Conserved Actin Cysteine Residues are Oxidative Stress Sensors That Can Regulate Cell Death in Yeast

Michelle E. Farah and David C. Amberg

SUNY Upstate Medical University, Department of Biochemistry and Molecular Biology,
750 E. Adams St., Syracuse, NY 13210

Abstract

Actin's functional complexity makes it a likely target of oxidative stress but also places it in a prime position to coordinate the response to oxidative stress. We show that ROS accumulation and premature chronological aging of cells lacking the oxidoreductase Oye2p can be completely suppressed by removing the potential for formation of its substrate, the actin C285-C374 disulfide bond. We further show that the clinically important antioxidant N-acetylcysteine can suppress the *oye2*Δ cytoskeletal and cell death phenotype. Since these two cysteines are co-conserved in all actin isoforms, we theorize that we have uncovered a universal mechanism whereby actin both senses and responds to oxidative load.

Introduction

The accumulation of oxygen-containing free radicals in the cell results in oxidative stress conditions that may cause apoptosis and cellular aging. The actin cytoskeleton is an early target of cellular oxidative stress [1]. In certain disease conditions, the oxidative state of actin in the cell is very different from normal. For example, in sickle cell crisis, a major factor that contributes to the inflexibility of irreversibly sickled cells (ISCs) is the formation of an intracellular disulfide bond between C284 and C373 of β-actin [2], [3]. We have previously shown that the yeast actin cytoskeleton is subject to the same form of oxidation as ISCs. Our finding that an oxidoreductase called Oye2p (Old Yellow Enzyme) regulates oxidation between C285 and C374 in *S. cerevisiae* suggests that actin oxidation takes place in all eukaryotic cells and that the actin cytoskeleton is subject to redox regulation [4]. That $act1^{C285A}$ and $act1^{C374A}$ bearing mutants are more resistant to oxidative stress than wild type strains, and that these alleles completely suppress the oxidative sensitivity of an old yellow enzyme null strain, proves that actin is a critical target for determining sensitivity to oxidative stress.

Actin's involvement in programmed cell death is becoming a central focus of apoptosis and cancer research [5]. Changing actin dynamics in lymphocytes by either stabilizing or destabilizing F-actin structures modulates apoptotic signaling upon withdrawal of growth factors [6]. In addition, during TNF-α apoptotic signaling, viral disruption at the β-actin locus dramatically reduces mitochondrial clustering and production of reactive oxygen species (ROS), indicating that actin participates in a programmed cell death program [7]. Furthermore, hyperstabilization of actin by addition

of a drug called jasplakinolide (Jas) induced apoptosis in HL-60 cells, and blocking of actin polymerization inhibited camptothesin-induced apoptosis suggesting that the polymerization status of the actin cytoskeleton is crucial to apoptotic initiation and progression [8].

In recent years, the yeast model has been emerging as an important system to better understand the ancestrally conserved mechanisms of regulated cell death. Yeast cells exhibit markers of apoptosis in common with mammalian cells such as chromatin condensation, DNA fragmentation, phosphatidylserine exposure and ROS accumulation [9]. In *S. cerevisiae*, decreased actin dynamics has been shown to increase the accumulation of ROS [10]. Since the redox state of actin is an important contributor to actin stability ([4], [2], [11]), in the current study we asked whether the oxidation state of actin's cysteines 285 and 374 was able to regulate cell death in *S. cerevisiae*. Our data suggests that the oxidoreductase Oye2p may be playing an anti-apoptotic role in the cell by protecting the actin cytoskeleton from oxidative hyperstabilization. Our data supports a model in which the actin cytoskeleton is a central signaling component that couples the accumulation of reactive oxygen species to programmed cell death.

Results

The Accumulation of ROS in cells lacking Oye2p is suppressed by *act1*^{C285A} and *act1*^{C374A}

ROS are central mediators of apoptosis in yeast and mammalian cells [9], [12], being both necessary and sufficient to drive cells into programmed cell death [9]. We

have previously shown that *oye2Δ* cells are sensitive to external sources of oxidative stress and that the recovery of their actin cytoskeleton and growth following oxidative stress is delayed in comparison to wild type cells. Here we asked whether *oye2*Δ cells have naturally high levels of ROS by quantifying the percentages of logarithmically growing cells that stain with the fluorescent, ROS indicator dye dichlorodihydrofluorescein diacetate H_2DCFDA. An example of a wild type versus an *oye2*Δ cell is shown in the inset of Fig. 1. To confirm that the ROS-sensing dye, H_2DCFDA, was specifically reacting with ROS in our cells, we showed that addition of the anti-oxidant N-acetyl L-cysteine (NAC) to the *oye2Δ* cells blocked H_2DCFDA-induced fluorescence (data not shown). As shown in Fig. 1, in comparison with wild type, there is approximately a 30% and 20% increase in the number of H_2DCFDA reactive *oye2Δ* and *oye3Δ* cells, respectively. In an *oye2Δ* strain, but not the *oye3Δ* strain, we were able to suppress increased ROS levels with either the $act1^{C285A}$ or the $act1^{C374A}$ alleles. These data suggest that Oye2p, but not Oye3p, is playing a specific role in protecting cells from an elevation of ROS that is in fact induced by the oxidation of the C285-C374 cysteine pair in actin. Consistent with these observations, our previous studies showed that Oye3p is unable to interact with actin and that *oye3Δ* cells have a normal actin cytoskeleton. The oye3Δ strain does show an elevation in ROS positive cells that appears to be unrelated to actin oxidation. Consistent with these observations, the *oye2Δ oye3Δ* $act1^{C374A}$ and *oye2Δ oye3Δ* $act1^{C285A}$ strains have ROS levels equivalent to that of the *oye3Δ* strain. To examine the role of a direct interaction between actin and Oye2p, in regulating the *in vivo* accumulation of ROS, we examined a strain expressing a mutant form of actin (act1-123p) that we previously showed has a reduced affinity for Oye2p [4].

As expected, the *act1-123* strain showed a comparable percentage (27%) of ROS positive cells to the *oye2*Δ strain.

Mitochondria have been recognized as the major source of ROS generation in the cell, specifically stemming from the electron transfer complexes of the respiratory chain [13]. To examine whether the mitochondrial respiratory chain is the source of oxygen-containing free radicals in our ROS-elevated mutants, we generated strains lacking mitochondrial DNA (rho^0). These strains were tested for lack of growth on glycerol (a non-fermentable carbon source) and confirmed by DAPI staining to be devoid of mitochondrial DNA (data not shown), and are therefore lacking a functional mitchondrial respiratory chain. As shown in Fig 1, ROS-accumulating cells in *oye2Δ* and *oye3Δ* mutants was decreased by approximately half, suggesting that half of the ROS in old yellow enzyme-lacking mutants are generated from an origin other than the electron transport chain. We stained the actin cytoskeleton of rho^0 *oye2oye3* cells in order to examine if this level of oxidative stress was sufficient to cause oxidation-mediated stabilization of the actin cytoskeleton. We found that there was a partial suppression of the hyperstabilized actin phenotype of the old yellow enzyme-lacking mutants (data not shown) consistent with the observed decrease in ROS levels. As with our rho^+ mutants, the number of rho^0 *oye2Δ* cells with elevated ROS was suppressed to wild type levels by both $act1^{C285A}$ and $act1^{C374A}$. This suggests that the oxidative status of these cysteine residues of actin, and the consequent stabilization of the actin network, are key events in the mechanism governing ROS generation.

Yap1p is a transcription factor that is translocated to the nucleus upon exposure to oxidative stress [14]. It contains a carboxy-terminal cysteine-rich domain (c-CRD) that

senses cytoplasmic oxidative stress and is necessary for cellular viability upon addition of external oxidants [15, 16]. Furthermore, age-induced cell death has been shown to be delayed in Yap1p over-expressing strains [17]. As a second important indicator of oxidative stress, we found that in approximately 32% of *oye2Δ* cells, GFP-Yap1p is localized to the nucleus in early stationary phase cells, whereas this nuclear localization pattern was observed in less than 1% of a comparable culture of wild type cells (Fig. 2). Importantly, in our double mutants *oye2*Δ $act1^{C285A}$ and *oye2*Δ $act1^{C374A}$ nuclear accumulation of Yap1p is suppressed. These results indicate that in the absence of Oye2p, normal cellular levels of ROS lead to actin oxidation and a perception that the cell is under oxidative stress. This actin oxidation appears to trigger an elevation of ROS and in this way, actin appears to be both a sensor and an effector of ROS elevation.

Chronological Aging of *OYE*-lacking mutants is suppressed by $act1^{C285A}$ and $act1^{C374A}$

It has previously been shown that aging yeast cells display an apoptotic phenotype, showing markers such as increased ROS accumulation, DNA degradation, extracellular phosphatidylserine exposure, and yeast caspase activation [17]. To determine if the observed ROS elevation in *oye*-deficient cells is triggering cell death, we monitored the viability of our strains maintained in YPD over a period of 18 days. We found that there was a rapid decline in the viability of our *oye2Δ* cells compared to wild type (Fig. 3). During 18 days at stationary phase, both the *oye2Δ* and *oye2*Δ *oye3Δ* cell viability dramatically decreases starting at day 4 reaching less than 20% by day 18. Note that the loss of OYE3 appears to have little additive effect, suggesting that only the loss of OYE2 alone is causing the observed loss of cell viability. Importantly, the premature

aging of the *oye2Δ (*and the *oye2*Δ*oye3*Δ strain) was suppressed by both *act1*^{C285A} and *act1*^{C374A} mutations. These data support our model that in the absence of Oye2p, oxidation of actin's cysteine residues, and the resulting elevation of ROS, leads to premature aging and cell death.

The antioxidant N-acetyl L-cysteine suppresses the actin defects and loss of viability observed in *oye*-lacking mutants

N-acetyl-L-cysteine (NAC) is an antioxidant that is emerging as an important therapeutic agent for the treatment of many conditions. Importantly, NAC is in stage II clinical trials as a treatment for sickle cell patients. It is believed to act by reducing the oxidation of thiol groups in the β-actin and spectrin of irreversibly sickled red blood cells restoring plasiticity to the cortical cytoskeleton [18], [19]. To ask if NAC could reverse the effects of actin oxidation in yeast, we examined the phenotype of oye2Δ cells following treatment of NAC. As can be seen in Fig. 4A, the characteristic actin organization defects of the *oye* deficient strain (hyperstabilization of actin cables and patches) were suppressed by the addition of NAC. We next tested whether the accelerated death of the *oye2Δ* cells could be suppressed by the addition of 31 mM NAC (Fig. 4B). The addition of NAC at day 4 increased the percentage of *oye2Δ* viable cells at day 5 from 78% to 92%. By day 8, addition of NAC at either day 4 or day 6 increased untreated *oye2Δ* viability from 63% to 82% and 86%, respectively. However, after day 8, the viability of both NAC-treated *oye2Δ* and wild type cultures rapidly drops off perhaps as a consequence of NAC or NAC byproduct toxicity. Nonetheless, it appears that NAC can

protect the actin cytoskeleton of yeast from oxidative stress and from actin-oxidation induced cell death.

Discussion

Actin is perhaps one of the most functionally diverse proteins in eukaryotic cells. Traditionally the actin cytoskeleton is thought of as playing structural and organizational roles as well as a being a direct participant in a wide range of motility processes. Recently actin has also been found to be involved in many nuclear processes as well including regulation of chromatin structure and transcription [20-22]. This functional diversity makes the actin cytoskeleton an ideal candidate for integrating signaling between diverse cellular processes. Previous work has not extended actin's functions to include signaling but the results presented here show that a distinct change in the actin oxidation state activates an oxidative stress response that can culminate in apoptosis. Concerning the work presented here, it has become appreciated that the proper regulation of actin dynamics is an important determinant of cell survival; hyperstabilization of actin filaments leads to an apoptotic phenotype followed by programmed cell death in both yeast and mammalian cells.

We have furthered these findings by showing that cysteines 285 and 374 of actin are important physiological sensors of intracellular oxidative stress and are regulators of programmed cell death during chronological aging. Specifically, the $act1^{C285A}$ and $act1^{C374A}$ alleles are able to suppress the intracellular accumulation of ROS, Yap1p nuclear localization, and accelerated cell death observed in *oyeΔ* cells. We have also shown that both the hyperstabilized actin phenotype and rapid decline of chronologically

aging *oye* cultures is suppressed by treatment with the clinically relevant antioxidant NAC. NAC is becoming recognized as an important treatment for HIV infection[23-25], cancer[26], neurodegenerative diseases[27] and many other diseases and conditions. It is believed to function by protecting against cell death/as a promoter of cell survival [28]. Importantly, it is currently in phase II trials for the treatment for sickle cell crisis[18]. Its efficacy in treating sickle cell crisis is based upon its ability to reduce C284-C373 oxidized actin and return proper plasticity to the RBC cytoskeleton[19]. The results reported here further suggest that NAC may act by suppressing an actin-induced cell death pathway. Erythrocytes are capable of undergoing a form of apoptosis known as eryptosis. In patients with sickle cell anemia, erythrocytes have been shown to have a 6 day half-life, compared to the 60 day half-life of normal red blood cells[29]. It is also worth noting that sickle cells generate twice the amount of ROS as control red blood cells, an observation similar to that of OYE-lacking mutants [30]. Future studies of the mechanism of action of NAC in the treatment of sickle cell anemia and other diseases may uncover that the oxidative state of actin plays a large part in the determination of survival of mammalian cells and that a key target of NAC is oxidized actin.

If actin's cysteines 285 and 374 can become disulfide-bonded and ultimately cause cell death, then why have they been co-conserved in all eukaryote actin isoforms? We theorize that it may be to aid in the destruction of unfit cells that have accumulated excessive ROS and intracellular oxidative damage. In support of this theory of adaptive aging, superoxide has been shown to mediate an altruistic aging and death program that leads to the death of over 90% of a yeast population to allow for the survival of fitter/healthier cells[31].

To begin to understand the cellular mechanisms by which ROS are being generated in *oye2*Δ cells, we have used cells lacking a functional respiratory chain to show that mitochondrial ROS account for approximately half of the total ROS accumulation in *oye2*Δ cells. Many studies have shown that mitochondria and actin functionally interact. Regulation of actin dynamics is required for proper mitochondrial inheritance, and for maintenance of mitochondrial morphology[32, 33]. The actin-depolymerizing protein gelsolin has been shown to inhibit the activity of the voltage-dependent anion channel (VDAC), the major channel in the outer membrane of mitochondria that regulates superoxide release, thereby preventing apoptotic mitochondrial changes in mammalian cells[34]. Actin itself has been shown to regulate apoptotic changes in *Neurospora crassa*, where monomeric actin, not the filamentous form, appears to regulate this channel's activity[35]. Yeast also contains the VDAC in their mitochondria[36]. One can easily imagine that the release of ROS through pores in the mitochondrial membrane of yeast and mammalian cells may be an event that is regulated by the dynamic state of actin. In our model, actin would be further targeted by mitochondrial ROS, and this would lead to greater stabilization of actin, and even more spilling of ROS from mitochondria in a positive feedback loop leading to cell death.

Although some of the molecular details remain to be elucidated, the work presented here is consistent with a model in which cellular oxidative load is measured via the oxidative formation of the C285-C374 disulfide bond of actin. Under normal levels of ROS, old yellow enzyme 2 is able to keep pace in reducing the disulfide but when the load becomes too high, or perhaps if Oye2p is inhibited, the resulting accumulation of oxidized actin results in stabilization of the actin cytoskeleton. This in turn triggers

further elevation of ROS, in part via spilling from the mitochondria, leading to greater oxidation and stabilization of actin, thereby establishing a positive feedback loop that ultimately leads to cell death.

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Table 1

Table 1. *Saccharomyces cerevisiae* strains		
Name	**Genotype**	**Source**
DAY111x112	*MAT***a**/*MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200*	D. Amberg
DAY127x128	*MAT***a**/*MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 oye2-Δ2::*HIS3*/ oye2-Δ2::*HIS3 *oye3-Δ2::*TRP1*/oye3-Δ2*::TRP1	Haarer and Amberg, 2004
DAY111	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200*	Haarer and Amberg, 2004
DAY119	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye2-Δ2*::HIS3	Haarer and Amberg, 2004
DAY123	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye3-Δ2*::TRP1	Haarer and Amberg, 2004
DAY128	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye2-Δ2*::HIS3 *oye3-Δ2*::TRP1	Haarer and Amberg, 2004
MDY11	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye2-Δ2*::HIS3 *act1* ^{C374A}::HIS3	This study
MDY13	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye2-Δ2*::HIS3 *act1* ^{C285A}::HIS3	This study
DAY173	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye2-Δ2*::HIS3 *oye3-Δ2::*TRP1 *act1* ^{C285A}::HIS3	Haarer and Amberg, 2004
DAY175	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 oye2-Δ2*::HIS3 *oye3-Δ2::*TRP1 *act1* ^{C374A}::HIS3	Haarer and Amberg, 2004
DAY169	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 act1* ^{C374A}*::*HIS3	Haarer and Amberg, 2004
DAY171	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 his3Δ200 act1* ^{C285A}*::*HIS3	Haarer and Amberg, 2004
BHY313	*MATα his3Δ1 lys2Δ0 uraΔ0 act1-123::klURA3 mfa1Δ::*P_{MFA1}*-sphis5+, can1Δ*	B. Haarer

***Table 1. Saccharomyces cerevisiae* strains utilized in this study**

Fig. 1

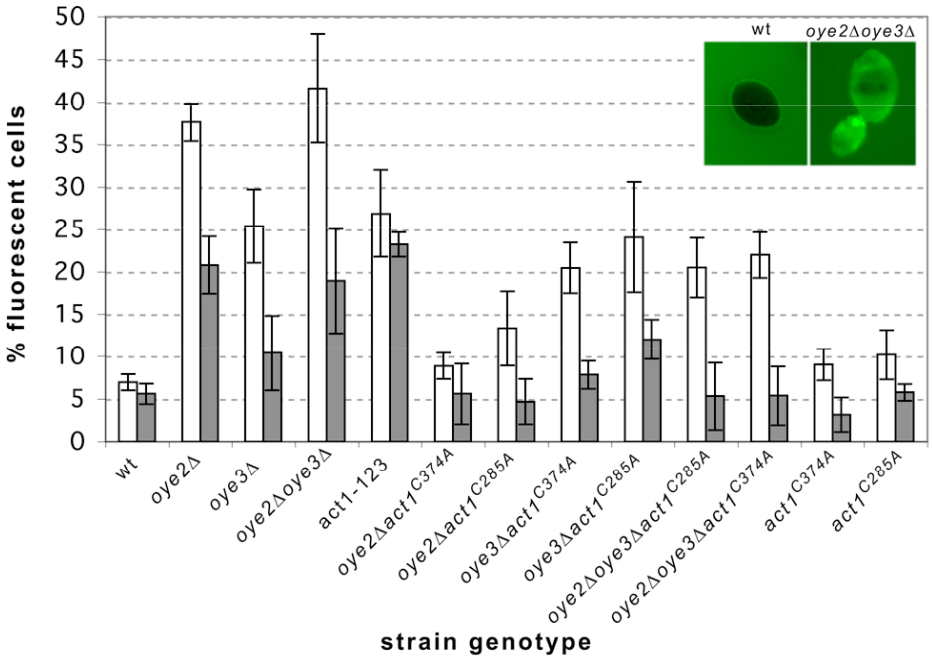


***Figure 1*. Actin oxidation in *oye*Δ cells leads to ROS accumulation.**

Logarithmically growing cells were stained for 3.5 hr with the ROS sensing dye H_2DCFDA and the percentages of fluorescent cells were determined. The white bars correspond to *rho*$^+$ cells and the black bars correspond to *rho*^{0} cells. The averages and standard deviations reported are for three to four experiments. An example of a ROS negative wild type cell is shown in the inset (left) and an example of a ROS positive *oye*Δ cells is shown in the inset (right).

Fig. 2

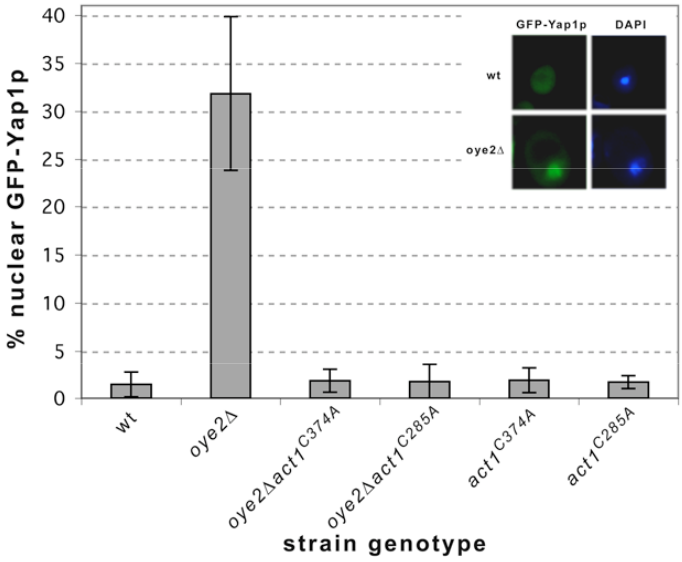


***Figure 2*. In the absence of Oye2p, actin oxidation activates the oxidative stress response.**

The oxidative stress responsive transcription factor Yap1p was expressed as a GFP fusion in various mutants and the percentages of cells with nuclear GFP-Yap1p was quantified by fluorescence miscroscopy. Nuclei were identified by DAPI staining. The results reported are for 3 experiments (N=500 per experiment). An example of a non-stressed wild type cell is shown in the upper inset and an example of an oxidatively stressed *oye2*Δ cells is shown in the lower inset.

Fig. 3

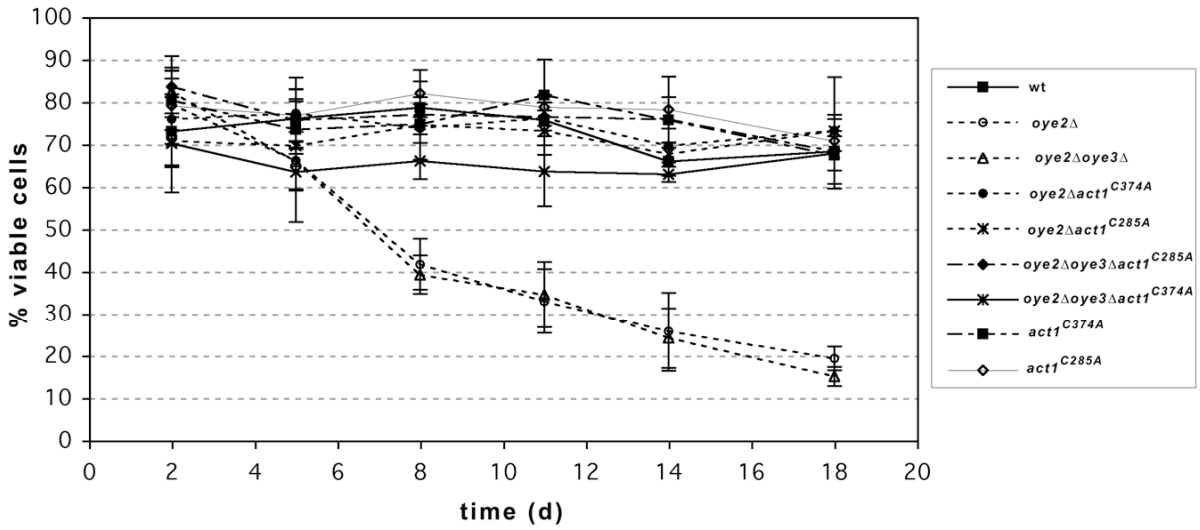
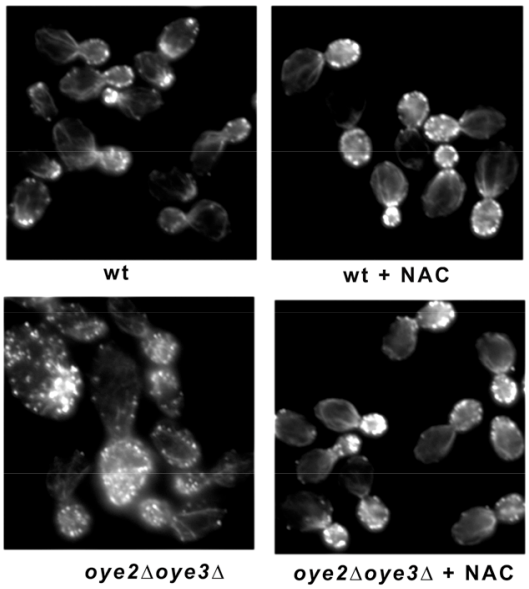


***Figure 3*. In the absence of Oye2p, actin oxidation leads to premature aging and cell death.**

Strains were grown to and maintained in stationary phase for 18 days. On the indicated days, cells were plated and the percentages of viable cells were determined based on colony forming units/total cells. The averages and standard deviations were calculated from 3 experiments where N=400, N being the number of cell bodies plated.

Fig. 4

A



B

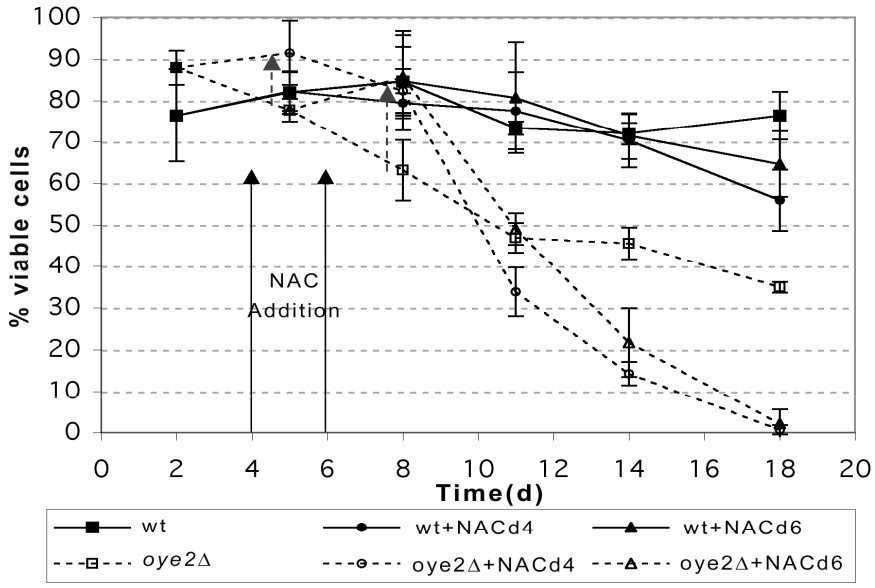


Figure 4. **N-acetyl-L-cysteine suppresses the actin organization and accelerated cell death phenotype of *oye2*Δ cells.**

(A) N-acetyl L-cysteine was added to logarithmically growing cultures at a final concentration of 31mM for 5.5 hr after which the cells were fixed, stained with rhodamine phalloidin and visualized by fluorescence microscopy. (B) N-acetyl L-cysteine (NAC) was added at a final concentration of 31 mM to stationary cultures on days 4 or 6 (indicated by arrows). Cells were removed at the indicated time points, dilutions were plated and percent viability was calculated based on colony forming units/total cell density. The arrows with dotted lines show the increases in viability at day 5 for NAC addition at day 4 and at day 8 for NAC addition at days 4 and 6.

Materials and Methods

Construction of strain, media conditions

All yeast strains used in this study are of the S288C background. Yeast strains are listed in table 1. Standard methods were used for growth, sporulation, transformation and genetic analysis of yeast [37].

Generation of rho^0 mutants

The method used to generate rho^0 strains is as described [38]. In addition to streaking all colonies to YP+ 3% glycerol to confirm lack of mitochondrial respiratory function, DAPI staining was performed as described to confirm the complete absence of mitochondrial DNA[37] .

Detection of accumulation of reactive oxygen species

Cultures were grown to logarithmic phase (around $3x10^7$ cells/ml) and reactive oxygen species (ROS) were detected *in vivo* with the ROS-sensing dye dichlorodihydrofluorescein diacetate (Sigma Inc.) as previously described [9], except the cells were incubated for 3.5 h. Cells that appeared fluorescent in the FITC channel were scored as ROS positive. 500 cells were quantified for each experiment. The experiment was repeated at least three independent times for all strains and the number of cells that appeared fluorescent was averaged and standard deviations were calculated.

Microscopy

The staining of the actin cytoskeleton, and microscopy methods were performed as described [37].

Localization of GFP-Yap1p

The *URA3*-marked, low copy CEN vector pGFP-Yap1p was obtained from the Moie-Rowley lab [14]. Transformed cells were grown in 3ml SC-Ura media until cells reach a density of ~1x10^8 cells/ml. Cells were stained with 0.5 μg/ml DAPI for 15 min and observed for GFP fluorescence on a Zeiss Axioskop 2 MOT microscope[37]. 200 cells of each strain were scored for nuclear GFP-Yap1p localization.

Actin Staining of N-acetyl-L-cysteine treated cells

While cells were growing in YPD in log phase, NAC was added to a final concentration of 31 mM and the cells were incubated for an additional 5.5h. Cells were fixed and stained with rhodamine phalloidin as described [37].

Determination of cell viability

A fresh colony of each strain was inoculated into 5ml of YPD in a well-aerated culture tube. For the length of the experiment, cultures were maintained on a rotating drum in a 30°C incubator. At the indicated time points, cells were gently sonicated with a Branson sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT), and duplicate serial dilutions were made in YPD. The cells were diluted by a total factor of 10^6 of which 100

μl was plated onto YPD media. Cell densities were obtained by making two 1:100 dilutions from each culture and then counting the cell numbers in duplicate in a hemacytometer. When a maximum cell density was obtained, this number was used as the cell density for all subsequent calculations of cell viability.

Cell viability was calculated as:

Average colony forming units/maximum average cell density = % viability at each timepoint

Each experiment was repeated three times.

For the viability experiments that utilized NAC, 5ml cultures of cells were grown to stationary phase and at day 2, the cultures were split into separate tubes (control and experimental). Cells were plated at days 2, 5, 8, 11, 14 and 18. An aqueous solution of NAC was added to a final concentration of 31mM to cultures at either day 4 or day 6. An equivalent volume of water was added to the control sample. The fraction of viable cells was calculated as described above.

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