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Disruption of aldo-keto reductase genes leads to elevated markers of oxidative stress and inositol auxotrophy in *Saccharomyces cerevisiae* ☆

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Abstract

A large family of aldo-keto reductases with similar kinetic and structural properties but unknown physiological roles is expressed in the yeast *Saccharomyces cerevisiae*. Strains with one or two AKR genes disrupted have apparently normal phenotypes, but disruption of at least three AKR genes results in a heat shock phenotype and slow growth in inositol-deficient culture medium (Ino^-). The present study was carried out to identify metabolic or signaling defects that may underlie phenotypes that emerge in AKR deficient strains. Here we demonstrate that pretreatment of a pentuple AKR null mutant with the anti-oxidative agent N-acetyl-cysteine rescues the heat shock phenotype. This indicates that AKR gene disruption may be associated with defects in oxidative stress response. We observed additional markers of oxidative stress in AKR-deficient strains, including reduced glutathione levels, constitutive nuclear localization of the oxidation-sensitive transcription factor Yap1 and upregulation of a set of Yap1 target genes whose function as a group is primarily involved in response to oxidative stress and redox balance. Genetic analysis of the Ino^- phenotype of the null mutants showed that defects in transcriptional regulation of the *INO1*, which encodes for inositol-1-phosphate synthase, can be rescued through ectopic expression of a functional *INO1*. Taken together, these results suggest potential roles for AKRs in oxidative defense and transcriptional regulation.

Keywords: Aldo-keto reductase; Inositol auxotrophy; Oxidative stress; Heat shock sensitivity

1. Introduction

Aldo-keto reductases (AKRs) comprise a superfamily of structurally similar proteins in plant, animal, and microbial cells [1,2]. Most AKRs catalyze the NADPH-dependent reduction of carbonyl compounds into their corresponding alcohol products. Aldose reductase (AKR1B1), a prototypical AKR, catalyzes the first step of the polyol pathway via the conversion of glucose into sorbitol. In humans, this pathway likely contributes to tissue pathogenesis associated with diabetes mellitus [3–5]. In addition to aldose reductase, structurally similar AKRs of unknown function are expressed in mouse (AKR1B7, AKR1B8) and human tissues (AKR1B10) [1]. Gene targeting approaches have been used to probe the function of aldose reductase. However, AR-deficient mice appear to be essentially free of morphologic or developmental abnormalities [6], suggesting that remaining AKRs that have overlapping catalytic activities can functionally compensate for aldose reductase.

As an alternative to the use of mammalian animal models to search for physiological roles of AKRs, we have turned to the budding yeast *Saccharomyces cerevisiae*. The genome of this simple eukaryote contains multiple AKR genes that encode proteins similar in structure to mammalian AKRs. Genes and their associated nomenclatures are shown in Table 1. Some of the yeast enzymes, such as AKR5G (encoded by YDL124w, Ref. [7]), AKR3A2 (YPR1, Refs. [8,9]), and AKR3C (ARA1, Ref. [10]), were initially isolated on the basis of a desired catalytic property, such as reduction of targeted carbonyl functional groups for biosynthetic strategies. Still others, including AKR2B6 (GRE3, Ref. [11]) and AKR3A1 (GCY1, Ref. [12]) were identified as gene products that are upregulated in response to hypertonic or

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Table 1
Aldo-keto reductases in *Saccharyomyces cerevisiae*

AKR designation[a]	AKR2B6	AKR3A1	AKR3A2	AKR3C	AKR5F	AKR5G
Gene name	*GRE3*	GCY1	YPR1	ARA1		
ORF designation	YHR104W	YOR120W	YDR368W	YBR149W	YJR096W	YDL124W
References	[11,58–60]	[12,13,60,61]	[8,9,13]	[10]	[13,60]	[13,60]

[a] Nomenclature for the AKR Superfamily is available at http://www.med.upenn.edu/akr/.

hyperosmotic challenge. In previous work, we determined that five of the six AKR gene products in *S. cerevisiae* have robust activity as NADPH-dependent reductases toward various aldehydes (e.g., *p*-nitrobenzaldehyde, DL-glyceraldehyde) which are also the substrates utilized by human AR [13]. Mutant strains deficient in one AKR gene have apparently normal phenotypes. However, mutant strains involving deletion of at least three AKR genes exhibited an increased sensitivity to heat stress, suggesting that one function of the yeast AKR genes is to protect against heat stress [13]. The heat sensitivity phenotype, which was more severe following disruption of additional AKR genes [13,14], could be rescued by ectopic expression of human aldose reductase (AKR1B1) or yeast *GCY1* (AKR3A1) but not catalytically inactive mutants of these enzymes.

In the present study, we have utilized this yeast model system to elucidate the mechanisms by which AKR deficiency leads to increased sensitivity to heat stress. We hypothesize that mechanisms leading to mutant phenotypes in the AKR null strain may be associated with the cellular toxicity of unmetabolized aldehydes and ketones, which have been shown to be potent electrophiles capable of depleting intracellular redox equivalents and which have genotoxic effects associated with aberrant transcriptional regulation [15,16]. At least two lines of evidence are consistent with this hypothesis. First, kinetic analysis of the yeast AKRs in gene deletion studies by us and other investigators showed that these enzymes can reduce a variety of structurally diverse aldehydes [7–9,13]. In addition, we previously showed that the mutant phenotype (heat shock sensitivity) of a triple AKR null strain (*gre3Δ/gcy1Δ/ypr1Δ*) could be rescued by ectopic expression of only the catalytically active human or yeast AKRs but not by their catalytically inactive mutants [13].

Our studies indicate that disruption of the targeted AKRs leads to a phenotype characterized by increased expression of enzymes involved with oxidative defense. We also observed that AKR-deficient yeast have a slow growth phenotype when cultured in inositol-free medium (Ino^-). Mutants demonstrated aberrant transcriptional regulation of *INO1*, *CDS1*, and *OPI3*, all of which are inositol-responsive genes associated with phospholipid biosynthesis [17,18]. These results point to potential roles for the AKRs in oxidative defense and transcriptional regulation.

2. Materials and methods

2.1. Strains, gene deletion, and growth conditions

Parental wild-type yeast strain (BY4741) and its isogenic single deletion mutants were obtained from Research Genetics (Carlsbad, CA). AKR mutant strains were produced as described previously [13]. A list of strains used in this study is compiled in Table 2. Growth media, culture conditions, and transformation of yeast by plasmids followed standard methods [19]. Phenotypic screenings of deletion mutant strains were guided as described [20].

2.2. Molecular cloning

Plasmid pRS316, which contains the reading frame encoding the GFP-YAP1 fusion protein, was obtained from Dr. Moye-Rowley (University of Iowa) and was used as template for generating an expression construct based on the GPD415 vector. Essentially, GFP-YAP1 coding sequences were amplified from PRS316 and subcloned into the centromeric GPD415 expression vector [21]. For ectopic *INO1* expression in the ARD10 mutant, the ORF of *INO1*, together with its transcriptional termination sequences, was subcloned into the episomal GPD415 plasmid. The original plasmid for *INO1*, contained in a genomic fragment in YEp315, was kindly provided by Dr. Susan Henry [17]. The structures of both expression constructs in the GPD415 vector, including the ligation sites and transcription control elements, were verified by DNA sequencing. Expression of *INO1* in ARD10 is controlled by the GPD promoter and its own termination sequence.

2.3. Heat shock assay

Saturated yeast cultures were harvested and diluted (100-fold) into fresh YPD or SC medium to resume growth to mid-log phase ($A_{600}=1.0$). At this point, cells were harvested again, washed and resuspended in water for heat shock treatment at 39 °C, 42 °C or 55 °C. The duration of heat shock treatment is given in the appropriate figure legends. Following heat shock, cells were either returned to liquid media or were serially diluted (10-fold) in water and spotted on YPD, SC or SC selective plates to recover for growth at 30 °C for 3 days.

2.4. Iodine staining and glycogen measurement

To stain for glycogen, culture plates were exposed to iodine vapor for 5 min. The intensity of brown color developed after exposure to iodine vapor is qualitatively proportional to glycogen levels in yeast colonies. For quantitative measurement of glycogen, 3×10^8 log-phase cells were isolated and treated according to the procedures described previously [22]. Essentially, this was done by the digestion of isolated glycogen with α-amylase and amyloglucosidase at 37 °C overnight. Absorbance readings (540 nm) of released glucose were compared to a glucose standard curve to calculate glycogen content from yeast cultures.

Table 2
Strains used in this study

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
ARD10	BY4742 *gre3Δ*::*HIS3 ypr1Δ*::*kanMX4 gcy1*::*URA3*	[13]
ARD11	BY4742 *gre3Δ*::*HIS3 ypr1Δ*::*kanMX4 gcy1*::*URA3 ydl124wΔ*::*LEU2*	[13]
ARD12	BY4742 *gre3Δ*::*HIS3 ypr1Δ*::*kanMX4 gcy1*::*URA3 ydl124wΔ*::*LEU2 yjr096wΔ*::*ble*^{r}	[13]
bcy1Δ	BY4742 *yil033cΔ*::*kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics
Ino1Δ	By4742 *yjl153c*::*kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Research Genetics

2.5. Real-time relative RT-PCR

Total RNAs were isolated from log-phase wild-type and AKR deletion mutant strains using RNeasy mini kit followed by on-column DNase digestion (QIAGEN, Valencia, CA). Genomic cDNA was synthesized from 2 μg total RNA using random primers and M-MuLV reverse transcriptase (Ambion, Austin, TX). PCR cycling reaction was performed using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the following parameters: first cycle: 3-min denaturation at 95 °C; second cycle with 45 repeats: 95 °C 15 s, 50 °C 30 s, 72 °C 30 s; third cycle: 95 °C 1 min; fourth cycle: 55 °C 1 min; fifth cycle: melting curve analysis. Negative PCR controls were mixtures not containing either M-MuLV reverse transcriptase or cDNA template. PCR fragments containing the targeted gene sequence were used as standards. The relative transcription of targeted genes between individual yeast strains were extrapolated from a standard curve after normalization with 18s RNA (Ambion, Austin, TX).

2.6. N-acetyl-L-cysteine (NAC) treatment and glutathione measurement

Saturated cultures from wild-type and AKR deletion mutant strains were diluted (100-fold) in SC medium with different concentration of NAC. The cultures were then grown to mid-log phase, harvested, and subjected to heat shock assay as described above. For total glutathione measurement, triplicate 50 ml log-phase cultures from each strain were grown in SC medium, harvested and washed in PBS two times, and resuspended in 300 μl pre-chilled 1% 5-sulfosalicylic acid and 8 mM HCl. The cells were broken by vigorous vortexing with glass beads and then incubated on ice for 30 min. Cellular debris and proteins were pelleted by centrifugation at 14,000 rpm for 10 min. The supernatant was collected, serially diluted as needed, and used to determine the total glutathione contents (GSH+GSSG) by a colorimetric reaction measured at 412 nm using the Ellman's reagent (Dojindo, Gaithersburg, MD). Known concentrations of GSH were used in the assays simultaneously with the yeast samples for calibration and generation of a linear regression curve, from which total GSH amount in the yeast samples was extrapolated. Data were presented as relative to total GSH content in the wild-type strain. Statistical analysis for significant difference was performed by unpaired *t*-test assuming samples with equal variances.

2.7. Immunofluorescence

Wild-type and ARD10 strains (*gre31Δ/ypr1Δ/gcy1Δ*) were transformed with the *GPD415-GFP-YAP1* expression vector. Transformants were selected based on expression of the *LEU2* gene. To detect cellular localization of GFP-Yap1 in living yeast, transformants were grown overnight in synthetic medium (*-Leu*). The next day, the cells from 2 ml cultures were harvested and resuspended in fresh medium with the same volume to resume growth for addition 3 h, or at that time were treated with 4.4 mM hydrogen peroxide for 30 min. The cell-permeant nucleic acid staining fluorescent dye SYTO61 (Invitrogen, Carlsbad, CA) was then added to the cultures at a final concentration of 5 μM for 20 min with mild agitation. Culture aliquots were then examined by confocal microscopy (Zeiss LSM510). Cells were first visualized under transmitted light mode and then visualized under 2-channel fluorescent mode using argon laser to excite GFP and HeNe2 laser to excite SYTO61. Emission of fluorescent signal was examined using BP 505–550 filter for GFP and LP650 filter for SYTO61.

3. Results

3.1. Oxidative stress in the pentuple AKR null strain

In previous studies, we demonstrated that rescue of the heat shock phenotype in triple AKR null strain, ARD10, can be achieved by transfection with expression vectors encoding a functional AKR such as human aldose reductase or yeast Gcy1 [13]. This rescue effect was lost with catalytically inactive AKR mutants, suggesting that the heat shock phenotype may result from accumulation of unmetabolized AKR substrates such as aldehydes and/or ketones. As a group, carbonyl-containing compounds are highly electrophilic and can reduce the levels of intracellular antioxidants such as glutathione in yeast cells [23,24]. To test for this possibility, we compared the total glutathione (GSH and GSSG) levels from cultures in log-phase growth of the pentuple AKR null strain, ARD12, and the wild-type strain. As shown in Fig. 1A, total glutathione level in the mutant yeast was reduced by approximately 30% from the level of the wild-type strain ($P=0.001$).

With decreased glutathione levels in the mutant, it is possible that the heat shock phenotype results from direct oxidative stress and/or disruption of cellular redox balance. We then tested whether treatment with an antioxidant would rescue the heat shock phenotype. Strains treated for 16 h in YPD medium containing *N*-acetyl-L-cysteine (NAC), a well-characterized antioxidant and a precursor for glutathione synthesis [25], were assayed for heat shock sensitivity. When grown in the presence of 25 mM NAC, heat shock sensitivity of the mutant strain was restored to that of the wild type (Fig. 1B). As expected, glutathione levels were also returned to near wild-type levels in mutant strain following culture in the presence of NAC ($P=0.0003$; Fig. 1A).

3.2. Nuclear localization of Yap1 in triple AKR null strain during normal growth

The leucine zipper oxidation-sensitive transcription factor Yap1 is important in regulation of general oxidative stress and

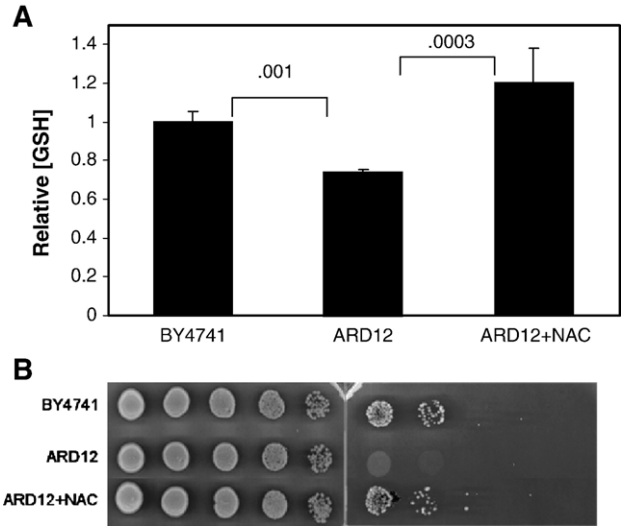

Fig. 1. Oxidative stress in the AKR deletion strain. (A) Intracellular glutathione levels were determined in log-phase cultures grown in synthetic medium from wild-type (BY4741), pentuple AKR null mutant (ARD12), and null mutant pretreated with *N*-acetyl-cysteine (ARD12+NAC). Differences in glutathione levels among strains were examined by a two-tailed *t*-test. Data are mean± standard error. *P*-values are shown between relevant comparison groups. (B) Pretreatment of ARD12 mutant with NAC rescued its heat shock phenotype. Strains shown in panel A were grown to log phase in YPD and were carried through a heat shock treatment for 10 min at 55 °C as described in Materials and methods. Serially diluted cultures were grown on YPD plates 30 °C for 3 days.

redox control genes [26,27]. Under oxidative stress, Yap1 drives targeted gene expression by translocating into the nucleus from its resting state in the cytosol [28]. In light of the observed rescue of the heat shock phenotype by NAC to the AKR null strain, we reasoned that the cellular localization of Yap1 might provide additional information about the state of oxidative stress in the AKR null strain. To this goal, wild-type (BY 4741) and triple AKR null (ARD10) yeast cells were transformed with an expression plasmid, which expresses the GFP-Yap1 as a fusion protein. No change in growth properties was observed following constitutive expression of the fusion protein, suggesting that its overexpression was not associated with any noticeable cellular toxicity. Cellular localization of the fusion protein was examined by confocal microscopy taken from cells during log-phase growth without nutritional starvation. As shown in Fig. 2A, fluorescence of GFP-Yap1 was distributed evenly throughout the cytoplasm in wild-type cells. In contrast, GFP fluorescence in the mutant strain was typically observed as a focal, condensed signal typical of nuclear localization (Fig. 2D). Nuclear localization of Yap1 in the mutant was tested by live staining the cells with cell permeant nucleic acid dye SYTO61 (Fig. 2B, E). Intense co-localization of the nuclear dye and GFP in the ARD10 triple AKR mutant (Fig. 2F) is in contrast to the even distribution of the GFP signal in both cytoplasm and nucleus of wild-type host cells (Fig. 2C). In a parallel experiment, treatment of wild-type and mutant log-phase cultures with 0.4 mM hydrogen peroxide for 30 min resulted in substantial nuclear localization of Yap1 (Fig. 2, panels G–I and J–L). Therefore, the high frequency of nuclear localization of Yap1 in mutant cells grown under basal culture conditions suggests that the mutant is indeed in a state of constitutive oxidative stress.

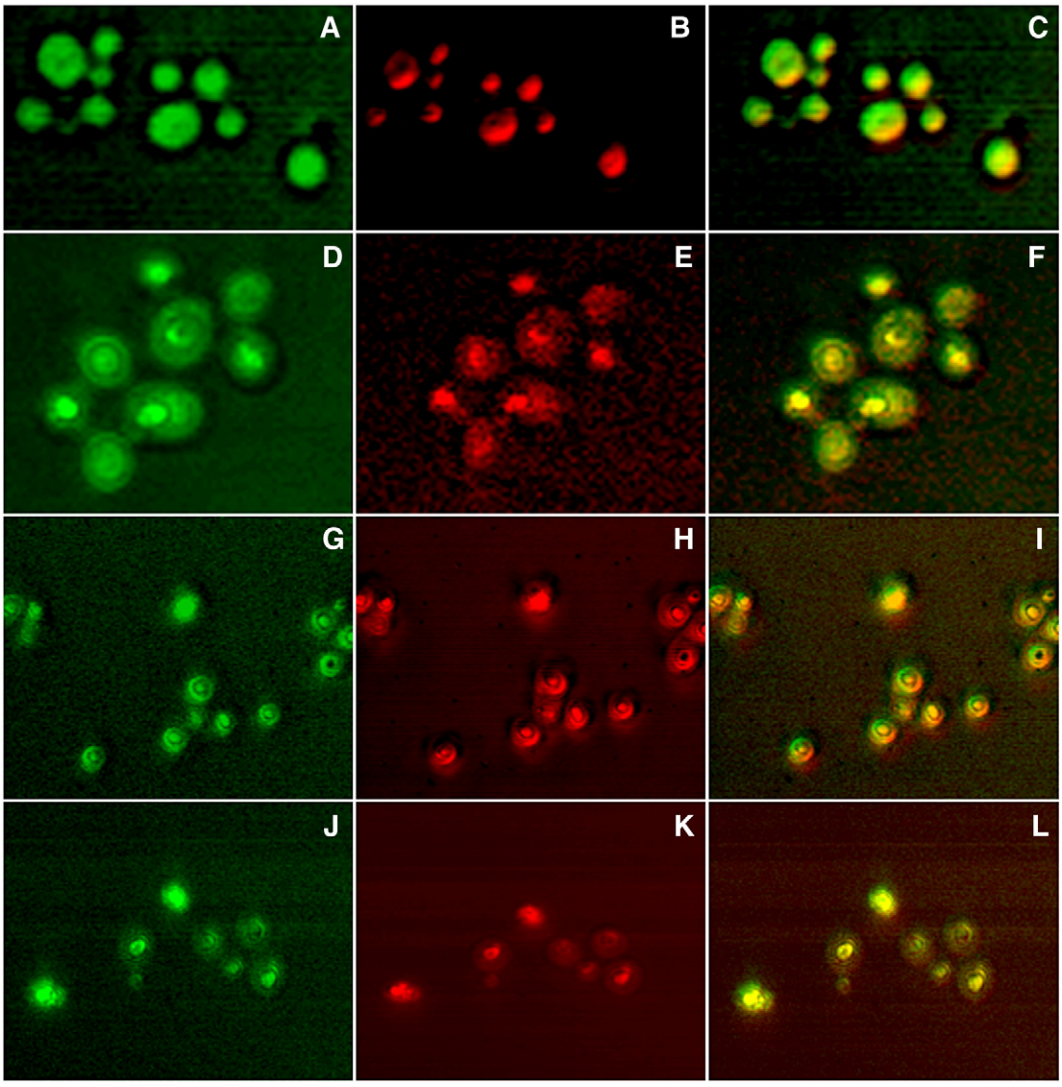

Fig. 2. Constitutive nuclear localization of Yap1 in AKR null mutant ARD10. Distribution of GFP-Yap1 in the BY4741 wild-type strain (panels A–C) and in the triple AKR null strain ARD10 (panels D–F) during log-phase growth and following treatment of wild-type (panels G–I) or ARD10 (panels J–L) with hydrogen peroxide as described in Materials and methods. Images showing localization of GFP-Yap1 (A, D, G, H) and nuclei by SYTO61 staining (B, E, H, K) were overlaid for comparison (C, F, I, L).

3.3. Oxidative stress regulatory genes are highly induced in the AKR null strains

In our previous studies using DNA microarray, we found that the triple AKR null strain was characterized by constitutive upregulation of a large number of heat shock-inducible genes [14,29]. The more severe heat shock phenotype observed in the pentuple AKR null mutant, together with the rescue effect by the antioxidant NAC and constitutive nuclear localization of Yap1, prompted us to compare the expression of general anti-oxidative stress and redox control genes such as *CTT1* (catalase T), *SOD1* (Cu,Zn superoxide dismutase), *GSH1* (gamma glutamylcysteine synthetase), *GSH2* (glutathione synthase), *TRX2* (thioredoxin), *TRR1* (thioredoxin reductase) [26,30–35] and general heat shock genes including *HSP26*, *HSP12*, *HSP104* [36,37] in our wild-type and AKR mutant strains. Transcript levels were measured in cells cultured under normal growth conditions (complete synthetic medium, 30 °C) and following a mild heat shock (37 °C). As shown in Fig. 3A, under normal growth conditions, virtually all stress associated genes examined in this data set showed some level of induction in the mutant strains compared to wild type. Overall, the pentuple AKR-deficient strain showed the most dramatic changes, particularly for the two redox control genes *GSH1* (γ-glutamylcysteine synthase, >6-fold) and *TRX2* (thioredoxin 2, almost 12-fold) and *HSP12* (heat shock protein 12, 6-fold). Most of these genes examined in this study were induced following heat shock treatment, but differences between wild-type and mutant strains following heat shock were relatively small (Fig. 3B). These results are consistent with our gene chip studies which indicated that the AKR mutant strains are characterized by constitutive upregulation of the stress response genes under normal growth conditions [14].

3.4. The triple and pentuple AKR null mutants show inositol auxotrophy for growth

In the course of screening our AKR mutant strains for metabolic or signaling defects, we compared the growth of AKR mutant strains in media with or without inositol supplementation. Inositol regulates the synthesis of phosphatidylinositol phospholipids and enzymes involved in the biosynthesis of phospholipids [17,38]. Dependence of growth on inositol supplementation can be used as a sensitive probe for defects in gene transcription [20]. A slow growth phenotype was observed when the triple AKR null strain ARD10 was cultured in inositol-free growth medium (Fig. 4). Similar to the heat shock phenotype, this moderate Ino$^-$ phenotype was enhanced with disruption of additional AKR ORFs, as shown for the quadruple AKR null strains ARD11 and the pentuple AKR null strain ARD12. Similar to its effect in an *INO1* null strain (*Ino1Δ*), ectopic expression of *INO1* rescued the Ino$^-$ phenotype in strain ARD10 (Fig. 4B).

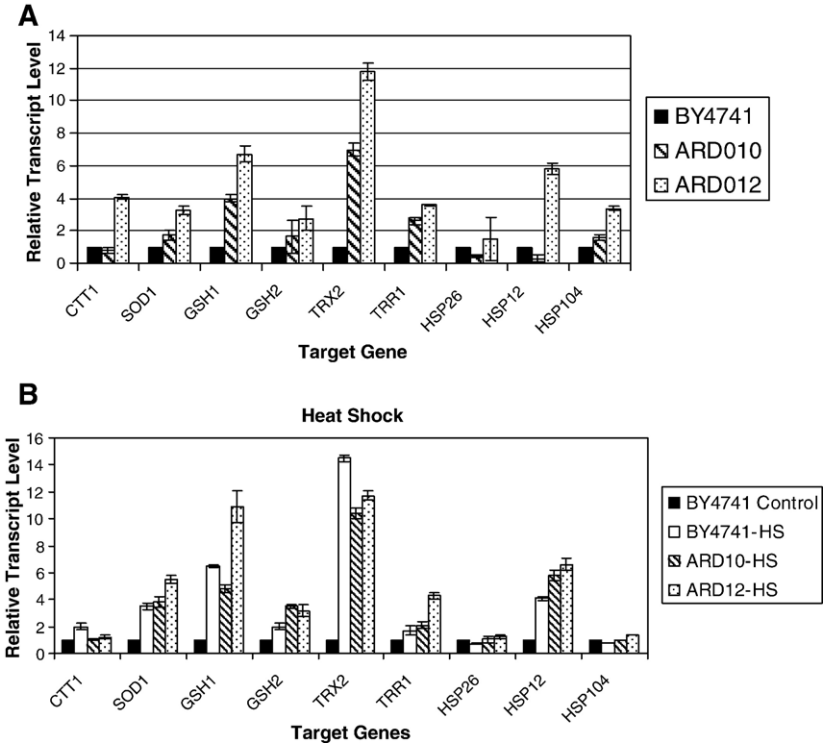

Fig. 3. Constitutive induction of stress related genes in AKR null mutants. Split log-phase cultures of wild-type (BY4741) and AKR mutant strains ARD10 and ARD12 were subjected to heat shock stress at 37 °C for 2 h or continued for growth at 30 °C for additional 2 h. Total RNAs were isolated from cells grown at 30 °C (panel A) or following heat shock (panel B). Targeted gene transcripts included CTT1 (catalase T), SOD (superoxide dismutase), GSH1 (γ-glutamylcysteine synthase), GSH (glutathione synthase), TRX2 (thioredoxin), TRR1 (thioredoxin reductase), HSP26, HSP12, HSP104 (heat shock proteins at subunit $M_r \sim 26{,}000$; 12,000; 104,000). Transcript levels were normalized to 18S RNA. Data are mean ± standard error and are expressed as values relative to the BY4741 wild-type strain grown at 30 °C.

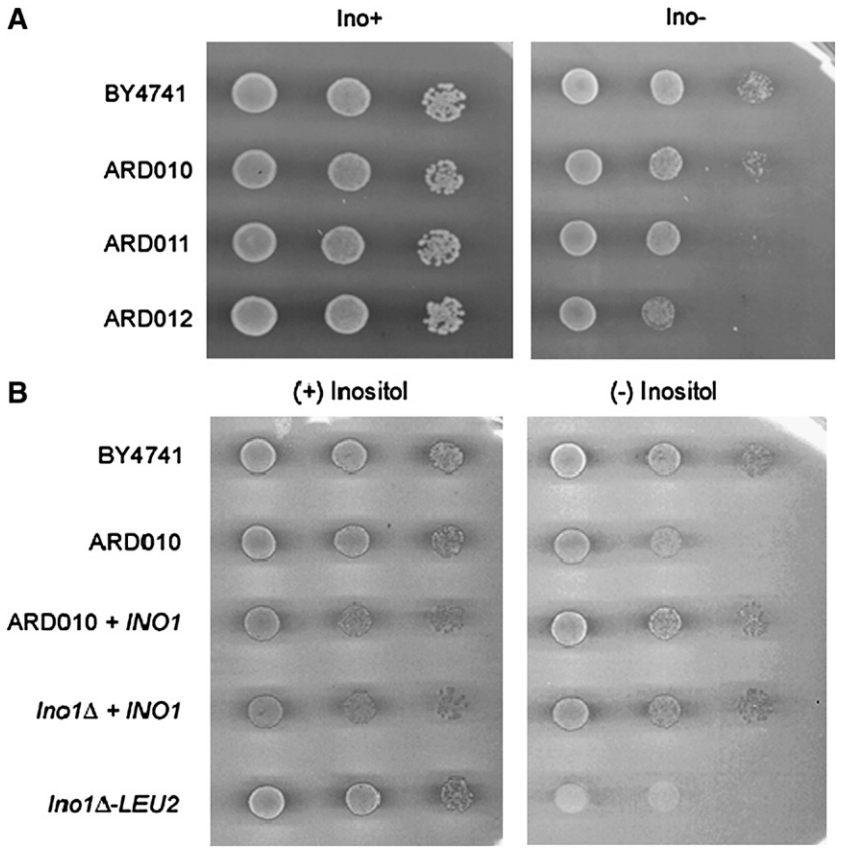

Fig. 4. The inositol auxotrophy phenotype in the AKR null mutants. (A) Cultures of wild-type strain BY4741 and AKR deletion mutants ARD10, ARD11 and ARD12 were grown overnight in SC medium with 55 μM *myo*-inositol. Cells were then harvested, washed in water, and serially diluted for spotting onto SC agar plates with (Ino^+) or without (Ino^-) 200 μM *myo*-inositol. Plates were incubated at 30 °C for 3 days. (B) Ectopic expression of a functional *INO1* in ARD10 rescues its inositol phenotype. ARD10 and *Ino1Δ* were transformed with *INO1-LEU2-GPD415*, or *Ino1Δ* with *LEU2-GPD415* as a negative control. Transformants were grown to mid-log phase in SC medium with 55 μM *myo*-inositol. Cultures were then harvested, serially diluted and grown on ±200 μM *myo*-inositol agar plates (-Leu) at 30 °C for 3 days.

An Ino^- phenotype can be associated with a defect in transcriptional regulation of the *INO1* gene [39]. Therefore, we examined *INO1* gene transcripts in mutant and wild-type strains. Similar levels of repression of *INO1* transcripts were found when wild-type and the triple AKR null strain (ARD10) were cultured in the presence of inositol (Fig. 5). However, induction of *INO1* transcript levels was approximately 3-fold lower in ARD10 than that of the wild-type cells when grown in the absence of *myo*-inositol. Levels of both *CDS1* and *OPI3*, genes which encode enzymes involved in phospholipid synthesis that are also regulated like *INO1* [38,40], were approximately 3-fold lower in ARD10 under inositol-free growth conditions as compared to wild type. Taken together, these data suggest that the Ino^- phenotype in the triple AKR deletion

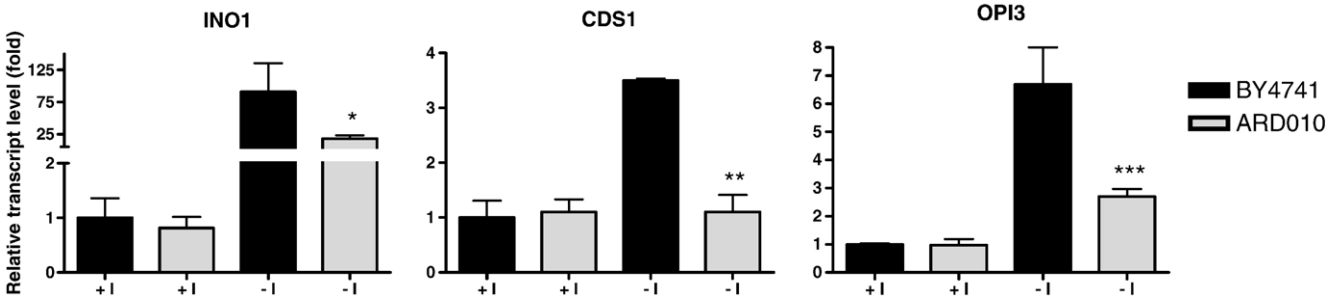

Fig. 5. Defects in transcriptional regulation of *INO1* and phospholipid synthetic genes in AKR null mutant ARD10. Overnight cultures of wild-type strain BY4741 and ARD10 mutant grown in SC medium with 55 μM *myo*-inositol were harvested and then resuspended in fresh SC medium at $OD_{600}=0.1$ with (+I) or without (−I) 200 μM *myo*-inositol to resume growth at 30 °C for 5–6 generations. Total RNA was then isolated from harvested cells to measure relative transcriptions of the following genes by real-time RT-PCR: *INO1* (inositol-1-phosphate synthase), *CDS1* (CDP-diacylglycerol synthase), and *OPI3* (phospholipid-*N*-methyltransferase). Data are mean±standard error from triplicate samples of gene and each strain. Differences in measured transcript levels between strains cultured without inositol supplementation were analyzed by *t*-test to yield the following *P*-values: *=0.038, **=0.018, ***=0.002.

mutant may result from a defect in transcriptional regulation of genes co-regulated with *INO1*.

3.5. Ras-PKA signaling in AKR null strains

We identified *PDE2* (phosphodiesterase) in preliminary studies to isolate possible suppressors of the heat shock phenotype in AKR-deficient strains (Chang and Petrash, unpublished results). This suggested to us that the heat shock phenotype could be associated with a defect that results in inappropriate activation of *Ras-PKA* signaling [41]. To test whether the heat shock phenotype in the AKR null strains is associated with PKA activation, we screened for informative phenotypes (e.g., reduced glycogen storage and growth sensitivity to nitrogen starvation) in the triple (ARD10) and pentuple (ARD12) AKR null mutants. First, as a qualitative assay, we compared the glycogen content of the wild-type and mutant yeasts by exposing colonies on YPD plates to iodine vapor. The *bcy1Δ* strain, which is known to have a higher PKA activity due to a mutation of the regulatory subunit of PKA, was used as a positive control. As shown in Fig. 6A, no noticeable difference in the intensity of iodine staining was observed between the wild-type and two AKR null strains, whereas glycogen staining for the *bcy1Δ* control strain showed marked glycogen depletion. This general pattern was confirmed by quantitative assay of glycogen content. Glycogen levels of ARD10 and ARD12 mutants were modestly lower than wild type, but the differences did not reach significance. In contrast, glycogen levels in the *bcy1Δ* control strain were significantly lower than wild type (Fig. 6B). These results indicate that the heat shock phenotype of the AKR null mutants is not associated with activation of PKA signaling.

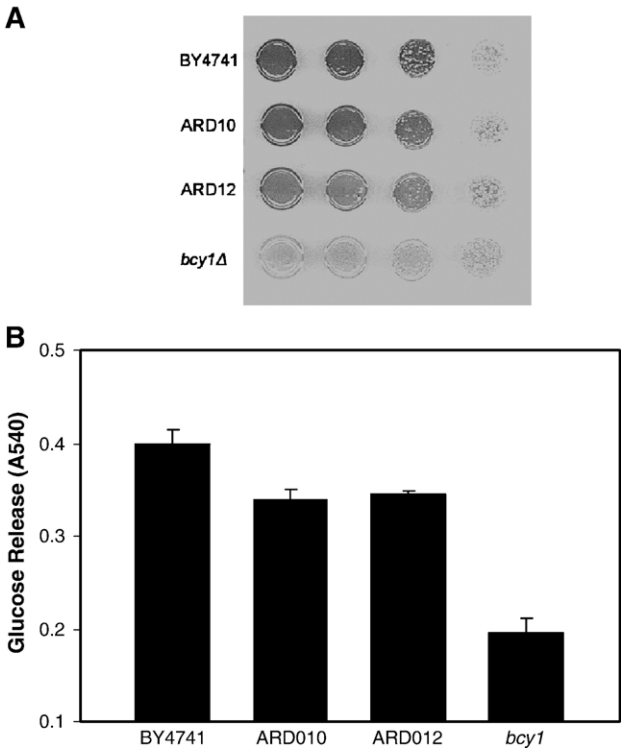

Fig. 6. AKR null mutants are not deficient in glycogen storage. (A) Glycogen staining of wild-type (BY4741) and AKR null mutants: colonies grown on YPD agar plates were inverted for exposure to iodine vapor for 5 min, after which images were collected. (B) Glycogen contents in wild-type and AKR null mutant strains. Glycogen was isolated from log-phase cultures grown in SC with glucose. Spectrometric absorbance of released glucose from breakdown of glycogen was measured as described in Materials and methods. Data are mean± 1 S.D.

4. Discussion

The biological roles of yeast AKRs are largely unknown. Previous studies of global stress response showed that a subset of the AKR genes, including *GRE3*, *GCY1* and *YPR1*, are induced under osmotic or oxidative stress [11,12,42]. Indeed, *GRE3* is recognized as one of three genes *de respuesta a estres* [11]. In previous studies, we showed that deletion of all of these putative stress-inducible genes (*gre3Δ/gcy1Δ/ypr1Δ*) does not result in a measurable oxidative or osmotic stress phenotype [13]. This suggests that the AKRs examined in our studies are redundant to other genes that function to protect against osmotic and oxidative stress. Like yeast AKRs, human aldose reductase (ARK1B1) shows strong osmotic induction as measured by gene expression at the RNA and protein levels in a variety of cell types [43]. However, aldose reductase-mediated synthesis of sorbitol from glucose is considered nonessential for osmotic balance due to contributions by other active osmolytes [44]. Therefore, the parallels between yeast and human AKRs may be extended to include a sensitivity to osmotic induction at the transcriptional level but without a clear identification of the functional utility.

This suggests that the emergence of the phenotype results from an inability of the mutant to metabolize the substrates. Strong phenotypic rescue to the AKR null strain, ARD12, is observed by its pretreatment with the antioxidant agent NAC, suggesting that the mutant phenotype is strongly linked to oxidative stress. Abnormal translocation of Yap1 to the nucleus of the triple AKR mutant ARD10 (*gre3Δ/gcy1Δ/ypr1Δ*) indicates that this strain is under oxidative stress even under normal log-phase growth conditions. Induction of oxidative stress and redox control genes would be expected to result from nuclear localization of Yap1. We favor the view that the protective role of NAC to increase cell survival rate following a severe heat shock acts in concert with the effect of Yap1 at transcriptional control. The nucleophilic thiol group in NAC may scavenge the free reactive aldehydes generated during cellular metabolism, or prevent their reaction with the endogenous nucleophilic amino acids in newly synthesized thiol-containing proteins and peptides.

Prior studies using oligodeoxyribonucleotide microarrays demonstrated that a large number of heat shock-responsive genes were constitutively upregulated in the triple AKR null strain. This work was extended in the current study to examine a subset of stress response genes in the pentuple AKR null strain (ARD12). We found markedly elevated transcript levels for all genes examined in the null strain under basal growth conditions as compared to wild type. Consistent with prior microarray studies with ARD10, we found that these genes were further induced only modestly or not at all when ARD12 was subjected

to a mild heat shock. These results indicate that even under basal conditions, the transcription profile of ARD12 is similar to that of a cell in the midst of a stress response.

Yap1, a bZIP DNA-binding protein, is a member of a large family of AP-1 transcriptional activators in *S. cerevisiae* [45]. Several studies have shown that Yap1 is involved with up-regulation of oxidative stress response genes following exposure to reactive oxygen species [26,46,47]. Promoter elements associated with most genes strongly induced in response to ROS have Yap1 binding sites, including cytosolic catalase (CTT1), cytosolic superoxide dismutase (SOD1), gamma glutamylcysteine synthetase (GSH1), thioredoxin 2 (TRX2), and thioredoxin reductase (TRR1) [48,49]. Redistribution of Yap1 from the cytoplasm to the nucleus is thought to be triggered by formation of an intramolecular disulfide in one or more cysteine-rich domains [34]. As such, the subcellular distribution of Yap1 is considered a good indicator for the redox state of the cell. Constitutive nuclear localization of the Yap1 in AKR null but not wild-type cells cultured under normal conditions further suggests that the AKR null strain is characterized by a chronic state of cytoplasmic oxidative stress.

In addition to the stress phenotype of heat shock sensitivity, deletion of all five yeast AKR genes also results in a slow growth phenotype when cultured in inositol-free media. This phenotype appears to be associated with the inability of this mutant to launch a full transcriptional induction of structural *INO1* gene under induced condition (-inositol). Given the important role of this gene product for the production of inositol-containing compounds, including inositol phospholipids [50], it would be interesting to understand whether there are changes in the abundance of phosphatidylinositol in the AKR deletion mutants. Alternatively, the Ino$^-$ phenotype with the associated defect in transcriptional regulation of *INO1* can also be indicative of one or more defect(s) linked to general transcription [51]. The mechanism behind this notion is consistent with *in vitro* studies which showed that malondialdehyde, a lipid-peroxidation-derived aldehyde, can form adducts with guanine in DNA [15]. Adduct formation can cause genomic lesions and block the transcription by RNA polymerase II [16] although the impact of DNA adducts involving malondialdehyde or base propenals [52] may be minimized by a transcription-coupled repair process [53]. In support of the idea of transcriptional defects in the AKR null mutant, we found that a hyperactive mutant allele of *Ras* (*Ras2*^{val19}) brings about a dramatic growth defect when introduced to the triple AKR mutant ARD10 under inositol-free growth conditions (Qing and Petrash, unpublished observations). Since genetic studies have shown that the *Ras* signaling in yeast is involved in regulation of RNA polymerase II-controlled transcription [54], it seems reasonable to speculate that the underlying mutation(s) causing a substantial Ino$^-$ phenotype in the pentuple AKR null mutant converges at a signaling step mediated by a hyperactive *Ras*. Since the null mutant does not harbor an activated PKA mutation (Fig. 6), the genetic interaction of its inositol phenotype with a hyperactive *Ras* appears to be independent of PKA.

Transcription of the *INO1* gene requires binding of Ino2p and Ino4p to an activating sequence that is located in the promoter region of *INO1* and other co-regulated genes involved in phospholipid biosynthesis [55]. *INO1* transcription is repressed by the negative regulatory protein Opi1p [56]. Inositol auxotrophy can result from abnormalities in *INO1* gene regulation and is often associated with defects in RNA transcription components including RNA polymerase subunits [51] and the TATA binding protein [57]. As regulation of *INO1* expression and defects leading to inositol auxotrophy are complex, further studies will be required to delineate possible mechanisms linking AKR gene mutations and the Ino$^-$ phenotype.

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