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# Fermentative metabolism impedes p53-dependent apoptosis in a Crabtree-positive but not in Crabtree-negative yeast

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Tumour cells distinguish from normal cells by fermenting glucose to lactate in presence of sufficient oxygen and functional mitochondria (Warburg effect). Crabtree effect was invoked to explain the biochemical basis of Warburg effect by suggesting that excess glucose suppresses mitochondrial respiration. It is known that the Warburg effect and Crabtree effect are displayed by *Saccharomyces cerevisiae*, during growth on abundant glucose. Beyond this similarity, it was also demonstrated that expression of human pro-apoptotic proteins in *S. cerevisiae* such as Bax and p53 caused apoptosis. Here, we demonstrate that p53 expression in *S. cerevisiae* (Crabtree-positive yeast) causes increase in ROS levels and apoptosis when cells are growing on non-fermentable carbon sources but not on fermentable carbon sources, a feature similar to tumour cells. In contrast, in *Kluyveromyces lactis* (Crabtree-negative yeast) p53 causes increase in ROS levels and apoptosis regardless of the carbon source. Interestingly, the increased ROS levels and apoptosis are correlated to increased oxygen uptake in both *S. cerevisiae* and *K. lactis.* Based on these results, we suggest that at least in yeast, fermentation *per se* does not prevent the escape from apoptosis. Rather, the Crabtree effect plays a crucial role in determining whether the cells should undergo apoptosis or not.

**Keywords.** Crabtree effect; escape from apoptosis; *Kluyveromyces lactis*; p53 in yeast; *Saccharomyces cerevisiae*; Warburg effect

## 1. Introduction

Warburg proposed that mitochondrial impairment is the driving force for tumorigenesis, based on the observation that tumour cells take up far more glucose than normal cells and ferment it to lactate even in the presence of oxygen (Warburg 1925, 1956). This phenomenon is known as Warburg effect or aerobic glycolysis. While aerobic glycolysis is a universal phenotype of tumour cells, it is now clearly established that tumour cells need not necessarily be defective in mitochondrial respiratory function (Fantin *et al*. 2006; Le *et al*. 2010; Schell *et al*. 2014; Senyilmaz and Teleman 2015). Following Warburg's observation, Crabtree proposed that glucose inhibits mitochondrial function in tumour cells (Crabtree 1928). Since then, studies carried out to understand Crabtree effect in tumours (Ibsen 1961; Guppy *et al*. 1993; Marin-Hernandez *et al*. 2006; Suchorolski *et al*. 2013) have revealed a plethora of different mechanisms. For example, mechanisms such as a competition between mitochondria and glycolytic enzymes for ADP (Gatt and Racker 1959; Weinhouse 1972; Diaz-Ruiz *et al*. 2009), changes in the phosphate potential (Sussman *et al*. 1980), changes in the permeability of outer mitochondrial membrane (Zizi *et al*. 1994), and glucose-induced increase in Ca+ ions (Wojtczak *et al*. 1999) have been proposed. More recently, fructose 1,6 bisphosphate at concentrations normally present in hepatoma cells was demonstrated to inhibit respiration of mitochondria isolated from normal rat liver cells (Diaz-Ruiz *et al*. 2008), suggesting that mitochondrial impairment is not a prerequisite for the induction of Crabtree effect. Despite these observations, the relationship between Warburg effect and Crabtree effect in

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promoting different aspects of tumorigenesis such as escape from apoptosis has remained an enigma.

*Saccharomyces cerevisiae* cells proliferating in presence of abundant glucose as a carbon source and sufficient oxygen exhibit similar fermentation values as observed in tumour cells (Warburg 1956) and is demonstrated to exhibit Crabtree effect (De Deken 1966). In *S. cerevisiae,* Crabtree effect in the guise of glucose repression has been extensively studied at the metabolic (Fiechter and Gmunder 1989), genetic (Zaman *et al*. 2008) and evolutionary levels (Dashko *et al*. 2014, Rozpedowska *et al*. 2011). Using yeast as a model, it was reported that Warburg effect in addition to inducing aerobic glycolysis may suppress apoptosis (Ruckenstuhl *et al*. 2009). More recently, metabolic and regulatory similarities have been reported between tumour affected organism and yeast colony which consists of two distinct layers of cell types (Cap *et al*. 2012). Because of the similarity in the regulation of glucose metabolism between *S. cerevisiae* and tumour cells, there has been a resurgence of attempts to use yeast as a model to understand metabolic and genetic basis of cancer (Fiechter and Gmunder 1989; Diaz-Ruiz *et al*. 2009, 2011; Li *et al*. 2009; Legiša 2014).

The tumour suppressor and the pro-apoptotic gene p53, was demonstrated to regulate the mitochondrial oxygen uptake thus linking Warburg effect to apoptosis (Matoba *et al*. 2006; Olovnikov *et al*. 2009). While yeast lacks the ortholog of p53, it induces the mitochondrial-mediated apoptotic pathway similar to what is present in higher eukaryotes (Ludovico *et al*. 2002). It was demonstrated that co-expression of p53, with CDC2Hs in *S. cerevisiae* growing in raffinose as the carbon source, lead to growth inhibition (Nigro *et al*. 1992). A similar study conducted in *S. pombe* also showed that expression of p53 alone was sufficient to cause growth retardation (Bischoff *et al*. 1992). Human pro-apoptotic factor Bax is reported to induce apoptosis in yeast (Ligr *et al*. 1998). A subsequent study demonstrated that expression of Bax induces apoptosis when *S. cerevisiae* cells are growing in lactate while its ability to induce apoptosis is delayed when cells are growing in glucose (Priault *et al*. 1999). In contrast, in *K. lactis*, the Bax induced lethality was suppressed when lactate was used as a carbon source (Kost'anova-Poliakova and Sabova 2005). An independent study reported that p53-induced apoptosis in *S. cerevisiae* cells when grown on galactose, another fermentative carbon source, for prolonged period of time (Hadj Amor *et al*. 2008). Thus, it appears that in the above study, growth of *S. cerevisiae* on ethanol, the end product of sugar metabolism, might be a permissive condition for p53-dependent apoptosis. However, this conclusion is in variance with the observation reported in *K. lactis* (Kost'anova-Poliakova and Sabova 2005).

To gain insights into the role of fermentative metabolism, Crabtree effect vis-à-vis mitochondrial role, if any, in p53-mediated apoptosis, we studied the effect of expression of p53 in *S. cerevisiae*, a Crabtree-positive yeast and *K. lactis* a Crabtree-negative yeast (Piskur *et al*. 2006), under varying experimental conditions. Our results suggest that p53 does not induce apoptosis when *S. cerevisiae* cells are grown under fermentative growth conditions, but induces apoptosis only when it grows under non-fermentative condition. In contrast, p53 causes apoptosis in *K. lactis* regardless of whether it is grown in presence of fermentative or non-fermentative carbon source. Thus, the escape from apoptosis observed only under fermentative condition in *S. cerevisiae* recapitulates what is normally observed in tumour cells. However, in *K. lactis* apoptosis occurs even during fermentative conditions, ruling out the possibility that fermentation *per se* is essential for apoptosis. Based on these and other results, we propose that it is the functional status of the mitochondrion that is primarily responsible for p53-dependent apoptosis.

## 2. Materials and methods

### 2.1 *Media and growth conditions*

Yeast cells were grown in minimal medium containing 0.67% (w/v) yeast nitrogen base (Difco) and ammonium sulphate mixture (1:3), 0.05% (w/v) of amino acid mixture (complete or drop out). The carbon sources were 3% (v/v) glycerol plus 2% (v/v) potassium lactate or 0.2% (w/v) sucrose or 2% (w/v) galactose or 2% (w/v) glucose (Amberg *et al*. 2005). Geneticin (G418) at a final concentration of 200 µg/mL was used in YPD (0.5% Yeast extract, 1% Peptone, 2% Dextrose) (Wach *et al*. 1994). For induction of p53 galactose was used at a final concentration of 2% when cells were grown on carbon source other than galactose. *E. coli* XL1 was grown in LB with ampicillin concentration of 75µg/mL for plasmid maintenance.

### 2.2 *Plasmids*

$P_{GAL10}$::p53 cassette was cloned into YIPlac204 as a 2.1 Kb *Kpn*I – *Sac*I fragment from pLS89 (Scharer and Iggo 1992) to obtain pAK1. Specific mutations in p53 ORF were generated by site directed mutagenesis. The plasmids used are given in the supplementary material.

### 2.3 *Strain construction*

Genetic manipulations of yeast strains were done by lithium acetate method as described by (Daniel Gietz and Woods 2002). Integration of $P_{GAL1}$::p53 cassette in *S.*

*cerevisiae* was performed by linearizing YIplac204 derivatives (see plasmid list) by *Eco*RV (located within the *TRP1* locus) restriction digestion followed by the transformation of the recipients to Trp+ prototrophy. Integration of $P_{KlGAL1}$::p53 cassette and corresponding empty vector into *K. lactis* was carried out by linearizing the YDp-U based plasmids with *Nsi*I, and picking up the Ura+ transformants. The yeast genes involved in apoptosis (*MCA1*, *NUC1*) and autophagy (*ATG1*, *ATG5*) were disrupted with *KanMX4* cassette amplified from pUG6 vector using appropriate primers. The other two apoptotic genes *AIF1* and *NMA111* were disrupted by *KanMX4* cassette amplified from genomic DNA of corresponding deletion strain procured from EUROSCARF. *ATG5* was deleted in *atg1*Δ strain (JDY35) with KanMX4 cassette amplified from pUG6 after the marker rescue using pSH47 as described in (Güldener *et al*. 1996). *TRP1* was disrupted in BY4742-1Δ using pL328 as described in (Blank *et al*. 1997) to obtain JDY1. BY4741 was crossed with JDY1 and the spores were segregated to generate JDY2 strain.

*Strains: E. coli* Strain: XL-1 blue [*F'::Tn10(TetR), proA+B+, LacIq, Δ(lacZ)M15/recA1, endA1, gyrA96(NaIr), hsdR17(rkmk)supE44,relA1*] is used to maintain all the plasmids. The Strains used are listed in the supplementary material.

### 2.4 *List of primers*

The list of primers used in this study is given in the supplementary material.

### 2.5 *Polymerase chain reaction*

The PCR condition for all the primers, except PJB522 and PJB523, for gene amplification comprised of initial denaturation at 95°C for 8 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. Final extension was carried out at 72°C for 10 min. For Primers PJB522 and PJB523, annealing was set at 48°C for 1 min and extension at 68°C for 2 min. Final extension was carried out at 68°C for 10 min.

### 2.6 *Spotting assay*

The cells were grown in minimal medium containing either glycerol or sucrose as a sole carbon source. The cells were harvested at mid log phase and $O.D._{600nm}$ of 1.0 was achieved for all the cultures by diluting the samples with sterile distilled water. Cultures were then diluted serially and 5 μL of each sample was inoculated onto the minimal medium.

### 2.7 *Preparation of cell extracts for p53 protein studies*

For preparing the yeast cell extracts for determination of p53 protein, the cells were grown in appropriate medium till an $O.D._{600nm}$ of around 0.5. The cells were then induced with 2% galactose (final concentration) and were incubated further for 3 h. The cells were then harvested by centrifugation at 10,640*g* for 2 min at 4°C and then washed once with cold distilled water. The cells were then resuspended in 100mM Tris-Cl (pH 7.4) and were mixed with PMSF, PIC. The cells were broken with the help of an equal volume of glass beads (diameter 0.45mm). The mixture was vortexed with glass beads for 45 seconds, followed by incubation on ice for 45 seconds. The cycle was repeated 6–7 times. The cell lysate was centrifuged at 17,982 *g* for 15 min at 4°C. The supernatant was transferred into fresh tube and was used for further analysis.

### 2.8 *Western blotting*

The cell extract was prepared as described above. The cell extract was mixed with 6X SDS PAGE loading dye and was boiled for 5 min in boiling water bath. The sample was then loaded onto 10% SDS poly acryl amide gel. The electrophoresis was carried out at 15 mA. Proteins were transferred from gel to nitrocellulose membrane at the current of 150 mA. The membrane was blocked with 1% milk in phosphate buffer saline (PBS) for 1 h. The blot was then probed with 1:500 diluted rabbit polyclonal IgG against human p53 (Sigma aldrich) and incubated for 1 h at room temperature. The Membrane was washed 3 times with PBST (Phosphate buffer saline Tween 20) for 10 min. The membrane was then subjected to 1:5000 diluted secondary antibody conjugated with alkaline phosphatase and incubated for 1 h at room temperature. The blot was developed using solution that contained NBT, BCIP, 50 mM $MgCl_2$, 0.1M NaCl, 0.1 M Tris-Cl buffer pH 9.5.

### 2.9 *Propidium iodide (PI) staining*

For preparing the yeast cell for cell death assay, the cells were grown in glycerol lactate medium till an $O.D._{600nm}$ of around 0.5. The yeast cells were induced with 2% final concentration of galactose. Water was added in the cultures as a negative control. $H_2O_2$ at the final concentration of 150 mM was used as a positive control. After incubating the cultures for 4 h (or else mentioned) at 30°C with shaking, the cells were harvested and washed twice with TE buffer containing 10 mM of Tris-Cl (pH 8.0) and 1 mM of EDTA (pH 8.0). The cells were resuspended in 200 μL of TE buffer. Cells were treated with RNAase A to the final concentration of 1 mg/mL and incubated at 37°C for 1 h. After that cells were washed twice with PBS (0.05M $K_2HPO_4$,

0.05M $KH_2PO_4$, 0.15M NaCl). The cells were then resuspended in 0.1 mL of PBS containing 50 µg/mL of propidium iodide and incubate at 4°C for 2 h. Cells were washed twice with PBS, resuspend in 0.1 mL of PBS and observed under fluorescence microscope under 100× objective.

### 2.10 *Measurement of intracellular ROS levels*

Intracellular Reactive Oxygen Species (ROS) were detected by using the oxidant-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA), Molecular Probes, as described by (Balzan *et al*. 2004). The cells grown in glycerol medium were reinoculated in glycerol medium at $O.D._{600nm}$ of 0.03 and were allowed to grow until it reached the $O.D._{600nm}$ of 0.5. The cells were then harvested, washed twice with sterile distilled water and were resuspended in 2 mL sterile distilled water. These cells were then used to inoculate appropriate medium (glycerol or sucrose with and without 2% galactose) at $O.D._{600nm}$ of 0.1 for the induction. The cells were incubated further for 4 h at 30°C on rotator shaker. $H_2O_2$ induction was used as a positive control while water was added to the cells as a negative control. Equal number of cells was aliquoted into a microcentrifuge tube. To this culture was added 2 µL of DCDHF-DA (Invitrogen) from a fresh 5 mM stock solution prepared in ethanol and incubate at 28°C for 20 min. The cells were then washed twice in sterile distilled water and resuspended in 1 mL of 50 mM Tris-Cl buffer (pH 7.5). Two drops of chloroform and one drop of 0.1% (w/v) SDS was added and the cells were vortexed for 20 sec. Incubated at room temperature for 15 min to allow the dye to diffuse into the buffer. Cells were pelleted and the fluorescence of the supernatant was measured using a spectrofluorometer with excitation at 490 nm and emission at 518 nm.

### 2.11 *Annexin V and PI staining for determination of apoptosis*

The annexin V and PI staining was performed for detection of apoptosis in cells using a kit from Invitrogen. The cells grown in glycerol medium were reinoculated in glycerol medium at $O.D._{600nm}$ of 0.03 and were allowed to grow until it reached the $O.D._{600nm}$ of 0.5. The cells were then harvested, washed twice with sterile distilled water and were resuspended in 2 mL sterile distilled water. These cells were then used to inoculate appropriate medium (glycerol or sucrose with and without 2% galactose) at $O.D._{600nm}$ of 0.1 for the induction. The cells were incubated for 4 h in the induction medium. $H_2O_2$ induction was used as a positive control while water was added to the cells as a negative control. The cells were then washed with PBS. The reagent was made fresh by mixing 20 µL of Annexin V and 20 µL of PI into 1 mL of dilution buffer provided in the kit. The cells were resuspended in 50 µL of this reaction buffer and incubated in dark at room temperature for 20 min. The cells were then analysed by fluorescence microscope.

### 2.12 *Analysis of oxygen consumption*

The oxygen consumption by the cells was determined using the procedure described in (Blom *et al*. 2000) with some modifications. The yeast cells grown in appropriate medium were harvested in the log phase ($O.D._{600}$ of 0.5) and then washed thrice with ice-cold distilled water. The wet weight of the pellet was determined and the cells were resuspended in oxygraph buffer [1% yeast extract, 0.1% $KH_2PO_4$, 0.12% $(NH_4)_2SO_4$ (pH 4.5)] at 10 mg cells/mL. Oxygen consumption rates of the cultures were measured using a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, U.K.). The rate of $O_2$ consumption was calculated from the slope of the plot of $O_2$ concentration versus time. The values are expressed as nano moles of $O_2$ consumed/mL/min/10 mg wet weight of cells.

### 2.13 *Marker rescue by induction of cre expression*

The protocol for marker rescue is modified from (Güldener *et al*. 1996). The cre expression vector pSH47 was transformed into the disruption strain containing *KanMX4* cassette flanked by *loxP* sites. The transformants were selected on ura drop out plate. The two independent transformants were then resuspended in 2mL YP-Galactose and were incubated for 2 h at 30°C under shaking. Around 200 cells from above culture were plated onto YPD plates and incubated for 2 days at 30°C. The colonies were then replica plated from YPD plate to the YPD plate containing G418 (200 mg/l G418). The colonies which did not grow on YPG-G418 plate were checked for loss of *KanMX4* cassette by diagnostic PCR. The Cre expression vector pSH47 was eliminated from the putative colonies by growing them into 25 mL YPD for 24 h at 30°C. The culture was then diluted and plated onto complete glucose plate to get around 200 colonies. After incubation at 30°C for 2 days, the colonies were replica plated onto ura drop out glucose medium and incubated for 2 days at 30°C. The colonies which were not growing on ura drop out plate were selected and used further.

## 3. Results

### 3.1 *Over-expression of p53 causes growth inhibition of Saccharomyces cerevisiae when grown on non-fermentable carbon source*

We decided to monitor the effect of over-expression of p53 on the growth of *S. cerevisiae* cells under fermentative and

non-fermentative conditions. Our attempts to express p53 from tetracycline-regulated promoter (Bellí *et al*. 1998) under above mentioned conditions were unsuccessful. Hence, we resorted to the conditional expression of p53 from galactose inducible promoter. For this purpose, a wild-type strain (AKY3) that can grow on galactose as a sole carbon source and its isogenic *gal1*Δ *gal7*Δ derivative (AKY1) which cannot utilize galactose as the sole carbon source, were used to integrate $P_{GAL1}$::p53 cassette (supplementary figure 1). The parent strains (AKY1 and AKY3) and their corresponding $P_{GAL1}$::p53 integrants (AKY2 and AKY4 respectively) were pre-grown in glycerol medium, serially diluted and spotted on the medium containing glycerol as the sole carbon source as well as glycerol plus 2% galactose. The two parent strains and their corresponding $P_{GAL1}$::p53 integrants grew on medium containing glycerol equally well (figure 1A, left panel). This suggests that integration of $P_{GAL1}$::p53 cassette does not alter the growth pattern when glycerol was used as the sole carbon source as under these conditions; p53 is not expected to be induced (see below for details). In contrast, on medium containing glycerol plus galactose, *gal1*Δ *gal7*Δ derivative in which $P_{GAL1}$::p53 is integrated (AKY2) showed reduced growth as compared to its parent strain (AKY1) lacking $P_{GAL1}$::p53 cassette (figure 1A, right panel). The wild type strain (AKY3) and its corresponding $P_{GAL1}$::p53 integrant (AKY4) grew equally well on glycerol plus galactose plates. The parental *gal1*Δ *gal7*Δ strain (AKY1) showed marginal growth inhibition on glycerol-galactose medium as compared to the wild type parental strain (AKY3). This is because, on glycerol-galactose medium the parent *gal1*Δ *gal7*Δ strain grows on glycerol as the sole carbon source, while it induces other GAL genes gratuitously in response to galactose, causing energetic burden leading to reduced growth and such phenotype has been previously observed (Ideker *et al*. 2001).

Expression of p53 could be detected as early as 1 h and at a galactose concentration as low as 0.5% (see supplementary figure 2 for the induction kinetics of p53 expression in response to time and galactose concentration). Western blot analysis indicated that p53 is expressed in both AKY2 and AKY4 only in response to galactose (figure 1B). Marginal increase in expression of p53 under identical conditions was observed in AKY2 as compared to AKY4. This could be because of the sustained presence of galactose in the medium as this strain cannot metabolise galactose. Based on these results, we infer that p53 inhibits the growth of *S. cerevisiae* cells when glycerol, a non-fermentable carbon source is metabolised as the sole carbon source.

It was necessary to determine whether the above phenotype observed on solid medium can be reproduced in the liquid medium as well. The p53 over-expressing strains, AKY2 and AKY4, pre-grown in glycerol medium were inoculated into medium containing glycerol (no p53 expression) and glycerol with 2% galactose (p53 expression). Growth was monitored by measuring the optical density (O.D. at 600nm) at various time intervals. The AKY2 strain attained a maximum cell density of 0.5 in glycerol-galactose medium while it attained a cell density of 2.0 in medium containing only glycerol (figure 1C), indicating that p53 indeed interfered with the growth of this strain. Note that this strain cannot utilise galactose as the sole carbon source because it lacks the galactose metabolising pathway. Hence, the growth inhibition that is observed can be a combined effect of p53 as well as the energetic burden borne by the cells because of the presence of galactose in the medium. Further, samples were collected at different time intervals and the cells were plated onto medium containing glycerol to determine the total viable cells. AKY2 grown in liquid glycerol medium showed around $10^7$ CFU/mL at the end of 24 h of growth (figure 1D). On the other hand, the same strain grown in glycerol-galactose medium had around $10^5$ CFU/mL at the end of 24 h of growth in liquid medium. In comparison, the wild type strain bearing the $P_{GAL1}$::p53 integration (AKY4), grew better in glycerol-galactose medium where p53 is expressed as compared to the same strain grown in glycerol medium where p53 is not expressed (figure 1E). AKY4 showed around $10^7$ CFU/mL in medium containing glycerol and glycerol plus galactose (figure 1F). The above results suggest that p53 inhibits cell growth when cells utilise glycerol but not glycerol plus galactose as the carbon source.

### 3.2 *Over-expression of p53 does not affect the growth of S. cerevisiae on fermentable carbon source*

Previous experiments clearly indicated that over-expression of p53 caused growth inhibition in AKY2 but not in AKY4 strain. It should be noted that unlike AKY2, AKY4 can metabolize galactose as a carbon source even if glycerol is provided as the alternate carbon source. Therefore, we surmised that the preferential utilization of galactose, a fermenting carbon source, prevents the ability of p53 to induce the growth inhibitory effect. To test this possibility AKY4 was grown in a medium containing galactose as a sole source of carbon. Interestingly, when galactose was used as a sole source of carbon, p53 was unable to cause the growth inhibition in AKY4 strain (figure 2A). This observation indicated that the p53 cannot inhibit the growth when cells are grown in a fermentable carbon source. To generalise this observation, we used sucrose as an alternative fermentative carbon source and analysed the growth phenotype. Both the yeast strains, AKY2 and AKY4 grown on sucrose, expressed p53 upon galactose induction (figure 2C). Both the strains were grown in sucrose till mid log phase, diluted serially and spotted onto synthetic medium containing sucrose with or without galactose. In spite of p53 expression upon galactose

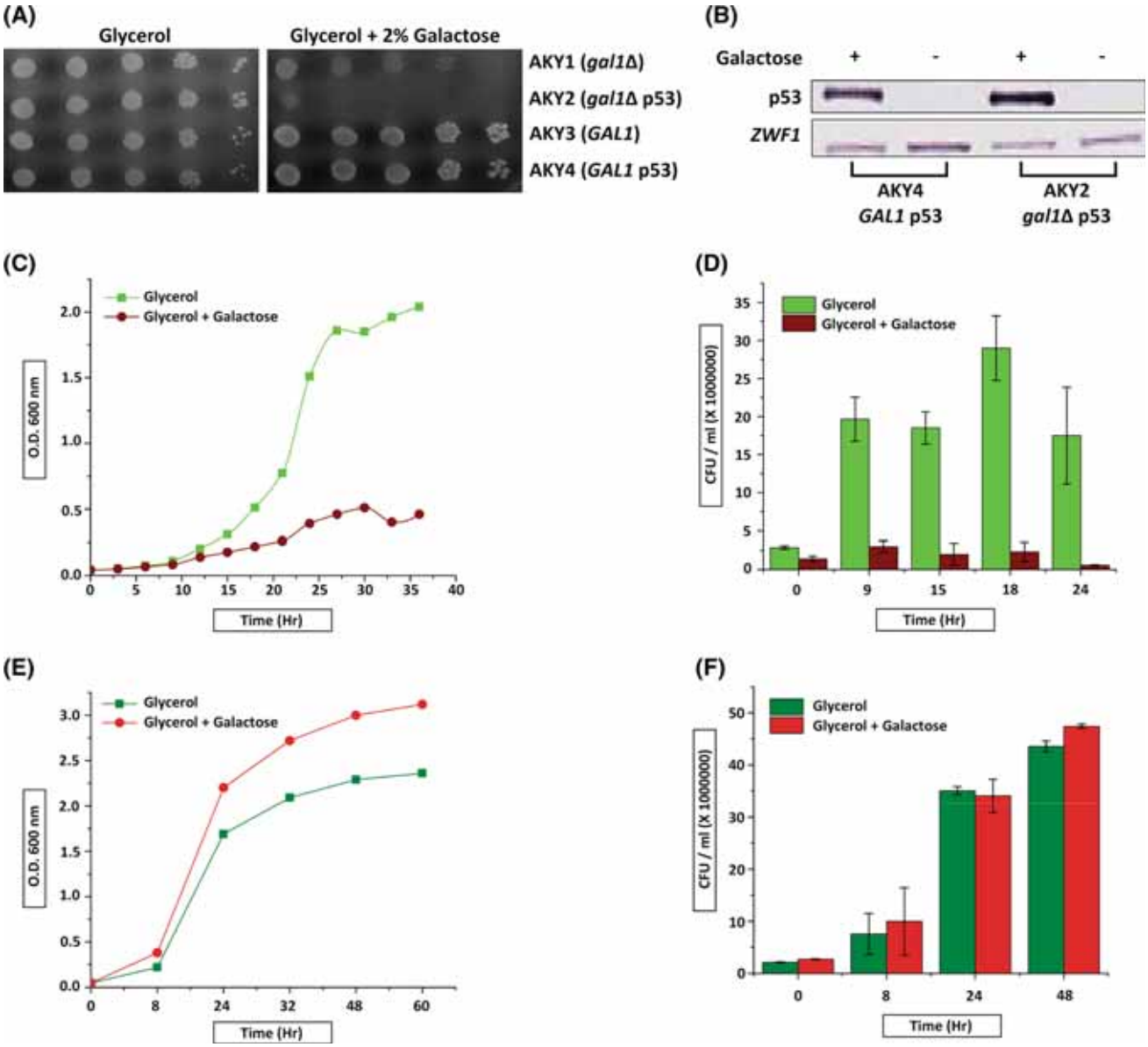


**Figure 1.** Phenotypic analysis of p53 over-expressing yeast strains on medium containing glycerol. (**A**) Overnight cultures of yeast strains grown in glycerol were diluted serially in sterile distilled water and 5μL of each sample was spotted onto medium containing glycerol with (right panel) and without 2% Galactose (left panel). (**B**) Cells were grown in glycerol medium till mid-log phase and induced with 2% galactose (final concentration). After 3hrs of induction the cell free extracts were subjected to detection of p53 expression using anti p53 antibody in a western blot analysis. Antibody against Zwf1 was used as loading control. (**C**) The time course of growth of AKY2 strain in glycerol medium (light green line) and glycerol medium containing 2% galactose (brown line). (**D**) CFU/mL of AKY2 strain growing in glycerol medium (light green bars) and glycerol-galactose medium (brown bars) as a function of time. (**E**) The time course of growth of AKY4 strain in glycerol medium (dark green line) and glycerol medium containing 2% Galactose (red line) as a function of time. (**F**) CFU/mL of AKY4 strain growing in glycerol medium (dark green bars) and cells growing in glycerol-galactose medium as a function of time (red bars). All the above experiments are performed at least thrice.

induction, both the strains did not show any growth inhibition phenotype when sucrose was used as a carbon source (figure 2B). Above observations indicate that the p53 is unable to cause growth inhibition when *S. cerevisiae* utilizes fermentable carbon source.

### 3.3 *Over-expression of p53 leads to cell death in non-fermentable but not in fermentable carbon source*

Results presented thus far clearly indicate that the p53 over-expression inhibits the growth of *S. cerevisiae* cells only

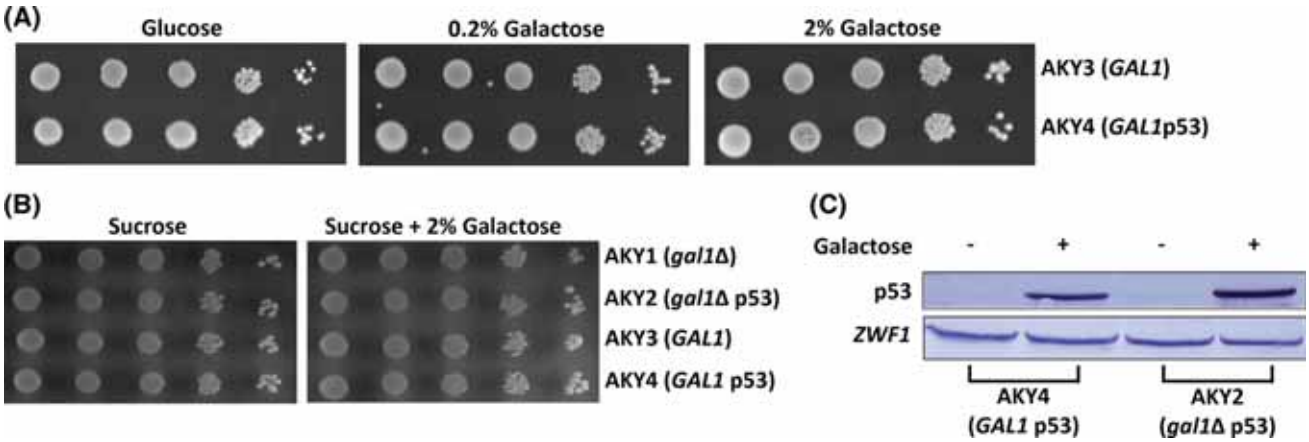


**Figure 2.** Phenotypic analysis of AKY2 and AKY4 on fermentative carbon source. (**A**) Overnight cultures of AKY3 and AKY4 strains grown in glycerol lactate medium were diluted serially in sterile distilled water and then spotted onto the medium containing galactose as a sole source of carbon. The growth phenotype was recorded after 48 h of growth at 30°C. Growth on medium containing glucose as a sole source of carbon was considered as a control. (**B**) Strains AKY2 and AKY4 along with their respective parental strains were grown in minimal medium with sucrose as a sole carbon source. Serial dilutions of the cultures were spotted onto sucrose and sucrose-galactose medium. (**C**) Cell free extracts obtained from sucrose grown cultures induced with galactose as described in materials and methods were subjected to western blot analysis and the blot was probed with p53 antibody. Zwf1p was used as a loading control.

when non-fermentable carbon source is used as the sole carbon source. To study whether this inhibition of growth is due to cell death or due to stasis, cells were subjected to propidium iodide (PI) staining. On PI staining, the cells which are dead show the signal while the live cells do not. In this experiment, cells treated with 150mM $H_2O_2$ (Ribeiro *et al*. 2006) were used as a positive control while cells treated with water were used as a negative control. For every sample, at least 300 cells were counted and the number of cells showing the signal is indicated as the percentage of dead cells.

Yeast strains AKY1, AKY2 and AKY4 grown in a non-fermentative carbon source i.e. glycerol medium, showed a death rate of approximately 95% when treated with $H_2O_2$ (figure 3A). The AKY1 did not show any significant increase in the number of dead cells upon galactose induction. The AKY2 strain showed around 3-fold increase in cell death when the cultures were induced with galactose as compared to the uninduced controls. AKY4 strain showed no significant difference in the number of dead cells between uninduced and galactose induced cultures.

Similar experiment was carried out using cells grown in sucrose medium which is a fermentative carbon source. All the above three cultures exhibited almost 95% cell death upon $H_2O_2$ treatment and less than 10% of dead cells in uninduced controls. As expected, AKY1 strain showed no significant difference in the number of dead cells in presence of galactose, where p53 is not expressed (figure 3B). Similarly, AKY2 strain as well as the AKY4 strain showed not more than 10% cell death, though both the strains express p53 protein. Above results clearly indicate that the strain which can utilize galactose does not show cell death upon p53 expression. The cell death occurs upon p53 expression only when cells are utilizing non-fermentable carbon source and not fermentable carbon source. These data indicate that the growth inhibition observed in the previous experiments is due to cell death.

We wanted to test whether the defective growth phenotype of the *gal1*Δ *gal7*Δ strain when p53 expression is induced is due to (a) non-specific effect of the growth conditions, (b) the genetic background of the strain used and (c) a functional p53. First, we monitored the growth of the above strain in synthetic medium containing ethanol as well as ethanol plus galactose (supplementary figure 3A) and rich medium containing glycerol as well as glycerol plus galactose (supplementary figure 3B). It is clear from these experiments that expression of p53 in non-fermentable carbon source either in synthetic or rich medium retards the cell growth.

To test the second possibility, we studied the growth inhibition phenotype induced by p53 over-expression in the strains obtained from Euroscarf. We integrated $P_{GAL1}$::p53 cassette into the Euroscarf wild type (BY4741) and *gal1*Δ (BY4742-1Δ) background as discussed under material and methods. The wild type Euroscarf strain (JDY4) over-expressing p53 did not show any discernible defective growth phenotype in medium containing glycerol plus galactose while over-expression of p53 in the *gal1*Δ background (JDY3) resulted in defective growth phenotype, similar to what was observed earlier (supplementary figure 4).

To test the third possibility, six different mutants of p53 were generated using site directed mutagenesis. The mutant ORFs were cloned and integrated into the AKY1 strain. The

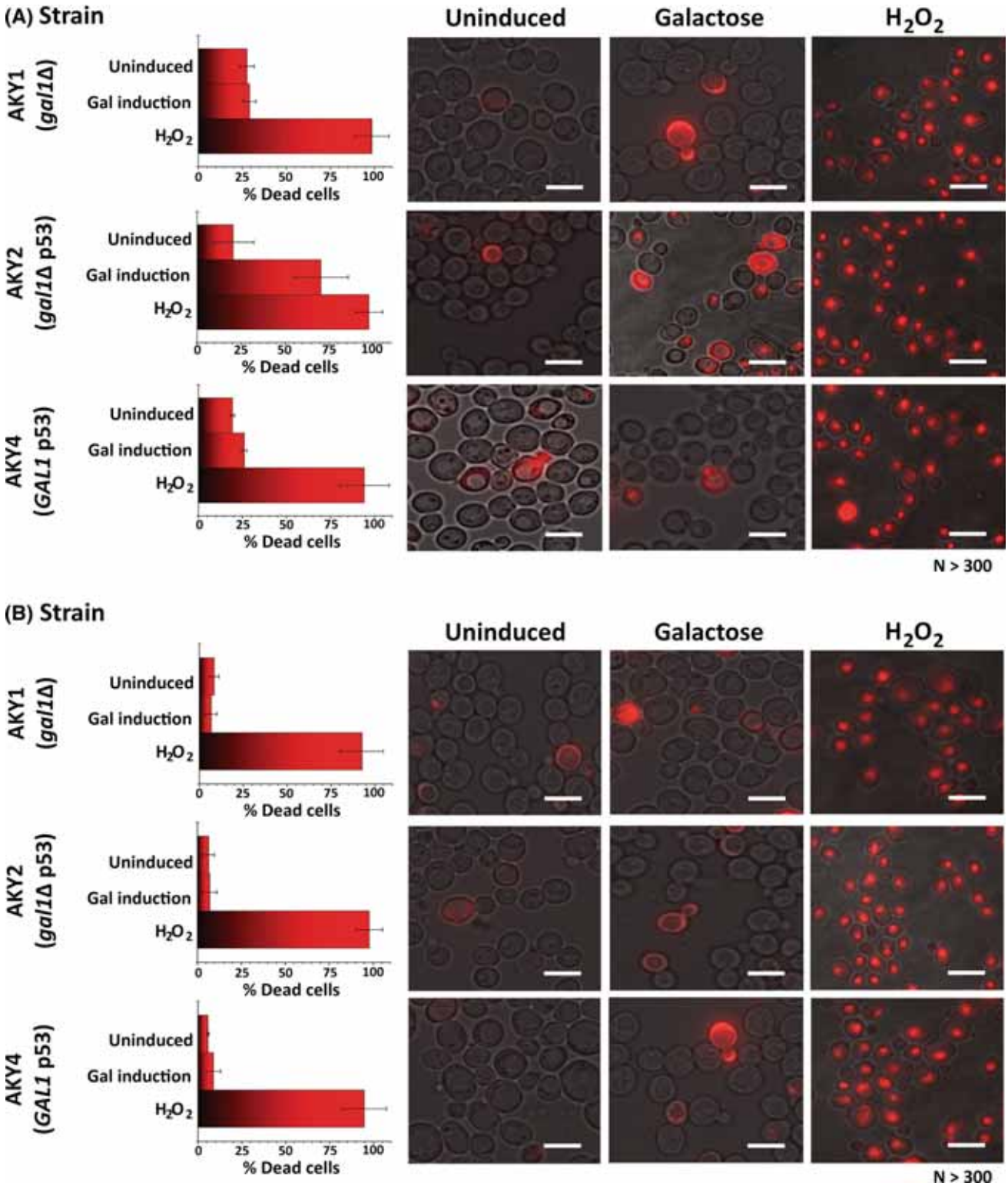


**Figure 3.** Analysis of cell death of p53 over-expressing yeast cells using PI staining. Strains, AKY1, AKY2 and AKY4 were grown in (**A**) glycerol or (**B**) sucrose and were analysed for the cell death in presence of galactose. $H_2O_2$ and water treatment was used as a positive and negative control respectively. The results of three independent experiments is represented where N > 300. The merged image for bright field and PI staining is shown for all the samples. Scale bar represents $\sim$5 μm.

growth phenotype of these strains harbouring mutant p53 ORF indicates that the p53-dependent growth inhibition exhibited by the *S. cerevisiae* is due to the functional p53 (supplementary figure 5). Inactivation of this function by mutation abrogates the ability to confer the growth inhibition suggesting that the protein function is important and it is not due to a nonspecific affect of expressing a heterologous protein.

### 3.4 *Over-expression of p53 causes cell death in Kluyveromyces lactis in a carbon source independent manner*

Thus far, the ability of *S. cerevisiae* (Crabtree-positive) cells to withstand the effect of p53 was correlated with the fermenting growth condition but not with non-fermenting growth condition. To further probe this correlation, we decided to monitor the effect of expression of p53 in *K. Lactis*, a Crabtree-negative species (Piskur *et al*. 2006; Dashko *et al*. 2014). That is, *K. lactis*, unlike *S. cerevisiae*, ferments glucose but unable to accumulate ethanol because of its inability to exert glucose repression of mitochondrial respiration. Thus, we surmised that if fermentation *per se* prevents p53-induced apoptosis in *S. cerevisiae*, then over-expression of p53 in *K. lactis* growing on galactose, a fermentable carbon source, should not cause cell death.

After confirming that the GAL switch in *K. lactis* strain JDY26 is functional (supplementary figure 6A), we integrated $P_{KlGAL1}$::p53 cassette or the vector backbone as described in methods. Expression of p53 was determined at different time intervals after induction with galactose by probing p53 with its antibody (supplementary figure 6B). The p53 protein expression and growth phenotype of p53 over-expressing *K. lactis* was compared to that of *S. cerevisiae* AKY4 strain, as both the strains can metabolize galactose as the sole carbon source. *K. lactis* strains JDY26 in which $P_{KlGAL1}$::p53 is integrated and JDY27 in which empty vector is integrated (control) were grown in medium containing glycerol or sucrose under p53 inducible and non-inducible conditions. Cell extracts were analysed for expression of p53 protein by probing with anti p53 antibody. *K. lactis* strain JDY26, showed expression of p53 upon galactose induction in glycerol as well as in sucrose, similar to what is observed in *S. cerevisiae* strain AKY4 under identical experimental conditions (figure 4A). We consistently observed that the extent of p53 expression is less in *K. lactis* as compared to that is *S. cerevisiae* (see figure 4A and supplementary figure 6B). The cultures were then analysed for growth phenotype on glycerol, sucrose and galactose medium under p53 inducing conditions. After serial dilutions the cultures were spotted onto glycerol medium and glycerol medium containing galactose. Unlike *S. cerevisiae* AKY4 strain, the *K. Lactis* JDY26 strain exhibited growth inhibition phenotype on glycerol-galactose medium (figure 4B). The control strain which does not express p53 remained unaffected upon galactose induction. It should be noted that AKY4 strain of *S. cerevisiae* does not show growth inhibition on glycerol-galactose medium (figure 1A), whereas the JDY26 strain exhibits severe growth inhibition on glycerol-galactose medium. This difference is observed despite the fact that both these strains can metabolize galactose, also the amount of p53 protein expressed is less in *K. lactis* than in *S. cerevisiae* (figure 4A). These observations suggest that the physiological status of a cell plays a far more crucial role in conferring the sensitivity towards p53-induced growth inhibition.

The *K. lactis* strains were also grown in minimal medium with sucrose, which is a fermentable carbon source. After serial dilutions, the cultures were spotted onto sucrose medium with or without galactose. Unlike *S. cerevisiae*, the growth of *K. lactis* strain expressing p53 was inhibited (figure 4C). The p53-dependent growth inhibition phenotype was also observed in *K. lactis* on galactose medium (figure 4D). The requirement of functional p53 for the above mentioned growth inhibition phenotype in *K. lactis* was established (supplementary figure 7) as was demonstrated in case of *S. cerevisiae* (supplementary figure 5).

To perform Propidium iodide staining for determination of cell death as a function of p53 expression, *K. lactis* cells over-expressing p53 were grown in glycerol medium till $O.D._{600nm}$ of around 0.8 and then induced with 2% galactose, water or $H_2O_2$. After 8 h of incubation cells were harvested and processed for PI staining. *K. lactis* strain with empty vector was used as a control. The number of dead cells in presence of $H_2O_2$ was around 85% in both the strains, while the strains showed only around 6% dead cells in presence of water. The JDY27 strain showed about 8% of cell death in presence of galactose, where p53 is not expressed (figure 5). The JDY26 strain showed $\sim$4-fold increase in the number of dead cells upon galactose induction as compared to the uninduced control. These results indicate that fermentation *per se* does not prevent the ability of p53 to induce cell death. We interpret this to mean that the functional status of mitochondria determines the ability p53 to induce cell death.

### 3.5 *Over-expression of p53 causes an increase in ROS production*

The results presented thus far indicate that p53 expression in *S. cerevisiae* leads to cell death only when cells are grown in non-fermentable carbon source where as in *K. lactis* p53 dependent cell death is independent of the growth conditions. We tested whether p53 expression results in increased ROS levels under the experimental condition where it induces cell death.

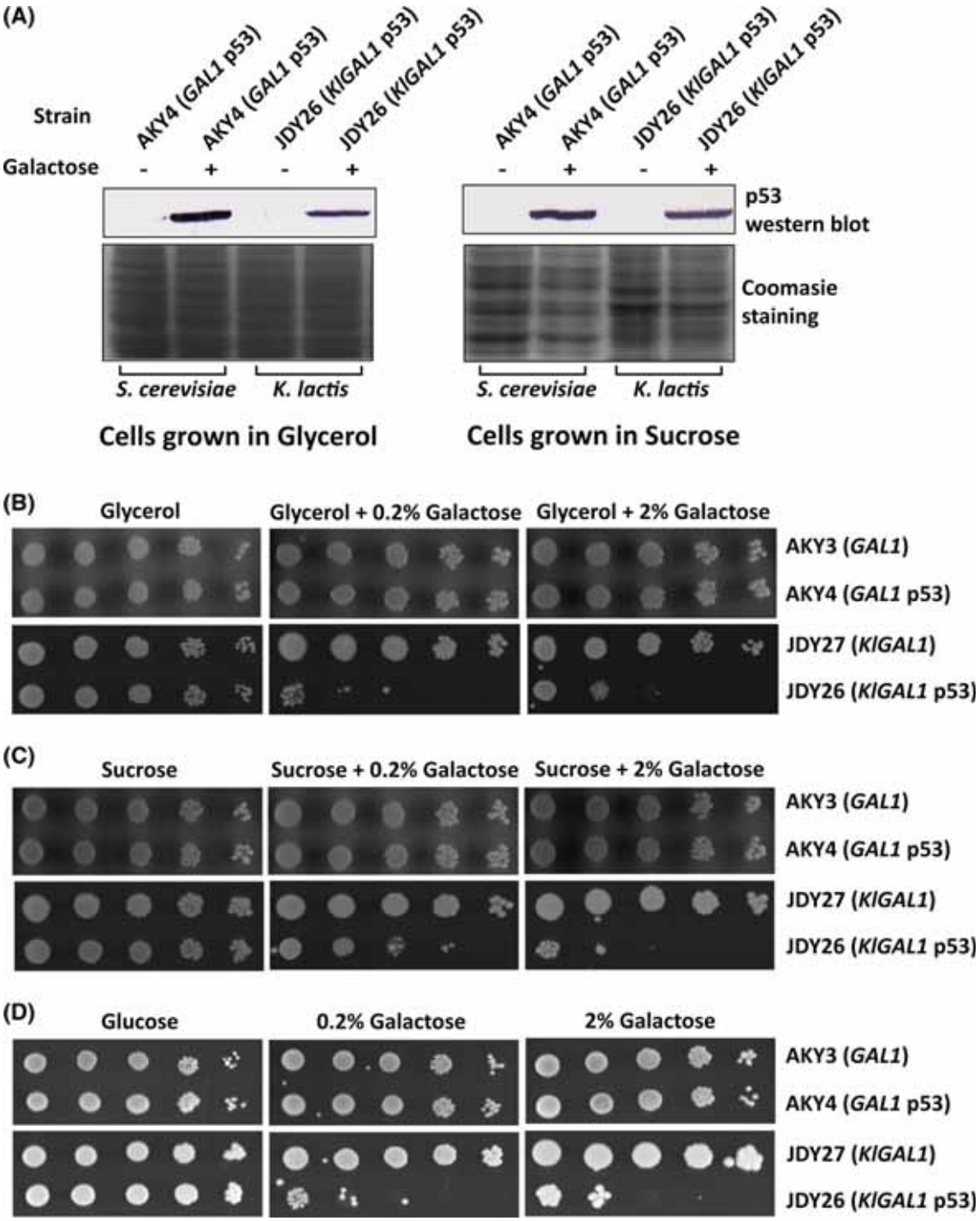


**Figure 4.** Effect of p53 over-expression on growth of *K. lactis*. (**A**) The yeast strains with p53 expression cassette were grown in glycerol or sucrose medium. At $O.D._{600nm}$ of 0.5 the cultures were induced with 2% final concentration of galactose and incubated further for 8 h at 30°C. The cells were harvested and the cell free extract was analysed for western blot analysis. The blot was probed with anti p53 antibody. Coomassie staining was performed to monitor equal loading of protein samples. (**B**) The overnight glycerol grown cells of *S. cerevisiae* (used as a control for the sake of comparison) and *K. lactis* were diluted serially and spotted onto glycerol and glycerol-galactose plate, (**C**) sucrose and sucrose-galactose plate and (**D**) Glucose and galactose. The results were recorded after 60 h of incubation at 30°C.

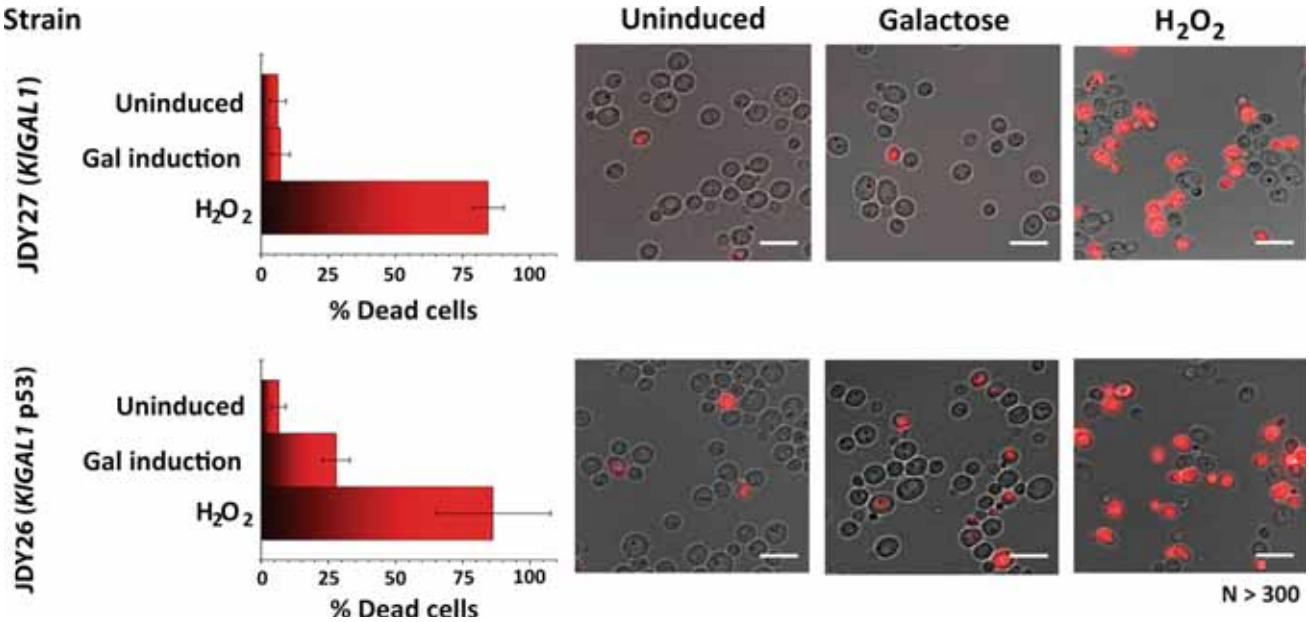


**Figure 5.** Cell death analysis of *K. lactis* strains grown in a medium containing glycerol. The *K. lactis* strains, JDY27 and JDY26 were grown in glycerol and analysed for the cell death in presence of galactose. $H_2O_2$ and water treatment was used as a positive and negative control respectively. The data of three independent experiments is represented where N>300. The merged image for bright field and PI staining is shown for all the samples. Scale bar represents ∼5 μm.

The AKY2 strain showed ∼3-fold increase in ROS levels upon galactose induction when grown in glycerol while the parental strain AKY1 did not show any significant increase in ROS. In contrast, S. *cerevisiae* strains AKY3 and AKY4 did not show any significant difference in the ROS levels. The p53 over-expressing strain of *K. lactis*, JDY26 showed ∼ 2-fold increase in ROS levels under the above experimental condition (figure 6A). As expected, galactose did not induce ROS production in a strain that does not carry $P_{GAL10}$::p53 cassette, indicating that galactose dependent p53 induction causes increased ROS level. Cultures treated with $H_2O_2$, which is known to increase ROS levels, showed increased levels of ROS. The ROS levels were also measured in all the strains grown in medium containing sucrose with or without galactose. In congruence with the phenotype exhibited by the yeast cells, only the *K. lactis* strain expressing p53, showed significant increase (∼2-fold) in the ROS levels as compared to the respective controls (figure 6B).

### 3.6 *p53-induced ROS generation is correlated with increase in oxygen consumption*

The contrasting phenotype conferred by p53 in *S. cerevisiae* and *K. lactis* under fermenting condition was unexpected. These results imply that fermentation *per se* is not the cause for the observed differences between *S. cerevisiae* and *K. lactis*. However, cell death is correlated to the ROS production which appears to be a function of mitochondrial activity. That is, when *S. cerevisiae*, grows on non-fermentable carbon source, mitochondria become indispensable, while mitochondria are indispensible in *K. lactis* regardless of the carbon source. Therefore, we surmised that probing the mitochondrial activity with respect to oxygen consumption would reveal the underlying physiological basis for the observed phenotypic difference. To determine whether the yeast cells expressing p53 show increased levels of oxygen consumption, rate of oxygen uptake by the cells was analysed using Clark's oxygen electrode. *K. lactis* cells expressing p53 (JDY26) shows ∼1.3-fold increase in oxygen uptake when grown on glycerol plus galactose (figure 7A) as well as when grown on sucrose plus galactose (figure 7B) as compared to respective uninduced controls. AKY2 strain displayed ∼1.2-fold increase in oxygen consumption when grown on glycerol plus galactose. There was no significant difference in the oxygen consumption by AKY2 when grown in sucrose plus galactose. AKY4 cells which metabolize galactose neither show death phenotype nor display increase in oxygen uptake as compared to its uninduced control (figure 7). From these results, we conclude that, p53 over-expression leads to increase in oxygen uptake in Crabtree-negative yeast cells independent of the carbon source used for the growth. In contrast, in Crabtree-positive yeast, this strictly depends on the mode of carbon metabolism. Here we find a direct correlation of the ROS produced and oxygen uptake.

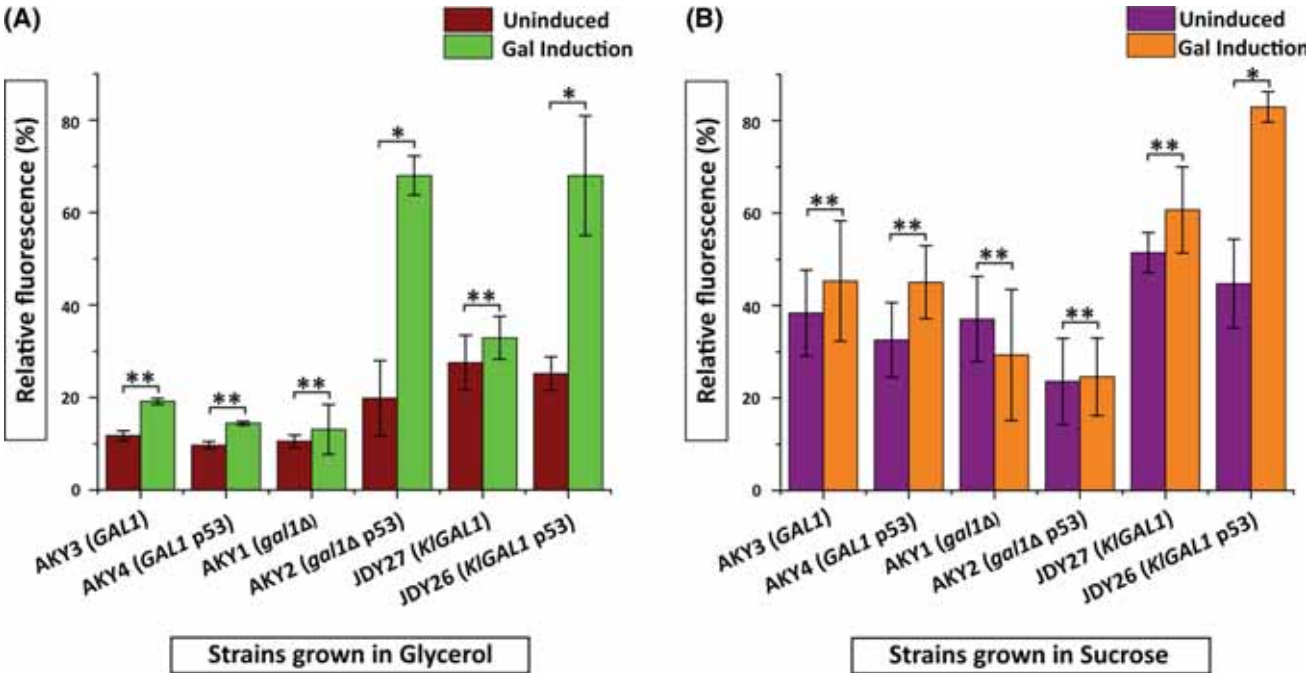


**Figure 6.** Determination of ROS levels upon galactose induction of p53: The p53 over-expressing strains and the respective parental strain (without p53) were grown in either (**A**) glycerol medium or (**B**) sucrose medium and induced with galactose at mid-log phase. The cells were then analysed for the ROS production. $H_2O_2$ induction was used as a positive control. The relative fluorescence is calculated for each sample by considering the fluorescence for $H_2O_2$ induced sample as 100%. * denotes $p < 0.05$, whereas ** denotes $p > 0.05$.

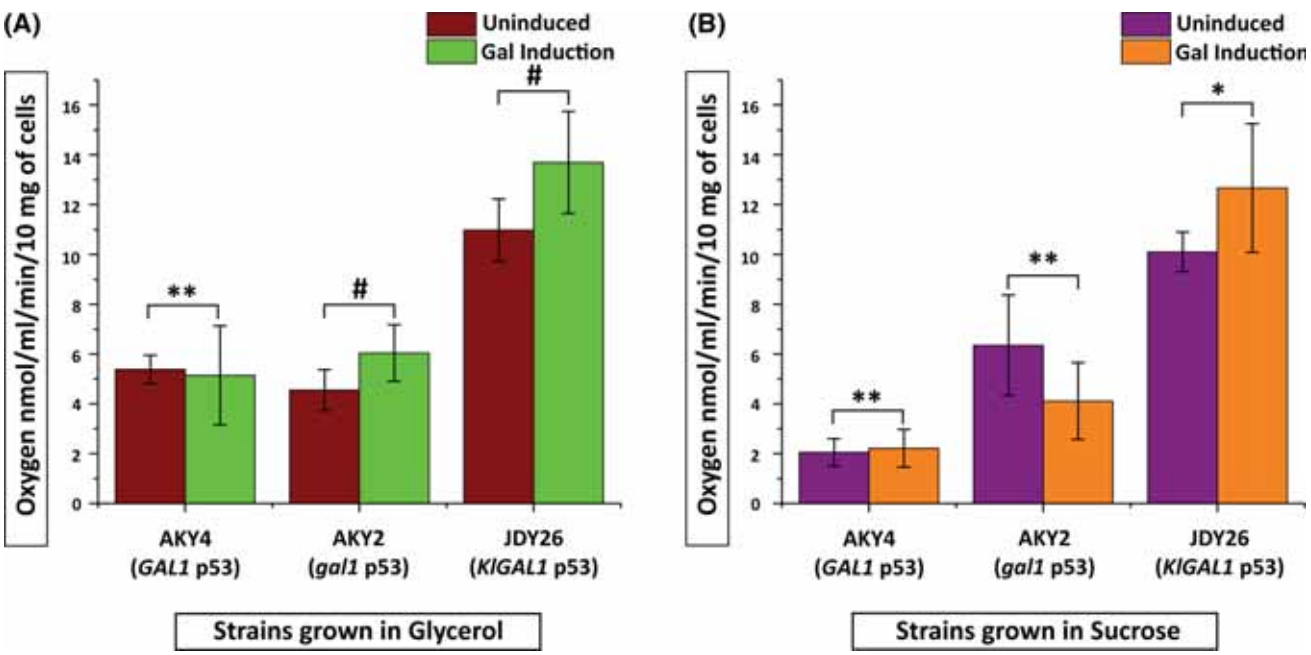


**Figure 7.** Oxygen consumption by yeast cells upon induction of p53. Yeast strains, AKY4, AKY2 and JDY26 were grown in either glycerol (**A**) or sucrose (**B**) until $O.D._{600nm}$ of 0.3 and then induced with 2% galactose and incubated further for 6 h. The rate of oxygen uptake per 10 mg wet weight of cells was monitored using Clark's electrode. A result of five independent experiments carried out in duplicates is presented. # denotes $p < 0.01$, * denotes $p < 0.05$, whereas ** denotes $p > 0.05$.

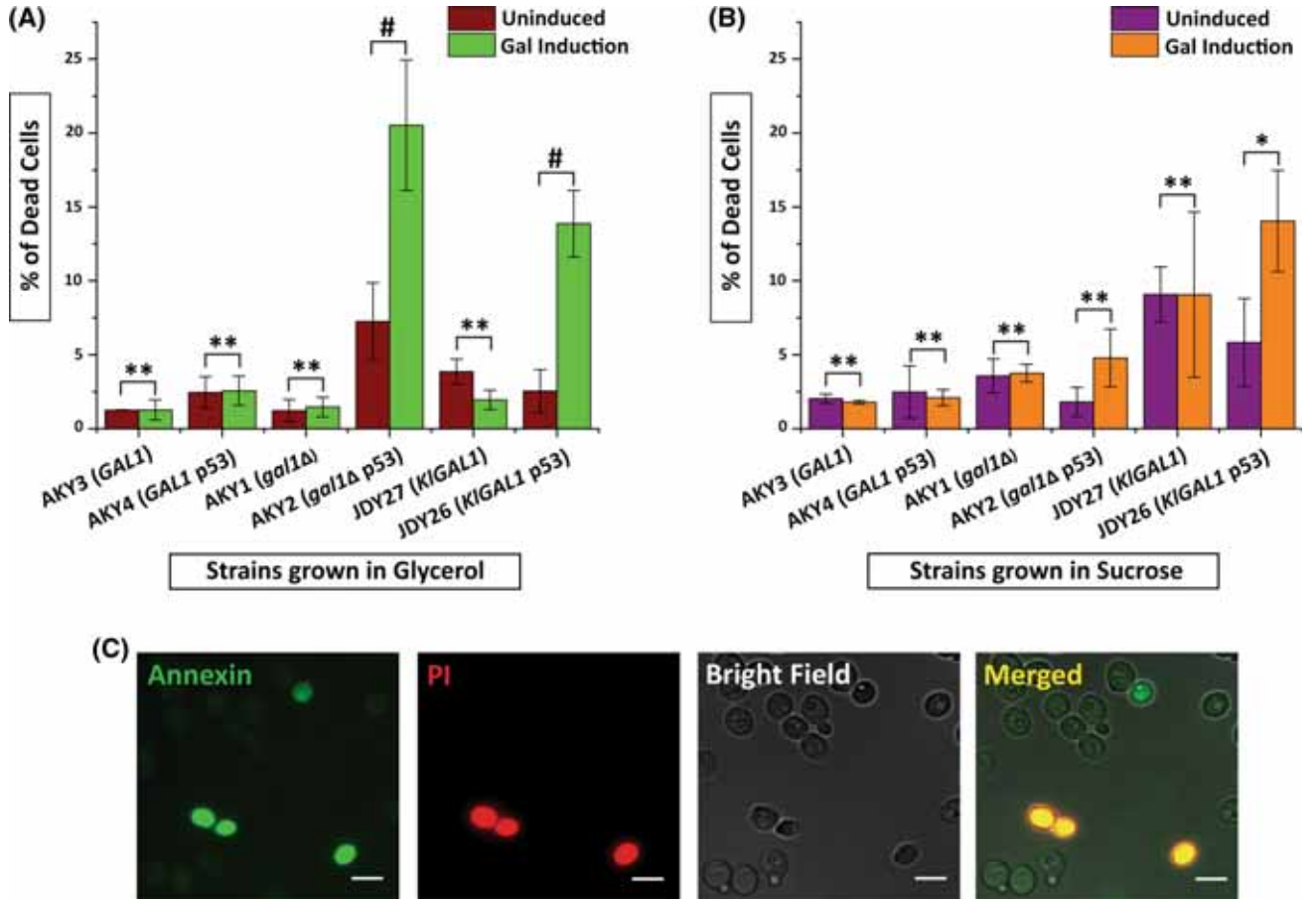


**Figure 8.** Analysis of cell death induced by p53 in yeast cells using Annexin V - PI staining: The p53 over-expressing strains of *S. cerevisiae* and *K. lactis* along with their parental controls (without p53) were grown in either (**A**) glycerol or (**B**) sucrose medium, harvested, washed with distilled water and re-inoculated in galactose containing induction medium at $O.D._{600nm}$ of 0.1. The cells were then analysed for the marker of apoptosis after 6 h of incubation in induction medium. Fluorescein coupled Annexin V staining of a cell indicates the early stage of apoptosis. The PI staining indicates the late apoptotic phase where cell membrane has started disintegrating. Unstained cells are the healthy, non-apoptotic cells. The data of three independent experiments is shown. The number of dead cells is displayed as the percentage of total cells ($N > 300$). # denotes $p < 0.01$, * denotes $p < 0.05$, whereas ** denotes $p > 0.05$. (**C**) Representative microscopic images for the yeast strain AKY2 are shown. Scale bar represents $\sim 5$ µm.

### 3.7 *p53 over-expression leads to exposure of phosphatidylserine (PS) on outer surface of cell membrane*

We monitored the exposure of phosphatidylserine to determine the status of apoptosis. The p53 expressing strains were subjected to Annexin staining as mentioned in Materials and Methods. Cells displaying green fluorescence represent the early apoptotic phase while those in red represent late apoptotic phase (figure 8C). After 6 h of galactose inductions, cells were subjected to Annexin staining. $H_2O_2$ treatment was used as a positive control. The *S. cerevisiae* strain AKY4 showed no significant difference in the number of dead cells after galactose treatment when grown in glycerol (figure 8A) or sucrose (figure 8B). This is in accordance with the growth phenotype shown by the strain. The AKY2 strain exhibiting growth inhibition on glycerol medium, showed $\sim$ 3-fold increase in the number of dead cells upon galactose induction within 6 h (figure 8A). No significant increase was observed in dead cell number for this strain when grown in sucrose (figure 8B). The *K. lactis* strain JDY26 showed $\sim$ 5-fold increase in the number of dead cells upon galactose induction when grown in glycerol and $\sim$ 2-fold increase upon galactose induction when grown in sucrose. The percentage of dead cells was almost similar after 10 h of galactose induction.

Our results demonstrate that the mechanism of p53-induced cell death is through apoptosis. To further corroborate that p53 causes apoptosis, we disrupted genes known to be involved in apoptosis of *S. cerevisiae* (supplementary

table 1). As expected, disruption of *AIF1* marginally abrogated p53-mediated apoptosis (supplementary figure 8). As this study was in progress, Leão *et al*. reported that p53 and p53 family proteins induce autophagic cell death in yeast *S. cerevisiae* (Leao *et al*. 2015). The possibility of p53-induced autophagic cell death was investigated by disrupting the genes known to be involved in autophagy. Even after the disruption of the genes involved in autophagy, p53-induced cell death was not rescued (supplementary figure 9), indicating that under the experimental condition described herein, p53 over-expression leads to apoptosis and not autophagy.

## 4. Discussion

Previously, it was attempted to link Warburg effect and mitochondrial functionality with that of growth and ROS in wild type and *rho0,* or *mgm1* or *oxa1* strains of *S. cerevisiae* (Ruckenstuhl *et al*. 2009). The result indicated that impairment of mitochondrial function provided a better survival value and this observation were correlated with ROS production. Moreover, prior logarithmic growth of wild type *S. cerevisiae* on glucose reduced the CFU on galactose or glycerol medium to the extent of 70%, suggesting that the initiation of colony growth was inhibited by mitochondrial respiration. However, glucose grown stationary phase cells did not show such drastic effects indicating the critical role played by functional state of the mitochondria.

In contrast to the above, we used galactose inducible system to express p53 and monitored its effect in respiro-fermentative (galactose, sucrose) and non-fermentative carbon source (glycerol) rather than glucose, which is only a fermentative condition. That is, in glucose grown cells, mitochondrial functionality is severely repressed (Johnston 1999) and the effect that p53 could have on mitochondrial function cannot be assessed. Such a possibility explains the previous result that over-expression of Bax was unable to induce apoptosis when cells were grown in glucose and not lactate (Priault *et al*. 1999). Unlike glucose, galactose exerts Crabtree effect to a lesser extent (Kruckeberg AL 2004) and thus the mitochondrial function is not abolished. Moreover, in cancer cells, mitochondria are known to be involved in respiratory function. Thus the observations made under respiro-fermentative condition (such as growth on galactose) seem to be more relevant than a condition in which mitochondrial function is completely obliterated (such as growth in glucose). Further, we compared the effect of p53 induced apoptosis under similar experimental condition in two different yeast species. This approach allowed us to correlate the differences in their physiology in addition to the differences that arise because of the variation in metabolism within a given species.

Based on our study, we suggest that p53 causes apoptosis only when *S. cerevisiae* is grown in a non-fermentable carbon source. In *S. cerevisiae*, the mitochondrial functionality is dependent on the carbon source unlike *K. lactis*. That is, *S. cerevisiae* consumes less oxygen in presence of carbon sources like galactose and sucrose as compared to non-fermentative carbon source like glycerol. In contrast *K. lactis* does not show such a difference (see supplementary figure 10). In our study, p53 was able to cause apoptosis in *S. cerevisiae* only when glycerol but not galactose or sucrose was used as a carbon source. This clearly indicates that it is the mitochondrial functionality that decides whether or not p53 can induce apoptosis. This is further supported by observation in *K. lactis*; p53 over-expression causes apoptosis independent of carbon sources.

As mentioned before, *K. lactis* although ferments sugars but does not accumulate ethanol because it is oxidized through mitochondrial oxidation (Piskur *et al*. 2006). If fermentation *per se* were to be responsible for escape from apoptosis, p53 should not have caused apoptosis in *K. lactis* under fermenting conditions. However, we observed that in *K. lactis* even under fermenting conditions p53 causes apoptosis unlike what is observed in *S. cerevisiae*. Thus the above differences between these two results probably arise because the mitochondria of *K. lactis* are more sensitive to p53 induced apoptosis as compared to that of *S. cerevisiae* as fermentation occurs in both strains.

*S. cerevisiae* response to p53 induced apoptosis appears to correlate to the oxygen uptake. That is, there appears to be a threshold level of oxygen uptake beyond which p53 is able to induce apoptosis (compare the oxygen uptake in galactose/sucrose to glycerol/ethanol supplementary figure 10). This difference can be attributed to difference in the gene expression when cells are growing in fermentable and non-fermentable carbon sources. Alternatively, when cells are growing in fermentable carbon source, sufficient reducing equivalents are produced to scavenge the limited amount of ROS. On the other hand, when cells are growing in non-fermenting conditions, not only ROS generated is more, but also the reducing equivalents generated may not be sufficient to scavenge the ROS. Thus, it appears that the difference in p53 mediated apoptosis in *S. cerevisiae* is a reflection of the net effect of ROS generation and the ability to scavenge the ROS. In contrast, in *K. lactis*, the reducing equivalents are insufficient to overcome the effect of ROS.

Based on our study, we speculate that *S. cerevisiae* is metabolically more akin to cancer cells in that during fermentation cells are not undergoing apoptosis. However, the fundamental difference being that in cancer cells, the mitochondria are functionally active in that they mainly oxidise glutamate than pyruvate derived from glucose. Unlike *S. cerevisiae*, *K. lactis* is more like a normal cell and it cannot subsist without mitochondria. Unlike *S. cerevisiae* but like humans, in *K. lactis*, the synthesis of orotate from

dihydroorotate is catalysed by dihydroorotate dehydrogenase (DHOD), a mitochondria linked enzyme. However, the DODH of *K. lactis* has not been characterised. Interestingly, inhibition of this function in human cancer cells was demonstrated to activate p53 (Khutornenko *et al*. 2010). Given the similarity between *K. lactis* and humans in this respect, it will be interesting to see whether similar mechanisms operate in *K. lactis* as well. Taken together, using Crabtree-positive and Crabtree-negative species of yeast, we provide an evidence that p53 dependent apoptosis is dependent upon the functional status of mitochondria and is independent of fermentation. If this is true, then the primary event that occurs during tumorigenesis could be escape from apoptosis brought about by the glucose dependent down regulation of mitochondrial function.

Normal quiescent mammalian cells oxidise glucose through mitochondrial oxidation and readily undergo apoptosis in response to cellular insult (Fulda *et al*. 2010; Barbour and Turner 2014; Childs *et al*. 2014). On the other hand, cancer cells mainly ferment glucose to lactate even in presence of oxygen and functional mitochondria but are resistant to apoptosis (Warburg 1956; Vander Heiden *et al*. 2009; Koppenol *et al*. 2011; Hall *et al*. 2013). Thus, the ability of cancer cells to escape from apoptosis despite having functional mitochondria is due to fermentation or not, is a fundamental question in cancer biology. While the mechanism is not clearly understood, there appears to be a strong functional link between Crabtree effect, aerobic glycolysis and escape from apoptosis (Diaz-Ruiz *et al*. 2011). As discussed in the introduction, several studies in the past have demonstrated the ability of human proteins to induce apoptosis in *S. cerevisiae*, *S. pombe* and *P. pastoris* under varying experimental conditions. These observations are unlikely to be fortuitous and therefore are likely to have biological implications in understanding the mechanisms by which cancer cells are known to escape from apoptosis. Our result that p53 induced apoptosis is prevented in *S. cerevisiae* by Crabtree effect provides a possible connection between these two phenomenon. This is in broad agreement to what has been observed in cancer cells (Diaz-Ruiz *et al*. 2008).

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