or without AA-treatment (Fig. 4A). Yet, deletion of *MKS1* failed to cause AA-PCD resistance: both the time course of GLU-Δ*mks1* cell viability and the percentage of GLU-*Δmks1* cells showing DNA fragmentation were indistinguishable from the ones measured in GLU-WT cells (Fig. 4C, D). RAF-Δmks1 resistance to AA-PCD was similar to that found in RAF-WT (not shown). Having established that *MKS1*-dependent activation of RTG pathway does not result in AA-PCD resistance in glucose-grown cells, we investigated whether glucose repression was somehow involved in the failure of RTG activation in preventing AA-PCD. To test this Δmks1 and WT cells were grown in 0.5 % glucose (0.5 % GLU-Δmks1 and 0.5 % GLU-WT cells), where glucose repression is not significant, and analyzed as for *CIT2* expression, cell viability and DNA fragmentation after AA-treatment (Fig. 4). 0.5% GLU-WT cells showed a moderate increase in their viability in 0–200 min range. Such an AA-PCD resistance proved to be higher in 0.5%GLU-Δmks1 cells (Fig. 4C). Interestingly TUNEL test assay showed that only 20% 0.5%GLU-Δmks1 cells showed DNA fragmentation after AA-treatment (Fig. 4D). RTG pathway was activated in 0.5%GLU-Δmks1 with respect to 0.5%GLU-WT cells, but at a lower level with respect to GLU-Δmks1 cells (Fig. 4A, B). Such activation was found to remain constant up to 200 min with or without AA-treatment as for GLU-Δmks1 cells.

3.3. Activation of RTG pathway due to cell shift to pH 3.00 takes place in yeast containing coupled mitochondria

After establishing that in raffinose-grown cells RTG pathway activation already occurs as a result shift to pH 3.00 along with previous

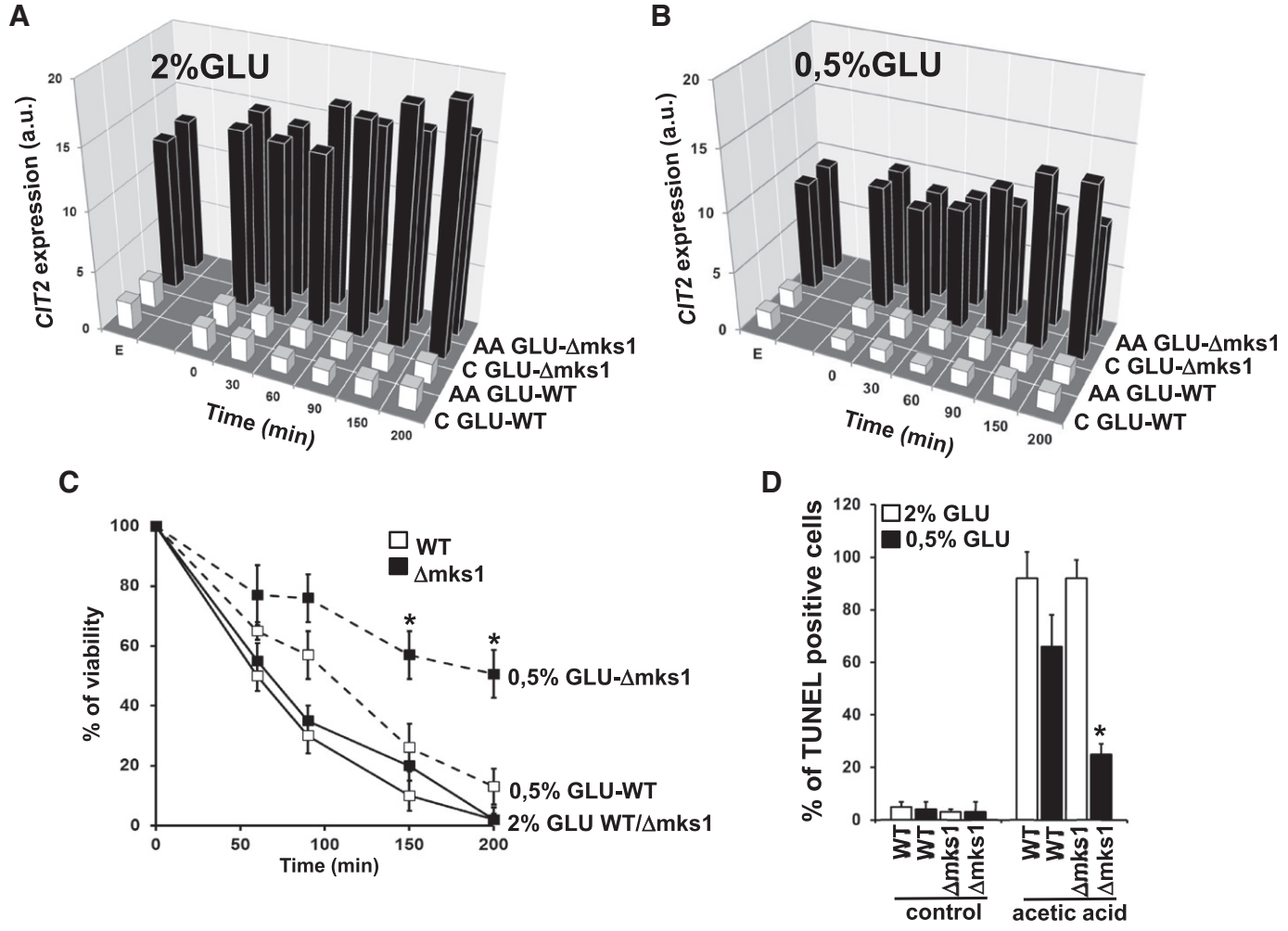

Fig. 4. The effect of *MKS1* deletion on *CIT2* expression and AA-PCD in W303-1B yeast cells grown in glucose. *CIT2* mRNA levels were measured by real-time PCR at the indicated times in WT and *Δmks1* cells grown either in 2% (A) or 0.5% (B) glucose (GLU), in the absence of acetic acid (C) or after acetic acid treatment (AA). E refers to exponential phase cells before starting AA-treatment. *CIT2* mRNA level, normalized to the level of *ACT1* mRNA, was reported in arbitrary units (a.u.). (C) WT (□) and Δmks1 (■) cells were treated with acetic acid in the presence of either 2% (solid lines) or 0.5% glucose (dashed lines) as carbon source. Cell viability was calculated at indicated times by measuring colony-forming units (cfu). 100% cell viability corresponds to the cfu at time zero. The means of six independent experiments with standard deviations are reported. (D) DNA fragmentation was detected by using the TUNEL assay. Percentage of TUNEL positive cells is reported at 150 min. At least 400 cells were analyzed in three samples from each of three independent experiments. WT, white bars; Δmks1, black bars; GLU, glucose.

observations that RTG pathway activation is currently associated with mitochondrial dysfunction [15,31], we investigated whether the change in the carbon source and the shift to low pH could have impaired the mitochondrial coupling. To this aim we first resorted to a procedure which allows for the detection of cells in which mitochondrial membrane potential was present. Thus we used the mitochondrial membrane potential-sensitive probe TMRM which accumulates into polarized mitochondria and equilibrate quickly [24,38,39]. The basic TMRM fluorescence of both RAF-WT and GLU-WT cells measured at pH 3.00 was largely decreased as a result of the addition of the uncoupler CCCP (20 μM), showing the $\Delta\Psi$ collapse (Fig. 5A). To confirm that no mitochondrial dysfunction barely takes place as a result of shift to pH 3.00, we monitored the ability of mitochondria to take up oxygen in a manner sensitive to the uncoupler CCCP. In an initial series of experiments we monitored the continuous oxygen uptake by exponentially growing (E) yeast cells incubated in their growth medium and checked whether the uncoupler CCCP could increase the rate of oxygen consumption. As expected, due to their glycolytic metabolism, GLU-WT cells can take up oxygen less effectively than RAF-WT cells can do. In a typical experiment oxygen consumption rate was 3 *versus* 11 nmol oxygen $\times$ min^{-1} $\times$ 10^7 $cells^{-1}$ in E GLU-WT and E RAF-WT cells, respectively (Fig. 5B). In E GLU-WT and E RAF-WT cells at neutral pH, 1.25 μM CCCP addition also resulted in an increase of the oxygen consumption rate from 3 and 11 up to 6 and 15 nmol oxygen $\times$ min^{-1} $\times$ 10^7 $cells^{-1}$, respectively. In any case 0.8 μM antimycin A, which inhibits the electron flow along the respiratory chain, proved to block oxygen consumption. The respiratory control ratios (RCR) were calculated in either E GLU-WT or E RAF-WT cells and expressed as the ratio between the rate of oxygen consumption due to addition of the uncoupler CCCP and that in the absence of the uncoupler (Fig. 5B). They were 2 and 1.4, respectively. After shift to pH 3.00 without acetic acid treatment (C), RCRs were 2.0 and 1.7 in GLU-WT and RAF-WT cells, respectively; mean RCR ($\pm$SD) values were 1.8 ($\pm$0.2) and 1.4 ($\pm$0.2) in E GLU-WT and E RAF-WT cells, respectively, *versus* 1.8 ($\pm$0.2) and 1.8 ($\pm$0.2) at pH 3.00. In parallel, we confirmed that RAF-WT cells show RTG pathway activation, as reported above.

3.4. Comparison of ROS production and cytochrome c release occurring in AA-treated raffinose- or glucose-grown cells

To gain a first insight into the mechanism by which AA-PCD is mostly prevented in RAF-WT cells by RTG pathway activation, but moderately occurs in RAF-Δrtg2 cells, investigation of how AA-PCD takes place should be necessary, as already made in GLU-WT cells. To this aim, we investigated two yeast AA-PCD features, namely ROS production and cyt *c* release, which represent an early (15 min) and late event (60–150 min), respectively, *en route* to AA-PCD of GLU-WT cells [6]. ROS levels were analyzed at 15 min AA-PCD by using H_2DCF-DA [40,41] (Fig. 6). In GLU-WT cells, as a result of AA-treatment a burst of ROS was observed at 15 min, with about 67 % of DCF positive cells with a very low ROS level measured in control cells. In RAF-WT at 15 min only about 22 % of DCF positive cells was found whereas in RAF-Δrtg2 cells about 68 % of DCF-positive cells were found.

Cyt *c* protein levels in both cytosolic and mitochondrial fractions obtained from GLU-WT, RAF-WT and RAF-Δrtg2 cells added or not with acetic acid were analyzed at 150 min by immunoblotting using anti-cyt *c* antibody (Fig. 7). Anti-Pgk1p or anti-Ilv5p antibodies were also used as cytosolic and mitochondrial matrix marker proteins respectively as in [26]. No difference in the released cyt *c* was observed in the cytosolic fraction of both RAF-WT and RAF-Δrtg2 cells after AA-treatment with respect to the same bands in the control cells as shown by densitometric analysis (about a 60% increase in both cases) (Fig. 7A). Accordingly, a decrease in mitochondrial cyt *c* content was observed after acetic acid treatment in both RAF-WT and Δrtg2 cells but to a lesser extent (about 20 %) than in the case of AA-PCD in GLU-WT (about 50%) (Fig. 7B). Notice that the release of cyt *c* into the cytosol of GLU-WT cells [26] was higher than in the case of RAF-WT or RAF-Δrtg2 cells (150 % vs. 60 %).

4. Discussion

We show here that W303-1B yeast cells have a different sensitivity to PCD induced in response to acetic acid dependent on the sugar used as a sole carbon source for their growth. In this investigation use

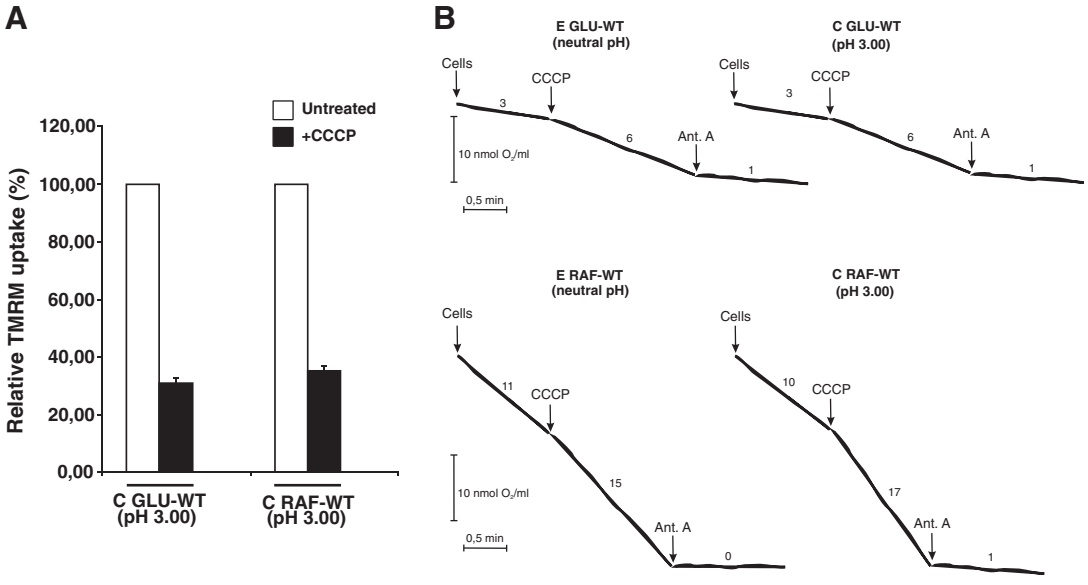

Fig. 5. Mitochondrial membrane potential and oxygen consumption by yeast cells grown either in glucose or raffinose. (A) Quantification of mitochondrial membrane potential (relative TMRM uptake) of exponential phase (OD_{600} = 0.6) GLU-WT and RAF-WT cells after shift to pH 3.00 (C) was assessed by flow cytometry using the probe TMRM. The geometric mean fluorescence intensities, obtained by analysing monoparametric histograms of TMRM fluorescence [FL-2 area (log)] of cells, were used to calculate relative TMRM uptake. Fluorescence of C GLU-WT and C RAF-WT cells at pH 3.00 was considered as 100 %. Cells were also treated with 20 μM CCCP. 30,000 cells were analyzed per sample and the results are the mean values of three independent experiments. (B) Oxygen consumption was continuously measured in GLU-WT and RAF-WT cells before (E) and after pH shift to 3.00 (C). When the rates, obtained as tangent to the progress traces, were constant, 1.25 μM CCCP was added and respiratory control ratio (RCR) calculated (see text). Antimycin A was added at the concentration of 0.8 μM. Numbers along the traces are rate of oxygen uptake expressed as nmol O_2 min^{-1} cell $number^{-1}$ from one representative experiment out of three experiments with different yeast cultures.

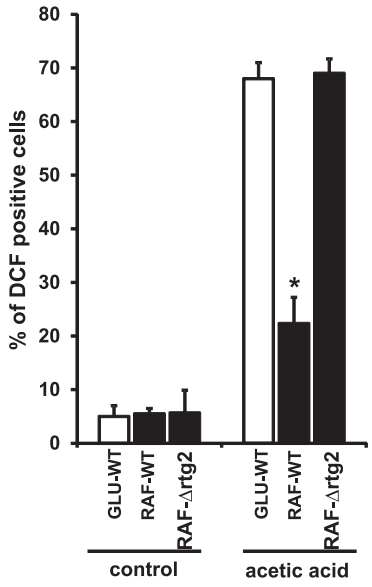

Fig. 6. Intracellular ROS levels in WT and Δrtg2 treated with acetic acid in raffinose. RAF-WT and RAF-Δrtg2 cells were incubated with H_2DCF-DA in the absence (control) or in the presence of acetic acid for 15 min. Cells were collected and DCF-stained cells were detected by confocal microscopy. Percentage of DCF-positive cells was calculated by analyzing at least 400 cells in three samples from each of three independent experiments. Student's t-test: statistically significantly different with (*) $p < 0.0005$ when comparing Δrtg2 with WT AA-treated cells. YPD, white bars; YPR, black bars.

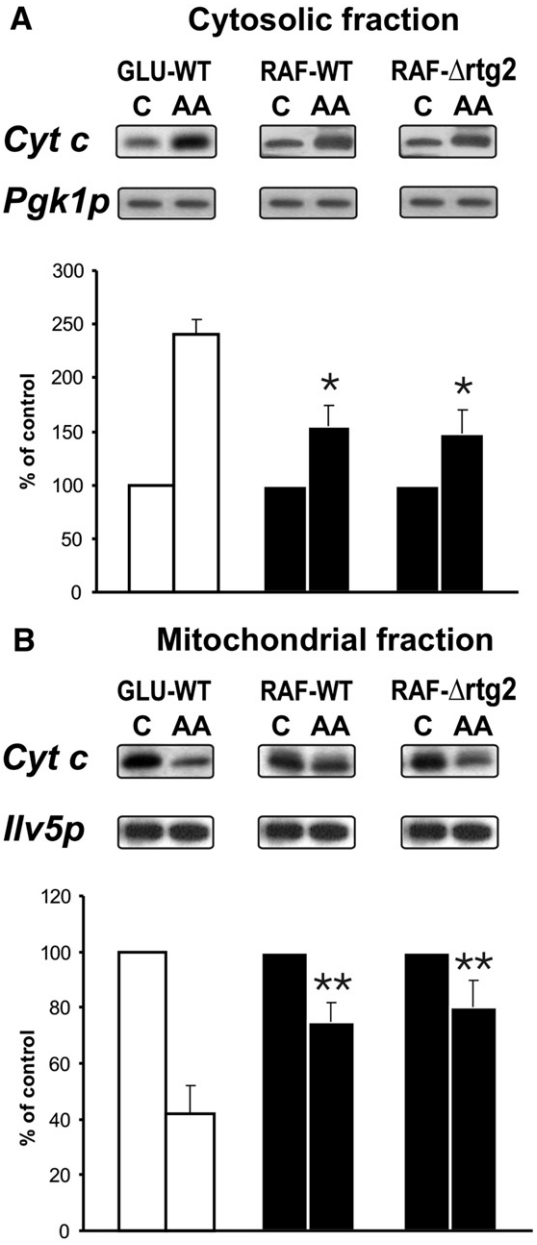

Fig. 7. Immunoblot analysis of the released cyt *c*. (A) Cytosolic and (B) mitochondrial fractions from WT and Δrtg2 cells treated with acetic acid (AA) or without acetic acid (C) in raffinose (RAF, black bars) for 150 min were analyzed by immunoblotting with anti-cyt *c* antibody. As a control, the two fractions from AA-treated (AA-PCD) or control WT cells in glucose (GLU, white bars) were similarly analyzed. The histograms report the cytosolic and mitochondrial amounts of cyt *c*, quantified by densitometric scanning of the film, normalized with antibodies against Pgk1p and Ilv5p, respectively, and expressed as a percentage of control cells, to which a value of 100 has been given. Reported values are the means of triplicate measurements and representatives of at least three different experiments. Student's t-test: statistically significantly different with * $p < 0.001$, ** $p < 0.01$, when comparing subcellular fractions from cells treated with acetic acid in raffinose with fractions from WT cells treated with acetic acid in glucose.

was made of either glucose or raffinose: differently from GLU-WT, RAF-WT cells are mostly resistant to AA-PCD (Fig. 1); accordingly, only in RAF-WT cells the activation of RTG pathway takes place in a manner dependent on a variety of genes including *RTG2*, *RTG3*, *MKS1*. *HAP4*, encoding the regulatory subunit of the Hap2,3,4,5 complex has no role in this case (Figs. 1–4). In particular, we show that AA-PCD resistance of RAF-WT cells occurs in a manner regulated by *RTG2*-dependent phosphorylation state of Rtg3p, but it is also regulated by *RTG2* itself (cfr Figs. 1–3). Interestingly, we show that constitutive activation of RTG pathway itself in GLU-Δmks1 cells does not result in AA-PCD resistance, but that partial resistance to AA-PCD occurs when 0.5% GLU-WT cells are used (Fig. 4). RTG pathway activation occurs in cells without apparent mitochondrial dysfunction where coupled mitochondrial respiration is still active (Fig. 5). Finally, we show that in yeast cells resistant to AA-PCD both ROS accumulation (in an *RTG2*-sensitive manner) and cyt *c* release, events which are markers of early and late AA-PCD respectively, occur. These points will be dealt with separately.

RAF-WT cells were found mostly resistant to AA-PCD with a simultaneous RTG pathway activation. That RTG pathway activation occurs in RAF-WT cells is shown by the up-regulation of RTG-target genes, i.e. *CIT2* and *IDH1*. Such a conclusion is definitely confirmed by the correlation found between RTG pathway activation and *i.* the effects of deletion of RTG pathway regulatory genes and *ii.* the Rtg3p phosphorylation state. Use of yeast cells lacking *RTG2* or *RTG3*, a positive regulator and a transcription factor of the RTG pathway, respectively, shows that lack of RTG pathway activation is accompanied by lack of AA-PCD prevention. In fact, when either *RTG2* or *RTG3* is deleted, the consequent failure in RTG pathway activation is accompanied by AA-PCD. The change in the Rtg3p phosphorylation state dependent on *RTG2* in raffinose-grown cells, treated with AA, shows that Rtg3p is regulated by *RTG2*, but *RTG2* itself is a contributor to AA-PCD resistance. Since partial AA-PCD resistance is still found in RAF-Δrtg2 and RAF-Δrtg3 cells, we are forced to assume that the change of yeast metabolism itself results in AA-PCD resistance in a manner independent on RTG pathway. The explanation of how this can occur needs further investigation. On the other hand, that the RTG pathway significantly contributes, but is not unique in determining AA-PCD resistance is shown by Fig. 4 in which the AA-PCD time courses of GLU-WT and in GLU-Δmks1 cells are essentially indistinguishable. This shows that AA-PCD induction is likely due to yeast metabolism under fermentative growth conditions as occurring in GLU-WT cells; in glucose-grown cells no AA-PCD resistance is possible even by constitutively activating RTG pathway, whereas under condition in which yeast respiration occurs (RAF-cells) RTG pathway activation contributes to AA-PCD resistance. Accordingly, the role of yeast metabolism in AA-PCD resistance is also shown in 0.5%

GLU-WT cells (Fig. 4). Under this condition, in which no glucose repression takes place, very low resistance to AA-PCD is found in 0.5% GLU-WT cells. In 0.5% GLU-Δmks1 cells, an increase in AA-PCD resistance is found due to AA-treatment; thus we could speculate that the lack of glucose repression itself favours AA-PCD resistance and it is a prerequisite for AA-stress resistance; yet another event is necessary, likely due to yeast/mitochondrial metabolism, for a rather complete AA-PCD resistance to occur. Such a hypothesis needs further investigation.

Since the genes encoding mitochondrial citrate synthase, aconitase and isocitrate dehydrogenase, switch between a dependence on Rtg1/3p, in cells whose mitochondrial respiratory capacity is reduced, and on Hap2,3,4,5p transcription complex in cells with robust mitochondrial function [34,42], we investigated the role of Hap2,3,4,5p transcription complex in AA-PCD resistance. This is excluded having found that both RAF-Δhap4 and RAF-Δrtg2Δhap4 cells do not differ from RAF-WT and RAF-Δrtg2 with respect to AA-PCD resistance. Thus, other transcription factors regulating glucose repression may be involved in AA-PCD resistance of RAF-WT cells.

We show that an RTG transient activation takes place as a result of cell pH shift to 3.00: the huge activation, about 200 % found immediately and up to 30 min after pH shift, decreases up to basal levels in 90-200 min time range. To date the RTG pathway activation is reported as strictly related to the occurrence of mitochondrial dysfunction; to the best of our knowledge we first show that RTG pathway activation can occur in yeast cells in which mitochondrial oxidative phosphorylation is active: cells shifted to pH 3.00 still maintain coupled mitochondria with RTG pathway activation. A first indication derives from the TMRM experiment which shows that both in GLU-WT and RAF-WT cells at pH 3.00 there is a cell population with coupled mitochondria exhibiting the TMRM fluorescence due to the probe binding to polarized mitochondrial membrane and that as a result of the addition of the uncoupler CCCP such a fluorescence decreases by about 75%. Given that one could argue that the fluorescence signal might involve unspecific probe binding to some cell membrane components [38,43] and since the experimental procedure does not exclude changes in the mitochondrial membrane potential along time, we resorted to a simple method in which the respiratory control ratio is measured by continuous monitoring of the oxygen uptake of the yeast cell suspensions. The point is that CCCP addition to cells grown in either glucose- or raffinose-medium shifted to pH 3.00, in which RTG pathway is active, results in an increase of the rate of oxygen consumption with a mean RCR equal to 1.8 in either cases, similar to that found in the controls with no RTG pathway activation.

We show that *en route* to AA-PCD early ROS production in GLU-WT [9,40,41] and in RAF-Δrtg2 cells was essentially unchanged, but it strongly decreases in RAF-WT cells. This strongly suggests that in RAF-cells, ROS production is largely under *RTG2* control. Since in RAF-WT cells, which are mostly resistant to AA-PCD, lower early ROS accumulation is detected with respect to that found in GLU-WT and RAF-Δrtg2 undergoing AA-PCD, we might assume that RTG pathway activation prevents AA-PCD by decreasing early ROS level accumulation. Whether this depends essentially on ROS production or increase in the scavenger system, including superoxide dismutase and catalase [40] remains to be established. Moreover, since RAF-Δrtg2 cells show partial AA-PCD resistance, we can conclude that ROS production itself is not sufficient to trigger complete AA-PCD in RAF-cells. However, in RAF-WT cells since AA-PCD resistance is high, while ROS production is about 30% of that in RAF-Δrtg2 cells, we might speculate that, even if it is not a committed step of AA-PCD, ROS level is too low thus preventing the processes necessary for AA-PCD to occur, yet it remains high enough to allow for the cyt *c* release. Thus in the light of Fig. 7, in which no difference in cyt *c* release occurs between RAF-WT and RAF-Δrtg2 cells, we have to assume that relatively low ROS production is required to cause cyt *c* release.

In RAF-cells resistant to AA-PCD cyt *c* release occurs at a lower extent with respect to that found in GLU-WT cells. However, given that the release of cyt *c* is essentially unchanged in resistant RAF-WT and partially resistant RAF-Δrtg2 cells, we conclude that other processes, different from cyt *c* release, occur accounting for the different sensitivity to AA-PCD. Perhaps differences in glucose and raffinose yeast metabolism account for differences in AA-PCD sensitivity. Thus AA-PCD resistance is not due to a reduction in cyt *c* release. Different hypothesis could be made to explain the role of cyt *c* release under condition in which AA-PCD is mostly prevented. One of them is that in this case the role of the released cyt *c* is similar to that already shown to occur *en route* to AA-PCD in yeast and in cerebellar granule cells [26,44]: released cyt *c* can work as a ROS scavenger and electron donor to the respiratory chain. The initial experiments carried out essentially are in favour of such a conclusion. On the other hand given that yeast AA-PCD can occur also independently of cyt *c* release [8,9], this could be the case for RAF-Δrtg2 cell AA-PCD. To gain further insight into this issue a detailed investigation of AA-PCD process in RAF-Δrtg2 cells similar to that carried out with GLU-WT cells is needed.

The current yeast RTG pathway scenario is that whatever mitochondrial dysfunction, including respiratory deficiency due to mtDNA depletion (ρ°), antimycin A-dependent inhibition of the electron flow along the respiratory chain, CCCP-dependent uncoupling but not to the loss of mitochondrial ATP synthesis themselves can trigger the RTG pathway [15], although ATP has been suggested as a candidate trigger of RTG signalling [45]. On the other hand, genetic evidence has also shown that loss of mitochondrial membrane potential can trigger RTG pathway extending replicative lifespan [46]. This paper shows for the first time that RTG pathway activation can occur also in yeast cells containing coupled mitochondria.

Having shown that RTG pathway activation contributes to AA-PCD resistance a question arises as to the other contributors to AA-PCD resistance. Another candidate signalling pathway could be that dependent on Hog1 stress-activated protein kinase, which is essential for the induction of diverse osmoadaptive responses in yeast and can control the Rtg1/3p transcriptional complex [47] and acetic-acid stress adaptation [48]. Interestingly enough, RTG-target gene expression can also be activated by exposure of cells to rapamycin, an inhibitor of the target of rapamycin (TOR) kinase signalling pathway that is involved in a broad range of cellular activities, including growth control, nutrient sensing and longevity [49]. This paper shows that RTG pathway activation decreases AA-PCD rate, which is in agreement with the possible role of TOR in causing AA-PCD [50] even though retrograde gene expression is separable from TOR regulation of RTG-responsive genes [51]. The RTG pathway can regulate factor/s involved in AA-PCD that is/are absent or inactive in RAF-Δrtg2 cells. Indeed, *RTG2* deletion could affect pathways different from the RTG pathway and could have opposite effect on the AA-PCD process. Indeed *RTG2* has been shown to exert its function in the epigenetic regulation of gene expression [52] and the mitochondria-vacuole cross-talk [32]. Another possible process involved in raffinose-grown yeast AA-PCD resistance is mitophagy, a process in which mitochondria are selectively removed by autophagy allowing cell adaptation to changing metabolic needs [53,54]. Indeed, RTG signalling has been shown to be important for mitophagy: *AUP1*, encoding a protein phosphatase localized in the mitochondrial intermembrane space, is required for efficient stationary phase mitophagy and RTG pathway activation through regulation of Rtg3p phosphorylation [55].

5. Conclusion

Here we give first experimental evidence of the contribution of the mitochondrial RTG-dependent retrograde response in determining resistance to yeast PCD. It is interesting to note that resistance to cell death is one of the hallmarks of cancer [56] and that mitochondria-to-nucleus retrograde response has been involved in tumorigenesis [57–59]. Our results, together with the role of RTG pathway in extending replicative life span in yeast [60] make yeast an attractive

model to investigate the relations between PCD and mitochondrial dysfunction in both physiological and pathological conditions [61,62].

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