p53 death signal is mainly mediated by Nuc1(EndoG) in the yeast Saccharomyces cerevisiae

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Introduction
The p53 tumor suppressor gene is a major barrier against cancer, preventing tumor development, and promotes apoptosis induced by chemotherapy (Whibley et al., 2009). p53 was first characterized as a transcription factor that, in response to several forms of stress (i.e. DNA damage, hypoxia, oncogenic activation), regulates the expression of genes involved in cell cycle arrest (p21\textsuperscript{waf1}, Mdm2), DNA repair (GADD45), and apoptosis (Bax, Puma, Noxa, p53AP1). In addition, evidences for transcription-independent p53 activities have been accumulating, especially for p53 function in the cytoplasm, where it can mediate a variety of distinct effects such as the regulation of cell cycle (Shinmura et al., 2007; Green & Kroemer, 2009; Galluzzi et al., 2010). Moreover, cytoplasmic p53 interacts with pro-apoptotic proteases of the Bcl-2 family, thus mediating mitochondrial membrane depolarization and triggering apoptosis (Chipuk et al., 2004; Galluzzi et al., 2011).

Recently, it has also been shown that the cytoplasmic pool of p53 represses autophagy through a direct molecular interaction with RB1CC1/Fip200, a protein that is essential for the very apical step of autophagy initiation (Maiuri et al., 2010; Morselli et al., 2011).

In the yeast Saccharomyces cerevisiae, the machinery of the basic apoptotic process seems to be conserved, and in recent years, several homologues of classical apoptotic regulators have been identified and characterized, that is, caspase (YCA1) (Madeo et al., 2002), AIF1 (Wissing et al., 2004), EndoG (endonuclease G, NUC1) (Buttner et al., 2007). Further, it has been recently identified a yeast protein called Ybh3p that harbors a functional BH3 domain that is characteristic of the mammal family proteins BCL-2 (Buttner et al., 2011).

Several studies have shown that yeast (that does not have p53 ortholog) may be an excellent model to study the multitude and growing roles of p53 as an apoptotic regulator (Greenwood & Ludovico, 2010). It has been reported that high level of p53 expression inhibits cell cycle progression in both fission and budding yeast (Nigro et al., 1992; Bureik et al., 1997), represses transcriptionally thioredoxin (TRX1/TRX2), a highly conserved...
multifunctional antioxidant protein family (Hadj Amor et al., 2008), fragmented mitochondrial tubular network (Abdelmoula-Souissi et al., 2011; Muscolini et al., 2011), and triggers cell death with major characteristics of apoptosis in S. cerevisiae (Hadj Amor et al., 2008; Coutinho et al., 2011; Mokdad-Gargouri et al., 2012).

Moreover, human p53 expression induces cell death also in the yeast Pichia pastoris (Abdelmoula-Souissi et al., 2012). We cloned the human gene, p53, under the control of Gal1-Gal10 vector, and we transformed it in both wild-type and S. cerevisiae strains mutated in genes involved in the programmed cell death (PCD) response. We then analyzed the effect of the p53 expression during an apoptotic stimulus (H$_2$O$_2$) and during aging.

**Materials and methods**

**Yeast strains plasmids and growth conditions**

The yeast strains used in this study are BY4741 (MAT a, ura3Δ0, leu2Δ0, met15Δ0, his 3A1) (Brachmann et al., 1998) and its derivative deletion strains yca1::kanMX4, aif1::kanMX4, and nuc1::kanMX4 obtained from EUROSCARF collection (Frankfurt, Germany). Cells were grown at 28 °C in S medium (yeast nitrogen base without amino acids) supplemented with 2% glucose (SD) or 2% galactose (SGal), with auxotrophic requirements added as needed. Solid media were supplemented with 2% Bacto Agar (Difco, Detroit, MI).

For heterologous expression of human p53, coding sequence was inserted under the regulated promoter Gal1-10 in the pESC-HIS vector (Stratagene) as described in the study by Muscolini et al. (2011).

Yeast transformation was performed by the lithium acetate procedure, as described by Gietz et al. (1992). Individual clones were grown overnight in SD (Gal1-10 repressing conditions) until exponential growth phase. To induce p53 expression, cells were washed three times with water and resuspended in minimal medium containing 2% galactose (SGal).

**Cell viability and treatments**

Cell suspensions (5 µL) containing c. 6 x 10$^6$ cells mL$^{-1}$ were poured on a thin layer of YPD agar on a microscope slide. A cover slip was placed over the samples, and after 24 h, viable and unviable cells were scored on the basis of their ability to form microcolonies (Palermo et al., 2007). For each experiment, we checked about 1000 cells for their ability to form or not microcolonies.

For chronological aging experiments, strains were grown in glucose-containing media (SD) to OD$_{600}$ = 0.4; then, after two washes in sterile distilled water, cells were resuspended in 2% galactose-containing media (SGal) for 20 h were harvested, and total proteins, extracted by the TCA method as previously described (Ossig et al., 1991), were separated on 10% acrylamide gel containing SDS. Western blot was performed following standard methods. p53 (IgG1-HRP; Sigma sc-126) antibody was purchased from Santa Cruz Biotechnology. Signals were revealed with Super Signal West Pico chemiluminescent substrate HRP (Pierce).

**Fluorescence microscopy**

For nuclear morphology, cells grown in SD and shifted to 2% galactose media (SGal) for 20 h were fixed with 70% ethanol and stained with DAPI (1 µg mL$^{-1}$) as described previously (Mazzoni et al., 2003). The presence of ROS was detected with DHR (Sigma Aldrich Co. D1054), as described in the study by Madeo et al. (1999).

**Results**

**Expression of p53 in yeast cells inhibits cell growth**

To understand the molecular pathways of p53 induction of cell death in yeast, we expressed human p53 in wild-type cells and in strains carrying mutations in genes involved in apoptotic cell death. The YCA1 gene (YOR197w) encodes a metacaspase that exhibits proteolytic activity homologous to mammalian caspases (Madeo et al., 2002). Most, but not all, apoptotic responses pass...
through the activation of this protease (Liang et al., 2008; Mazzoni & Falcone, 2008). AIF1 (YNR074c) and NUC1 (YJL208C) encode mitochondrial proteins that show 41% and 62% similarities with human AIF and EndoG, respectively (Wissing et al., 2004; Buttner et al., 2007).

All these strains were transformed with pESC-HIS vector containing human p53 gene and, as a control, with the empty plasmid (Muscolini et al., 2011). All strain cells were initially grown on glucose-containing minimal medium (SD) to keep the Gal1/Gal10 promoter in a repressed state and then transferred in 2% galactose-containing minimal medium (SGal) for 20 h to induce p53 expression. As shown in Fig. 1a, both wild-type (BY4741) and the tested mutant strains (yca1, aif1, nuc1) expressed human p53 after the galactose induction (lanes 4, 8, 12, and 16), while the presence of glucose completely inhibited p53 expression (lanes 3, 7, 11, and 15).

To test the effect on cell growth of p53 expression, we performed a drop assay on plates containing as carbon source 2% glucose (SD) and 2% galactose (SGal). As shown in Fig. 1b, in the presence of glucose medium (SD), a condition that does not allow the protein expression, there was no difference between strains expressing p53 and those expressing the empty plasmid. On the contrary, in the presence of galactose medium (SGal), growth of wild-type strain expressing p53 (BY4741 + p53) was completely inhibited. The expression of p53 blocked cell growth also in yca1 mutant gene, suggesting that cell growth inhibition does not require the metacaspase function. Cell growth was only partially inhibited in aif1 and nuc1 mutants, indicating a role for these genes in mediating the inhibition of cell growth.

**p53 expression renders cells more susceptible to cell death induced by H2O2**

To study the effect of the expression of p53 on cell viability and apoptosis, we compared the percentage of viability, measured by clonogenicity, after 20 h from the expression of the protein in galactose (Fig. 2a). In the wild-type and yca1 strains, the overexpression of p53 resulted in a reduction of about 50% viability. In the case of aif1 and nuc1 mutants, the loss of viability was quite low, about 35%, suggesting that these strains are resistant to p53-induced cell death. Aif1p and Nuc1p/EndoG are two mitochondrial proteins that are released from this organelle upon an apoptotic stimulus. The fact the aif1 and nuc1 mutants were more resistant, compared with the wild type, to p53 expression, suggests that Aif1p and Nuc1p are involved in p53-promoted cell death. On the contrary, p53-induced cell death is only partially dependent on active metacaspase Yca1p.

We then analyze cell viability in the presence of p53 proteins upon an apoptotic stimulus such as hydrogen peroxide treatment. The comparison of cell viability after 3 mM H2O2 treatment (Fig. 2b) has first pointed out

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**Fig. 1.** p53 expression inhibits yeast cell growth. (a) Western blot analysis of protein extracts from BY4741 (lanes 1–4), yca1 (lanes 5–8), aif1 (lanes 9–12), and nuc1 (lanes 13–16) yeast strains containing p53 and the control vector p-ESC-HIS. Cells grown in 2% glucose (repressing conditions, SD) were shifted for 20 h in minimal medium containing 2% galactose (inducing conditions, SGal). Ponceau red staining of membranes is also shown for quantization of transferred proteins. (b) Dilution spot assay of the indicated strains on plates containing 2% glucose (SD) and 2% galactose (SGal). Plates were recorded after 3 days of incubated at 28 °C.
differences between mutant and wild-type strains containing the plasmid control. In fact, all the mutated strains have a higher resistance to hydrogen peroxide, according to their role in mediating the programmed cell death (PCD) process. The expression of p53 leads to increased sensitivity to apoptotic stimuli, with the exception of nuc1 mutant, lacking the gene homologous to mammalian EndoG nuclease, which showed almost the same viability of cells expressing the control vector. The same results were obtained normalizing to the vector-expressing cells or to the untreated cells (see Supporting Information, Fig. S1).

These results suggest that at least in the yeast S. cerevisiae, cell death induced by an apoptotic insult in the presence of p53 is mainly mediated through the action of Nuc1p/EndoG.

Although it has been previously shown that expression of p53 in S. cerevisiae leads to cell death associated with apoptotic markers, the yeast backgrounds and p53 expression plasmids used in this study are different from those used in the previous study. Therefore, we looked at some phenotypes such as nuclei morphology, by DAPI staining, and accumulation of ROS, by DHR123 staining, upon p53 expression. As can be seen in Fig. 3b, cells expressing p53 showed higher percentage of fragmented nuclei, compared with cells expressing only the vector. Anyway, nuc1-p53-expressing cells showed the lowest percentage of fragmented nuclei, compared with all other considered strains.

We also determined the amount of ROS by incubation with dihydrorhodamine 123 (DHR; Schulz et al., 1996). Upon p53 expression, wild-type (BY4741), yca1, and aif1 strains showed about 30–40% of ROS-positive cells (Fig 3d). However, the fraction of DHR-positive cells was reduced to about 20% in nuc1 p53-expressing cells, a value only slightly higher than that shown by the nuc1 cells containing the vector (p-ESC-HIS).

**Effects of p53 on chronological life span**

Long-term cultivation causes an aging process in the whole yeast culture, called chronological aging (Fabrizio & Longo, 2003), which leads to physiologically induced apoptosis in yeast (Herker et al., 2004). In mammalian cells, p53 can both promote and prevent aging. In fact, in the cytoplasm, p53 exerts direct autophagy-inhibitory functions, thus favoring aging (Morselli et al., 2011). At the same time, p53 also inhibits mTOR, stimulating autophagy and promoting life span extension (Feng & Levine, 2010). Although the molecular mechanisms of this double activity are not known, they probably depend on the type or extent of the p53-inducing stress, and reactive oxidative species (ROS) likely play a prominent role (Vigneron & Vousden, 2010).

Therefore, we investigated the effect of p53 expression in chronological aging of wild-type and yeast strains mutated in genes involved in PCD. We observed that in all strains, at day 1, viability was lower in p53-expressing cells compared with those expressing the control vector (p-ESC-His). Within the analyzed strains, this effect was higher in the wild-type and yca1 strains (Fig. 4a and b) as the viability at day 1 of p53-expressing cells was reduced at least 50% compared with vector-expressing cells. This effect was less evident in aif1 and nuc1 p53-expressing cells, which showed a viability of about two-thirds of the vector-expressing cells (Fig. 4c and d).

Cell death kinetics were faster in p53-expressing wild-type cells, especially in the first days. In particular, at day 3, cells expressing the empty vector displayed a reduction of about 15%, while in cells expressing p53 the reduction was about 45% (Fig. S1D). The same was also true for p53-expressing yca1 and aif1 cells, a little bit later in time; in particular, the yca1 strain showed a loss of viability at day 6 of about 50% and 70% for p-ESC-HIS and p53-expressing cells, respectively. Concerning aif1, at day 4, the reduction in viability was about 30% and 70% for empty vector and p53-expressing cells, respectively.
This was not true for nuc1 p53-expressing cells, which showed a slower death kinetic until day 6 (Fig. 4d), while, as cells become more aged, the contribution of p53 to cell death becomes more important and the speed of cell death increases. The same results were obtained normalizing to the vector-expressing cells or to the untreated cells (see Supporting Information).

Discussion

The p53 phosphoprotein is probably the best-known tumor suppressor, being p53 lost or mutated in about half of all human cancers. The genetic tractability of budding yeast, its ease of manipulation, and the wealth of functional genomics tools available render it a good model to study the functions of this protein in a simple organism. In the present work, we highlight that the overexpression of human p53 blocks cell growth and causes the loss of viability in about half of the cell population, suggesting that the overexpression of p53 may play a role in removing old or damaged cells in yeast cell population.

This effect, pointed out in wild yeast strains, was attenuated in strains mutated in genes encoding component of the yeast apoptotic-like pathway. The strains that showed the higher resistance to p53 expression were mutated in AIF1 and NUC1 genes, which code for the homologues of mammalian Aif1 and EndoG, respectively.

The p53 expression made cells more susceptible to cells death induced by H2O2, an apoptotic inducer, except for the nuc1 mutant, which showed very little difference in cell viability between strains expressing p53 and the control plasmid, pointing to Nuc1p as an important protein mediating cell death induced by H2O2 in the presence of p53.

We also checked the effect of p53 during yeast chronological aging, as it has been described a dual role for p53 in promoting and preventing cellular aging. We observed that in wild-type strains, the presence of p53 slightly accelerates cell death during the first days, while in the terminal phases of aging it does not influence cell death kinetics. This could be explained with the recent described role of p53 in inhibiting autophagy, usually
recognized to be a cell response to prevent aging (Morselli et al., 2011). On the contrary, the nuc1 mutant expressing p53 showed slower cell death kinetics in the first days, compared with cells carrying the vector. In this mutant, the presence of p53, instead, accelerated cell death in the last part of cell growth.

The cause of this is not clear, and one can speculate that it could be linked to the intracellular physiology and ROS status: In fact, galactose is partially consumed by respiration (Fendt & Sauer, 2010), and the inactivation of NUC1 inhibits apoptotic cell death when mitochondrial respiration is increased, but enhances necrotic cell death when oxidative phosphorylation is repressed (Buttner et al., 2007).

In conclusions, it seems that p53-mediated cell death in yeast is in intimate connection with the mitochondrial response, as also supposed in other studies (Abdelmoula-Souissi et al., 2011; Coutinho et al., 2011; Muscolini et al., 2011). For this reason, we plan to explore in future more functions of p53 through the use of multiple mutants involved in mitochondrial morphology and post-translational modifications linked to the p53 cytoplasmic function.

**Authors’ contribution**

V.P., E.M. and C.P. contributed equally to this work.

**References**


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Statistic analysis of viability.