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Genome-wide screen for inositol auxotrophy in *Saccharomyces cerevisiae* implicates lipid metabolism in stress response signaling

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Abstract

Inositol auxotrophy (Ino$^-$ phenotype) in budding yeast has classically been associated with misregulation of *INO1* and other genes involved in lipid metabolism. To identify all non-essential yeast genes that are necessary for growth in the absence of inositol, we carried out a genome-wide phenotypic screening for deletion mutants exhibiting Ino$^-$ phenotypes under one or more growth conditions. We report the identification of 419 genes, including 385 genes not previously reported, which exhibit this phenotype when deleted. The identified genes are involved in a wide range of cellular processes, but are particularly enriched in those affecting transcription, protein modification, membrane trafficking, diverse stress responses, and lipid metabolism. Among the Ino$^-$ mutants involved in stress response, many exhibited phenotypes that are strengthened at elevated temperature and/or when choline is present in the medium. The role of inositol in regulation of lipid metabolism and stress response signaling is discussed.

Keywords

Yeast; Inositol auxotrophy; Inositol; Lipid metabolism; Stress response

Introduction

Inositol serves as a precursor of inositol-containing lipids and inositol phosphates, which play essential roles in signaling, membrane trafficking, and membrane identity in eukaryotic cells (De Camilli et al. 1996; Lemmon 2003; Jesch and Henry 2005; Majerus and York 2009). Most eukaryotic organisms, including the budding yeast, *Saccharomyces cerevisiae*, are able to synthesize inositol de novo (Majumder et al. 1997; Michell 2007; Majerus and York 2009), starting with the conversion of glucose-6-phosphate to inositol-3-phosphate (Chen and Charalampous 1964a, b). The first report of isolation of inositol auxotrophs (Ino$^-$ mutants) in yeast identified ten independently segregating loci among 52 independently generated mutants (Culbertson and Henry 1975; Culbertson et al. 1976). The majority of these mutants proved to be alleles of *INO1*, later shown to be the structural gene encoding

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inositol-3-phosphate synthase (Donahue and Henry 1981; Dean-Johnson and Henry 1989). Two other loci, *INO2* and *INO4*, each represented about 9% of the original Ino$^-$ mutants. The *INO2* and *INO4* loci were later shown to encode basic helix loop helix transcription factors that form a heterodimer that binds to the repeated upstream activation sequence, UAS_{INO}, found in the promoter of *INO1* and coregulated genes (Carman and Henry 1989; Kodaki et al. 1991; Lopes and Henry 1991; Lopes et al. 1991; Bailis et al. 1992; Nikoloff et al. 1992; Ambroziak and Henry 1994; Nikoloff and Henry 1994; Bachhawat et al. 1995; Schüller et al. 1995; Schwank et al. 1995). Subsequently, mutants with more general defects in RNA polymerase II (RNA-Pol II) transcription were found to have Ino$^-$ phenotypes (Henry and Patton-Vogt 1998) due to low levels of *INO1* expression (Nonet and Young 1989; Scafe et al. 1990a, b).

INO1 is the most highly regulated of a group of genes containing the inositol-sensitive upstream activating sequence, UAS_{INO}, in their promoters (Greenberg and Lopes 1996; Carman and Henry 1999). *INO1* and other UAS_{INO}-containing genes are maximally repressed when both inositol and choline are present in the growth medium, but inositol alone is sufficient to achieve 30-fold or more repression of *INO1* (Hirsch and Henry 1986). The addition of choline when inositol is present results in an additional several-fold repression of *INO1* expression (Hirsch and Henry 1986; Lopes et al. 1991; Jesch and Henry 2005; Jesch et al. 2006). Phosphatidic acid (PA), a precursor of all glycerophospholipids in yeast, serves as the metabolic signal for derepression of *INO1* and other UAS_{INO}-containing genes (Henry and Patton-Vogt 1998; Loewen et al. 2004). Opi1p, a known repressor of *INO1* (White et al. 1991), is localized to the endoplasmic reticulum (ER) by direct binding to PA (Loewen et al. 2004) and to the VAP homolog Scs2p (Loewen et al. 2003). Upon addition of inositol to the growth medium of wild-type cells, phosphatidylinositol (PI) synthesis increases dramatically, resulting in consumption of PA. Loss of the ER pool of PA causes Opi1p translocation to the nucleus where it represses the transcription of *INO1* and coregulated genes (Loewen et al. 2004). The *INO1* gene is also repressed in stationary phase and under conditions of nitrogen or zinc depletion, even when inositol is absent (Carman and Zeimetz 1996; Griac and Henry 1999; Carman and Henry 2007).

Mutants defective in several stress signaling pathways, including the glucose response pathway (Hirschhorn et al. 1992; Ouyang et al. 1999; Shirra and Arndt 1999), the unfolded protein response (UPR) pathway (Nikawa and Yamashita 1992; Cox et al. 1993), and the protein kinase C–cell wall integrity (PKC–CWI) pathway (Nunez et al. 2008) exhibit Ino$^-$ phenotypes. Because of their Ino$^-$ phenotypes and/or reduced *INO1* expression, it was assumed that the signaling pathways defective in these mutants were involved in regulating *INO1* transcription. However, the role of each of these pathways in *INO1* expression is complex. For example, while PKC–CWI mutants *pkc1Δ*, *bck1Δ*, and *slt2Δ/mpk1Δ* all exhibit Ino$^-$ phenotypes, the *slt2Δ/mpk1Δ* mutant is not defective in expression or regulation of *INO1* or other UAS_{INO} containing genes, but instead exhibits alterations in PC and neutral lipid homeostasis (Nunez et al. 2008). Thus, while the PKC–CWI pathway is not involved in expression or regulation of *INO1*, it is essential for survival and lipid homeostasis in cells growing in the absence of inositol.

Interestingly, the Ino$^-$ phenotype of PCK-CWI mutants are strengthened at 37°C in the presence of choline (Nunez et al. 2008), growth conditions known to influence PC synthesis and turnover (Dowd et al. 2001). Growing cells in the presence of choline or shifting cells from 30 to 37°C causes increased flux through the CDP-choline pathway (Dowd et al. 2001), one of the two PC biosynthetic pathways present in yeast. Moreover, growth under these conditions results in increased deacylation of CDP-choline-derived PC, which may provide wild-type yeast with the flexibility to adjust their PC acyl chain composition in response to changing environmental conditions (Boumann et al. 2003). However, the

membrane stress imposed by inositol limitation may be exacerbated by PC synthesis through the CDP-choline pathway (Fernandez-Murray et al. 2009).

Seeking to develop a comprehensive understanding of the diverse cellular processes that influence *INO1* expression or are required for growth when inositol is limiting, we conducted a genome-wide phenotypic screening for mutants exhibiting Ino^- phenotypes. Based upon our previous observation that the Ino^- phenotype of PKC–CWI mutants is significantly intensified at higher temperatures in the presence of choline (Nunez et al. 2008), we carried out our current screen under these growth conditions. We report the identification of 385 mutations not previously reported to confer an Ino^- phenotype. The addition of choline and/or growth at 37°C increased the strength of the Ino^- phenotypes of a subset of these mutants. Mutations affecting key members of a number of stress response pathways, including the high-osmolarity glycerol (HOG) pathway, are among the newly identified Ino^- mutants. Ino^- phenotypes were also detected in mutants defective in transcription, protein modification, membrane trafficking, lipid metabolism, and diverse stress responses.

Materials and methods

Strains and plasmids

The homozygous diploid deletion collection [BY4743 strain background; *MAT***a**/α *his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0* (4741/4742)] was purchased originally from Research Genetics (now Invitrogen Corporation). This deletion set consists of a collection of 4,741 strains in which a single non-essential open reading frame (ORF) has been disrupted in each strain. Complete details regarding the collection construction can be found at sequence. http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html.

Other strains were constructed in the BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) strains, which are parent strains of BY4743. BY4741 and BY4742 were originally derived from the S288C strain background (Brachmann et al. 1998). We noted that the *ino1Δ* strain provided in the original Research Genetics collection has growth deficiencies that are not ascribable to deletion of the *INO1* (S. Jesch, unpublished). Therefore, a single gene disruption in the *INO1* was separately created in the BY4742 background strain by replacing targeted ORF with the *HIS3* marker by PCR-mediated gene replacement using the pFA6a-His3MX6 template (kind gift from M. Longtine) as described previously (Longtine et al. 1998).

Media and growth conditions

YPD liquid medium consisted of 1% yeast extract, 2% bactopeptone, and 2% glucose. Chemically defined synthetic complete media lacking inositol and choline (I^-C^- media) used in this study was described by (Jesch et al. 2005), except that threonine was omitted. I^-C^- media contains (per liter): 20 g of glucose, 5 g of ammonium sulfate, 1 g of potassium phosphate, 0.5 of g magnesium sulfate, 0.1 g of sodium chloride, 0.1 g of calcium chloride, 0.5 mg of boric acid, 0.04 mg of cupric sulfate, 0.1 mg of potassium iodide, 0.2 mg of ferric chloride, 0.4 mg of manganese sulfate, 0.2 mg of sodium molybdate, 0.4 mg of zinc sulfate, 2 μg of biotin, 400 μg of calcium pantothenate, 2 μg of folic acid, 400 μg of niacin, 200 μg of *p*-aminobenzoic acid, 400 μg of pyridoxine hydrochloride, 200 μg of riboflavin, 400 μg of thiamine hydrochloride, 20 mg of adenine sulfate, 20 mg of arginine, 20 mg of histidine, 60 mg of leucine, 230 mg of lysine, 20 mg of methionine, 20 mg of tryptophan, and 40 mg of uracil. Where indicated, I^- media was supplemented with 75 μM *myo*-inositol (I^+) and/or

1 mM choline (C^+). For example, I^+C^+ medium contains 75 μM inositol and 1 mM choline, whereas I^-C^- medium lacks both inositol and choline. Solid media contained 2% agar.

In preliminary experiments leading to the final screening, we observed that the presence of threonine in the I^+C^- medium as described by Jesch et al. (2005) increased the lag phase of growth of a large number of mutant strains derived from S288C, including those in the BY4743 background, by about 10–12 h at 30°C. When threonine was omitted, this lengthening of the lag phase was not observed. Therefore, in order to compare growth of strains over comparable time intervals with and without inositol, threonine was excluded from all synthetic media used in this study. Similar inhibition of growth in the presence of threonine in synthetic complete media was previously reported by Shirra et al. (2001).

The standard protocol used in the mutant screening was as follows: Each frozen master-plate was thawed completely, and cells were resuspended to homogeneity. A 96-pin microplate replicator (V & P Scientific, Inc) was used to transfer a 2-μl aliquot from each well in a master plate to a corresponding well containing 800 μl of YPD, plus G418 (200 μg/ml) in a deep-well microtiter plate. The deep-well plate was incubated for 2 days at 30°C. A 2-μl aliquots from each well from the YPD + G418 cell culture were then inoculated into a second deep-well plate containing 800 μl of YPD per well and incubated at 30°C for 15 h. A 1:50 dilution of the 15-h culture was carried out by transferring 2-μl aliquots from each well into 100 μl of I^-C^- media to dilute any carryover of inositol or choline from the YPD culture. Finally, 2 μl from each 100 μl dilution was transferred to Nunc Omni plates containing the following solid media: YPD, I^+C^+, I^+C^-, I^-C^-, I^- C^+, and 2% agar. Plating was carried out in duplicate to control for variability in inoculation volumes, and plates were incubated for 4 days at 30°C or 37°C. Each plate was photographed on days 2 and 4 using a digital camera to create a permanent record. In all cases, only wells that gave the same result on duplicate plates were scored in the final tally of Ino^- phenotypes shown on Tables 1 and 2. Questionable cases and mutants of interest were reassessed in subsequent spotting assays. Growth of each strain on I^-C^- or I^-C^+ medium was scored visually relative to growth of the same strain on I^+C^- or I^+C^+ medium at the equivalent growth temperature, conducted independently by two different investigators in two separate blind screenings. On Tables 3 and S1, a score of “S” (strong) indicates no visible growth on media lacking inositol. A score “W” (weak) indicates some residual growth on I^- medium, but substantially less than on I^+ medium, while “VW” (very weak) indicates some growth on I^- medium, but still visibly less than on I^+ medium. Some strains had strong Ino^- phenotypes at 30°C, but failed to grow at all on I^+C^+ media, I^+C^- media, and/or YPD at 37°C. Such strains are denoted as “NG” (no growth) at 37°C. This scoring system is described in detail in the legend of Table 3 and Table S1, where the assigned scores of all Ino^- mutants identified in this study are listed. Several examples of Ino^- phenotypes, as detected in the original screening, are shown in Fig. 1, as are several examples of variable growth on I^-C^+ medium that were not validated in the duplicates plates and/or in the subsequent spotting assays.

Spotting assay

Inositol auxotrophy was confirmed for a subset of 73 strains detected in the original screen as having Ino^- phenotypes under one or more growth conditions using a standard spotting assay. Overnight cultures were grown in I^+C^- medium at 30°C. The cultures were diluted back to OD_{600} = 0.15 in 10 ml of the same medium and allowed to grow to mid-logarithmic phase at 30°C. Cells were harvested at OD_{600} = 0.5 and washed in I^-C^- medium at 30°C. A series of tenfold dilutions were made in a microtiter plate and a 5-μl aliquot of each dilution was spotted onto I^+C^-, I^+C^+, I^-C^-, and I^-C^+ media with 2% agar. The plates were incubated at 30°C and at 37°C. Strains presenting a growth defect in I^-C^- or I^-C^+ medium in comparison with their growth in I^+C^- or I^+C^+ medium were considered inositol auxotrophs.

Mutant identification and functional classification

Identification of the gene deleted in each strain that exhibited an Ino$^-$ phenotype was accomplished by comparison with a reference spreadsheet of the homozygous diploid deletion collection supplied by Invitrogen Corporation. Description and gene name information about particular ORFs was obtained from the Saccharomyces Genome Database (SGD) (Cherry et al. 1998; Issel-Tarver et al. 2002; Hirschman et al. 2006) (http://www.yeastgenome.org/). Genes that confer an Ino$^-$ phenotype when deleted were functionally classified (clustered) by biological process using the Gene Ontology (GO) Slim mapper tool in SGD (Ashburner et al. 2000) (http://www.yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). The *P* value was calculated by performing a hypergeometric test followed by Benjamini and Hochberg false discovery rate correction (Benjamini and Yekutieli 2001; Maere et al. 2005). The *P* value is defined as the probability of seeing at least *x* number of genes out of the total *n* genes in the list annotated to a particular GO term, with respect to the proportion of genes in the whole genome that are annotated for that GO Term.

Results

419 mutants from the genome-wide collection of deletions of non-essential genes confer Ino$^-$ phenotypes

We screened the entire set of 4,741 strains from the *Saccharomyces cerevisiae* homozygous diploid knockout collection for inositol auxotrophy. Each yeast strain that was screened carried a unique deletion of a single, non-essential gene. The goal of our screen was to identify all mutants present in the knockout collection that exhibit inositol auxotrophy under one or more of the following three conditions: I$^-$C$^-$ and I$^-$C$^+$ media at 30°C, and I$^-$C$^+$ medium at 37°C. These growth conditions were chosen based on our previous finding that certain mutants exhibit Ino$^-$ phenotypes that are progressively strengthened by growth at higher temperatures and/or in the presence of choline (Nunez et al. 2008). For example, the *lcb3Δ* mutant, which lacks the sphingolipid long-chain base-1-phosphate phosphatase, grew well on I$^+$C$^-$ medium at 30°C but grew visibly weaker on I$^-$C$^-$ medium (scored as very weak, VW). In medium containing choline (I$^-$C$^+$), even greater growth reduction was observed (scored as weak, W) and on I$^-$C$^+$ medium at 37°C, no visible growth was observed (scored as strong, S).

The total number of mutants displaying an Ino$^-$ phenotype increased when grown in the presence of choline and at 37°C. In I$^-$C$^-$ medium at 30°C, we identified 162 Ino$^-$ mutants, including 27 already reported in the literature. This number increased to 219 Ino$^-$ mutants in I$^-$C$^+$ medium at 30°C and 399 Ino$^-$ mutants in I$^-$C$^+$ medium at 37°C (Table 2). Virtually all mutants that failed to grow on I$^-$C$^-$ medium at 30°C also failed to grow at 37°C on I$^-$C$^+$ medium. A small number of mutants exhibiting Ino$^-$ phenotypes at 30°C failed to grow on any media at 37°C, including I$^+$C$^+$ and YPD. The full list of all the mutants identified in the screen is listed in Table S1, and all mutants described in the “Results” or “Discussion” are listed in Table 3.

Figure 1 illustrates a typical set of plates from the screen at 30°C and 37°C, comparing growth on I$^-$C$^+$ medium to growth on I$^+$C$^+$ medium. Panel A shows the Ino$^-$ phenotypes at 30°C of the *ino2*Δ and *doa4*Δ deletion mutants, both of which were previously reported (Donahue and Henry 1981;Henry and Patton-Vogt 1998). Panel B, shows phenotypes at 37°C of *vps41Δ* and *ydr049wΔ* mutants reported in this study to exhibit Ino$^-$ phenotypes. Full descriptions of the phenotypes of these mutants, including phenotype scoring under different growth conditions, can be found in Table S1.

In all, a total of 419 strains carrying deletions of non-essential genes were observed to exhibit growth impairment of varying degrees in the absence of inositol (Ino$^-$ phenotype). Among the 419 Ino$^-$ mutants identified, 385 had not previously been reported to have this phenotype (Table 1). The screening also confirmed 34 Ino$^-$ mutants previously reported to exhibit an Ino$^-$ phenotype, providing validation of the methods used in the current study (Tables 1, S1). In addition, rescreening of 73 randomly selected Ino$^-$ mutants identified in the current study confirmed the inositol auxotrophy phenotype of all 73 strains. This indicates that the false-positive rate among the Ino$^-$ mutants identified in our screen is below 2%. Another nine mutants previously reported to have an Ino$^-$ phenotype could not be confirmed in our screening. One such mutant did not exhibit an Ino$^-$ phenotype under any of the conditions tested, while the remaining eight mutants either failed to grow in YPD medium or were absent from the collection. These nine mutants are listed in Table S1, as are all of the previously reported mutants with Ino$^-$ phenotypes confirmed in this study.

Gene ontology classification of non-essential gene deletions conferring Ino$^-$ phenotypes

Each non-essential gene mutation that exhibited an Ino$^-$ phenotype was grouped according to the gene ontology (GO) Slim classification (Ashburner et al. 2000) into 44 GO categories and ranked according to GO category enrichment for each growth condition (Fig. 2; Table S2). The current screening revealed that many categories of mutants showed progressive strengthening of their Ino$^-$ phenotypes in response to the exposure to exogenous choline and elevation of growth temperature to 37°C. For example, genes in the GO category “response to stress” were enriched relative to other categories following addition of choline and shift of the growth temperature to 37°C, suggesting that stress response pathways are critical for survival in cells grown without inositol and at higher temperatures. Likewise, genes present in the GO category “transcription” were highly enriched only at 30°C. Overall, the greatest enrichment of Ino$^-$ mutants was observed in the following GO categories: response to stress, protein modification process, and signaling process (Fig. 2; Table S2). Phenotypes of mutants specifically discussed in the remainder of the “Results” are documented in Table 3 in the approximate order of the groupings described below.

Ino$^-$ phenotypes are associated with mutations affecting general RNA-pol II mediated transcription

Ino$^-$ phenotypes associated with 21 mutants defective in subunits of RNA-Pol II (Nonet and Young 1989; Scafe et al. 1990a, b), the TATA binding protein (Arndt et al. 1995), and subunits of various transcriptional coactivation, and nucleosome remodeling complexes were previously reported. We report Ino$^-$ phenotypes associated with 30 additional mutants affecting these complexes and several other related transcriptional complexes. The cause of the Ino$^-$ phenotype in these strains is most likely due to reduced transcription of *INO1*, whose expression is necessary in cells growing in the absence of inositol. Many of these mutants are defective in genes clustering in the GO category “transcription” as well as “RNA metabolic process,” “Response to stress,” “Protein modification,” “Chromosome organization,” and “DNA metabolic process” (Fig. 2; Table S2).

Ino$^-$ phenotypes have been described for mutants defective in nucleosome remodeling, including SWI/SNF (Peterson and Herskowitz 1992) and INO80 chromatin remodeling complexes (Ebbert et al. 1999; Shen et al. 2003a; Fernandez-Murray et al. 2009) (Tables 3, S1). We report Ino$^-$ phenotypes in *rsc1Δ* and *rsc2Δ* mutants, affecting the Remodel the Structure of Chromatin (RSC) complex (Chai et al. 2002; Bungard et al. 2004), which is related to SWI/SNF complex (Tables 3, S1) and *ies1Δ*, *ies2Δ*, *ies4Δ*, *ies5Δ*, and *nhp10Δ* mutants, which affect INO80 complex (Tables 3, S1).

Mutants defective in histone acetylation were previously reported to exhibit Ino$^-$ phenotype, including mutants in the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex (Gansheroff et al. 1995; Roberts and Winston 1996; Horiuchi et al. 1997). In the current screening, we report that six additional SAGA mutants, *ada2Δ, ngg1Δ, gcn5Δ, sgf73Δ, spt3Δ,* and *spt8Δ*, have Ino$^-$ phenotypes (Tables 3, S1). Mutations affecting the SET complex, which is involved in histone deacetylation, RNA-Pol II transcription and repression of sporulation genes (Pijnappel et al. 2001) and response to secretory stress (Cohen et al. 2008) were reported to have an Ino$^-$ phenotype. We confirmed that *set1Δ, hos2Δ, snt1Δ*, and *sif2* exhibit a weak Ino$^-$ phenotype at 30°C and report a strong Ino$^-$ phenotype for these mutants at in I$^-$C$^+$ at 37°C. We also observed an Ino$^-$ phenotype for *hda3Δ*, defective in a subunit of HDA1, a histone deacetylation complex (Carman and Zeimetz 1996; Wu et al. 2001) (Tables 3, S1).

In addition, we report Ino$^-$ phenotypes for mutants in complexes affecting ubiquitination and methylation of histones. These mutants include *swd3Δ, bre2Δ, sdc1Δ, spp1Δ*, and *swd1Δ*, defective in subunits of the COMPASS complex involved in histone H3 methylation (Miller et al. 2001; Krogan et al. 2002) (Tables 3, S1). The Swd3p subunit is shared with the related histone H2B ubiquitination complex (Weake and Workman 2008), and mutations in three additional histone H2B ubiquitination subunits, Bre1p, Lge1p, and Rad6p, also confer Ino$^-$ phenotypes (Tables 3, S1).

Mutations in several genes that interact with the carboxy-terminal domain of RNA-Pol II were previously reported to have Ino$^-$ phenotypes (Koleske et al. 1992; Betz et al. 2002). We report that mutations *sin4Δ/med16Δ, srb5Δ/med18Δ, soh1Δ/med31Δ*, and *pgd1Δ/med3Δ*, affecting the SRB/MED transactivation complex, *cdc73Δ* and *leo1Δ* mutations affecting the Paf1 complex, and *dbf2Δ* and *caf4Δ* mutants affecting CCR4-NOT subunits (Tables 3, S1) exhibit Ino$^-$ phenotypes (Tables 3, S1).

Mutations affecting protein modification pathways confer Ino$^-$ phenotypes

ALG7, which encodes UDP-*N*-acetylglucosamine-1P transferase (Hanson and Lester 1980; Hanson and Lester 1982), is essential for viability and is necessary for the synthesis of branched oligosaccharides during N-linked glycoprotein biosynthesis (Lehle et al. 2006) (Fig. 3). The reaction catalyzed by Alg7p is inhibited by tunicamycin (Takatsuki and Tamura 1971), a drug well known for its effect in triggering UPR signaling in the ER (Cox et al. 1993). Moreover, wild-type cells treated with sub-lethal doses of tunicamycin exhibit inositol auxotrophy (Fernandez-Murray et al. 2009). Deletion mutations affecting several subsequent steps in the N-linked glycosylation pathway confer Ino$^-$ phenotypes, including *alg6Δ*, *alg8Δ* and *die2Δ/alg10Δ*, and *alg9Δ* (Fig. 3; Tables 3, S1) as well as *hoc1Δ* (Neiman et al. 1997) and *mnn2Δ* (Yip et al. 1994) mutations, affecting protein glycosylation in the Golgi (Tables 3, S1). In addition, we found that the *pmt1Δ* and *pmt2Δ* mutations, which affect protein *O*-mannosylation, also confer Ino$^-$ phenotypes (Tables 3, S1). Pmt2p is specifically required for *O*-mannosylation of Mid2p and Slg1p/Wsc1p (Philip and Levin 2001), both of which are sensors in the PKC–CWI pathway (Fig. 4a), to be described in a subsequent section.

Biosynthesis and addition of the glycosylphosphatidylinositol (GPI) anchor to target proteins in the ER involves modification of PI by step-wise addition of sugars and ethanolamine phosphate (Orlean and Menon 2007; Pittet and Conzelmann 2007). Although mutations in any of the non-essential genes of this pathway did not result in Ino$^-$ phenotypes, deletion of the *ARV1* gene, required for the efficient delivery of an early GPI intermediate to the first mannosyltransferase involved in GPI synthesis (Kajiwara et al. 2008), results in an Ino$^-$ phenotype (Tables 3, S1). In addition, deletion of several structural

genes encoding specific GPI-anchored proteins result in an Ino$^-$ phenotype, including *gas1Δ*, *kre1Δ*, *utr2Δ*, *fig2Δ*, and *spi1Δ* (Tables 3, S1).

Ino$^-$ phenotypes were also detected in mutants defective in N$^\alpha$-terminal protein acetylation (Driessen et al. 1985; Polevoda and Sherman 2002), a modification that affects about half of the abundant proteins in yeast (Polevoda and Sherman 2003). Mutations in two subunits of NatA N-terminal acetyltransferase complex, Ard1p and Nat1p, confer Ino$^-$ phenotypes (Tables 3, S1). Significantly, Ino1p is partially acetylated and NatA is responsible for this activity (Perrot et al. 2008). Finally, the *shr5Δ* mutant, defective in protein palmitoylation (Nadolski and Linder 2007) has an Ino$^-$ phenotype (Tables 3, S1). Shr5p is a subunit of a palmitoyltransferase, that together with Erf2p, palmitoylates Ras2p (Nadolski and Linder 2007), a protein that plays a significant role in several important signaling pathways (Fig. S1).

Mutations resulting in defects in membrane trafficking pathways confer Ino$^-$ phenotypes

Several temperature sensitive mutants, defective in essential genes involved in the secretory pathway, have previously been reported to have Ino$^-$ phenotypes at temperatures that are semipermissive for growth (Tables 3, S1). These include *sec13-1* (Gilstring et al. 1999;Chang et al. 2004), defective in a COPII vesicle component required for budding from the ER (Barlowe 2002) and *sec14-1* (Kearns et al. 1997;Chang et al. 2004), defective in a PI/PC transporter (Skinner et al. 1993) required for exit from the Golgi (Novick et al. 1980). We report that deletions of 51 additional non-essential genes encoding products involved in membrane trafficking and organelle homeostasis that exhibit Ino$^-$ phenotypes (Fig. 3). In addition, we also confirmed four non-essential genes involved in membrane trafficking previously reported to have an Ino$^-$ phenotype (Table 3; Fig. 3). These genes are largely grouped within the GO category “transport” but are also found in the categories “cell cycle” and “vesicle mediated transport.” (Fig. 2; Table S2).

We found mutations affecting multiple steps spanning early, middle, and late steps in the secretory pathway pathway that exhibited Ino$^-$ phenotypes, including two mutants in translocation of nascent proteins into the ER, two mutants in ER-to-Golgi trafficking, two mutants in Golgi-to-ER retrieval, five mutants intra-Golgi trafficking, and nine mutants in trafficking out of the Golgi, and four mutants involved in protein trafficking to the vacuole (Fig. 3; Table 3). We also found four mutants involved in endocytic trafficking that exhibit Ino$^-$ phenotypes (Fig. 3; Table 3). Mutations in organelle homeostasis also exhibited Ino$^-$ phenotypes, including 11 mutants in ER-associated protein degradation (ERAD) pathway and 12 mutants in vacuolar acidification (Fig. 3; Table 3). While this paper was under review, a similar study reported Ino$^-$ phenotypes associated with mutations in genes involved in vacuole acidification (Young et al. 2010).

Mutants with defects in a number of major stress response pathways exhibit Ino$^-$ phenotypes

Prior to this screening, Ino$^-$ phenotypes had been reported for mutants in several signaling pathways, including the UPR (Nikawa and Yamashita 1992) the PKC–CWI pathway (Nunez et al. 2008), and the Glucose Response Pathway (Hirschhorn et al. 1992; Shirra et al. 2001). The current study expands the number of mutants shown to have Ino$^-$ phenotypes in certain stress response pathways, including the PKC–CWI pathway (Fig. 4a). Mutants exhibiting Ino$^-$ phenotypes are also reported here for the first time in the HOG pathway (Fig. 4b): the target of rapamycin (TOR) pathway (Fig. 4a), the cAMP-Protein Kinase A (PKA) and the calcineurin, and filamentous growth pathways (Fig. S1; Tables 3, S1). These pathways collectively respond to stress caused by extreme growth conditions and also respond in an interconnected fashion to stress via pathway cross talk (Fuchs and Mylonakis 2009). The

majority of mutations in these pathways conferring Ino$^-$ phenotypes are associated with the GO categories, “response to stress,” “RNA metabolism,” “protein modification,” “transcription,” and “response to chemical stimulus” (Fig. 2; Table S2).

The HOG pathway, which contains a mitogen-activated protein kinase (MAPK) cascade (Fig. 4b), responds to multiple stress conditions, including high temperature (Winkler et al. 2002), high osmolarity (Hohmann et al. 2007), oxidative stress (Staleva et al. 2004), and high salt (Posas et al. 2000). We found that deletion of the *HOG1* gene, which encodes a MAP kinase, confers an Ino$^-$ phenotype, as do the HOG pathway mutations, *msb2Δ, sho1Δ, ste50Δ, ptc1Δ*, and *nbp2Δ* (Fig. 4b; Tables 3, S1). Deletion of the *SIC1* gene, a Hog1p target that is a negative regulator of the Cdc28p-Cln3p cyclin-dependent kinase complex (Fig. 4a, b) results in an Ino$^-$ phenotype (Tables 3, S1). In total mutations in four genes that promote cross-talk between cell cycle regulation and the HOG pathway exhibit Ino$^-$ phenotypes.

Previously we reported the Ino$^-$ phenotypes of mutants in the well-characterized PKC–CWI pathway (Levin 2005), including *pkc1Δ, slt2Δ/mpk1Δ, bck1Δ, and rlm1Δ* (Fig. 4a) (Nunez et al. 2008; Fernandez-Murray et al. 2009). However, in the course of this screening, many additional mutants related to PKC–CWI signaling were found to have Ino$^-$ phenotypes. These mutants include *mkk2Δ* defective in one of two redundant MAPK kinases, as well as mutants affecting upstream sensors and regulatory components, *slg1Δ/wsc1Δ, mid2Δ, zeo1Δ, rom2Δ, sac7Δ, ack1Δ*, and *spa2Δ*, depicted in Fig. 4a and documented in Tables 3 and S1. Other Ino$^-$ mutants related to PKC–CWI signaling detected in this screen include *fks1Δ, smi1Δ/knr4Δ, bck2Δ, drs2Δ, ypk2Δ*, and *cln3Δ* (Fig. 4a).

The TOR pathway (Fig. 4a) regulates metabolism and cellular growth in response to nutrient availability, as well as environmental stress, via two distinct multiprotein complexes, TORC1 and TORC2 (Torres et al. 2002;Wullschleger et al. 2006). Deletion of the *TOR1* gene confers an Ino$^-$ phenotype, while *TOR2* is essential (Giaever et al. 2002). However, *slm1Δ* and *avo1Δ* mutations, which affect the activity of the TORC2 complex, also have Ino$^-$ phenotypes (Fig. 4a;Table 3,S1). The TORC1 Complex has been implicated in response to heat stress, aging, cell size, and ribosome biogenesis (Morano and Thiele 1999;Kaeberlein et al. 2005;Wei et al. 2009) as well as cell wall integrity (Heinisch et al. 1999;Torres et al. 2002;Ho et al. 2005) (Fig. 4a). We also found that mutations in calcineurin, which is involved in calcium stress response (Zhang and Rao 2008) results in Ino$^-$ phenotypes (Figs. 4b, S1; Tables 3, S1). The interconnected cyclic PKA and filamentous growth pathways (Fig. S1) also play major roles in stress resistance to high temperature and nutrient availability. The *gpb2Δ/krh1Δ, gpb1Δ/krh2Δ*, and *sok2Δ* mutants affecting these pathways display Ino$^-$ phenotypes (Fig. S1).

Mutations in genes involved in the metabolism of diverse lipid classes, including sphingolipids, ceramides, phospholipids, fatty acids, and sterols confer Ino$^-$ phenotypes

Our screening methodology was, in part, validated by detection of mutations in genes affecting lipid metabolism already reported to confer the Ino$^-$ phenotype. These mutations include *ino1Δ, ino2Δ, ino4Δ* (Culbertson and Henry 1975; Donahue and Henry 1981), *sac1Δ* (Fig. 5) (Whitters et al. 1993; Rivas et al. 1999), *nte1Δ* (Fig. 5) (Nunez et al. 2008; Fernandez-Murray et al. 2009), *scs2Δ* (Kagiwada et al. 1998; Loewen et al. 2003, 2004; Brickner and Walter 2004), and *scs3Δ* (Hosaka et al. 1994) (Tables 3, S1). However, the current study has significantly extended the list of mutations affecting lipid metabolism that confer an Ino$^-$ phenotype, revealing Ino$^-$ phenotypes for the first time in mutants defective in structural genes encoding enzymes of sphingolipid and sterol metabolism (Fig. 5). Mutations in structural genes encoding enzymes of lipid metabolism that result in Ino$^-$ phenotypes occur in the five interconnected pathways, including glycerophospholipid, glycerol, phosphoinositide (PIP), sphingolipid, and the sterol metabolism (Fig. 5; Tables 3).

While the genes defective in these mutants cluster primarily in the GO category lipid metabolism (Fig. 2; Table S2), many are also found in the GO categories “RNA metabolic process,” “transcription,” “cellular homeostasis” and “cellular carbohydrate metabolism.”

We report that the glycerol pathway mutations *gpd1Δ, gpd2Δ, sct1Δ*, and *fps1Δ* confer Ino^- phenotypes (Tables 3, S1). Gpd1p and Gpd2p are NAD-dependent glycerol-3-phosphate dehydrogenases that produce glycerol-3-phosphate (Gro-3-P), which serves as a precursor for synthesis of PA, which serves as the signal for expression for *INO1* (Loewen et al. 2004). The *SCT1/GAT1* and the *GPT2/GAT1* genes encode Gro-3-P acyltransferases that catalyze the reaction of acyl CoA and Gro-3-P to produce lyso-phosphatidic acid (Lyso-PA), the immediate precursor of PA (Zheng and Zou 2001). Among these mutants potentially affecting PA production, only the *sct1Δ* mutant has an Ino^- phenotype. However, lyso PA can also be made from dihydroxyacetone phosphate (DHAP) (Fig. 5). In yeast, four gene products Gre3p, Ypr1p, Gcy1p, and Ara1p are associated with $NADP^+$ dependant glycerol dehydrogenase (GDH) activity responsible for conversion of glycerol into dihydroxyacetone (DHA), the precursor of DHAP. Triple mutant strains carrying deletions of three of the four genes responsible for GHD activity in yeast are Ino^- (Chang and Petrash 2008). We report that the *gre3Δ* and *ypr1Δ* single deletions confer Ino^- phenotypes when grown at 37°C in I^-C^+ medium, and the *gre3Δ* mutant also displays an Ino^- phenotype in I^-C^+ medium even at 30°C (Tables 3, S1; Fig. 5).

The inositol containing lipids of yeast include, in addition to PI, PIPs, and inositol containing sphingolipids, all of which are derived from PI (Fig. 5). The *PIS1* gene encoding synthase is essential, but a *pis1* mutant, which expresses a partially active PI synthase, is able to synthesize sufficient levels of PI to enable it to grow only when supplied with high levels of exogenous inositol (i.e., it behaves as an Ino^- mutant) (Nikawa et al. 1987). The *sac1Δ* mutant, defective in PI4P phosphatase, also exhibits an Ino^- phenotype (Whitters et al. 1993) but reportedly still expresses the *INO1* gene (Rivas et al. 1999). PI4P is the precursor of $PI(4,5)P_2$, which, as previously discussed, plays an essential role in both PKC–CWI and TORC2 signaling (Figs. 4a,5) (Audhya and Emr 2002;Tabuchi et al. 2006). Fab1p, located in the vacuolar membrane, is a PI3P 5-kinase that generates $PI(3,5)P_2$, the levels of which rise dramatically upon osmotic stress (Bonangelino et al. 2002). We report here for the first time that the *fab1Δ* mutant displays a strong Ino^- phenotype at 30°C (Fig. 5; Tables 3, S1).

Turnover of $PI(4,5)P_2$ by phospholipase C, Plc1p, yields diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Fig. 5). IP_3 is further phospholylated in a series of sequential reactions to yield inositol polyphosphates and diphosphate inositol polyphosphates which, in turn, mediate signaling regulating diverse cellular functions, including vacuole morphology and the response to salt stress (Dubois et al. 2002;Alcazar-Roman and Wente 2008;Demczuk et al. 2008). Two mutations affecting inositol polyphosphate metabolism, *arg82Δ*, and *kcs1Δ*, confer Ino^- phenotypes. The *arg82Δ/ipk2Δ* mutant was previously reported to have reduced *INO1* expression (Shen et al. 2003b). The *kcs1Δ* strain exhibits a strong Ino^- phenotype at 37°C, but *arg82Δ*, which is Ino^- at 30°C, fails to grow entirely, even in YPD medium at 37°C (Tables 3, S1).

The yeast complex sphingolipids inositol-phosphorylceramide (IPC), mannosyl-inositol-phosphorylceramide (MIPC), and mannosyl-diinositol-phosphorylceramide ($M[IP]_2C$) contain inositol. PI serves as the donor for the inositol phosphate moiety in these lipids in a reaction that also generates DAG (Fig. 5). De novo sphingolipid biosynthesis is required for heat stress response (Cowart et al. 2003), and sphingolipid metabolism may be regulated by TORC2 signaling (Fig. 4a) (Tabuchi et al. 2006), suggesting that sphingolipid levels may contribute to stress response signaling during inositol deprivation. Several mutations

affecting ceramide metabolism, *ypc1Δ, sur2Δ, lcb3Δ and dpl1Δ*, and *scs7Δ*, confer Ino^- phenotypes (Fig. 5; Tables 3, S1).

On the other hand, ergosterol synthesis (Fig. 5) does not contribute to the production of PA, use PA or PI as a precursor, or consume inositol. The pathways for sterol and glycerolipid synthesis, however, do share the common precursor acetyl-CoA (Fig. 5), which is also used in protein acetylation. The *ERG2, ERG3, ERG4, ERG5*, and *ERG6* genes are not essential for growth under normal conditions, but mutants carrying deletions of these genes all exhibit abnormal sterol compositions and also display alterations in sphingolipid composition (Guan et al. 2009). The *erg2Δ, erg3Δ, erg4Δ, erg5Δ*, and *erg6Δ* mutants all exhibit strong Ino^- phenotypes at 37°C, and all but *erg5Δ* have weak Ino^- phenotypes at 30°C (Tables 3, S1). The Ino^- phenotype of *erg6Δ* is shown in Fig. 6 in a spotting assay. At 30°C in I^-C^- medium, *erg6Δ* exhibits some residual growth (Fig. 6a), but at 37°C in I^-C^- medium it has an Ino^- phenotype as strong as that of the *ino1Δ* mutant (Fig. 6b).

Discussion

This study considerably expands the number of genes in yeast known to be necessary for growth in the absence of the phospholipid precursor, inositol. While these mutations affect a wide range of biological processes, they are heavily enriched in a discrete number of functional categories, including transcription, membrane trafficking, lipid metabolism, and stress response pathways. These results suggest that the Ino^- phenotype, which is classically associated with misregulation of lipid metabolism, is not limited to functions directly involved in regulating the *INO1* gene. Rather, defects in numerous stress response pathways processes conferred Ino^- phenotypes, suggesting that future work should focus on the identification of relevant lipid signals that mediate between inositol-dependent lipid metabolism and stress response signaling. We will discuss several mechanisms that may underlie the Ino^- phenotype, thus providing at least a partial explanation as to the number and range of functions that are deficient in these mutants.

Significant deficiency in Ino1p activity or *INO1* transcription results in inositol auxotrophy

The most direct cause of an Ino^- phenotype is lack of inositol-3-phosphate synthase (Ino1p) activity, as in the *ino1Δ* mutant. The *ino1Δ* mutant, which sets the benchmark for a strong Ino^- phenotype, shows no residual growth in the absence of inositol in plate assays at any temperature. The *ino2Δ* and *ino4Δ* mutants defective in the specific transcription factors required for expression of *INO1* and other UAS_{INO} containing genes (Ambroziak and Henry 1994) exhibit equally strong Ino^- phenotypes (Loewy and Henry 1984). Defects in RNA-Pol II mediated transcription, chromatin remodeling, and histone modification can also result in Ino^- phenotypes because of the sensitivity of *INO1* transcription to defects in general transcription (Scafe et al. 1990a). However, only a very small proportion of the mutants in this category have Ino^- phenotypes as strong as *ino1Δ, ino2Δ or ino4Δ* (Table 3). Variability in the strength of Ino^- phenotypes among mutants defective in RNA-Pol II transcription is presumably due to different levels of residual *INO1* transcription. However, direct assessment of *INO1* transcription has been carried out only in a subset of such mutants, and it remains an open question as to why *INO1* transcription is especially sensitive to sublethal mutations affecting RNA-Pol II transcription. Importantly, expression of several other highly regulated genes, such as *GAL10*, are similarly affected by defects in RNA-Pol II transcription (Scafe et al. 1990a), suggesting that this phenomenon is not unique to *INO1*, but may be a more general characteristic of highly regulated genes.

Mutations affecting diverse aspects of lipid metabolism confer Ino⁻ phenotypes

The signal for derepression of *INO1* and other UAS_{INO} containing genes involves sensing PA levels in the ER by the Opi1p transcriptional repressor. When PA levels are high, Opi1p is tethered to the ER by binding to both PA and Scs2p in the ER (Loewen et al. 2004). Consistent with this model of *INO1* regulation by Opi1p, the *scs2Δ* mutant exhibits an Ino⁻ phenotype (Hosaka et al. 1992) (Tables 3, S1), presumably due to continuous repression of *INO1* by Opi1p. Defects in lipid metabolism have the potential to influence *INO1* expression by raising or lowering PA production or its utilization, thereby influencing the degree of binding of the Opi1p repressor to the ER membrane and, as a consequence, the level of expression of *INO1* and other UAS_{INO}-containing genes (Henry and Patton-Vogt 1998; Loewen et al. 2004; Carman and Henry 2007). Mutations that lead to reduced PA levels should have the effect of lowering *INO1* expression, possibly to the extent of creating an Ino⁻ phenotype (Carman and Henry 2007). Likely candidates for this mechanism among the Ino⁻ mutant strains depicted in Fig. 5 include the *gpd1Δ* and *gpd2Δ* mutants, defective in homologous genes encoding glycerol- 3-phosphate dehydrogenases (Larsson et al. 1993; Eriksson et al. 1995), which catalyze the major route for the synthesis of Gro-3-P, precursor of lysoPA, the immediate precursor of PA. The *sct1Δ* mutant (Zheng and Zou 2001) defective in one of two acyl transferases responsible for the conversion of Gro-3-P to lysoPA (Fig. 5) should also have the effect of lowering PA levels. However, the correlation of *INO1* expression to PA levels has not yet actually been documented in any of these mutants.

Defects in other lipid metabolic steps also produce Ino⁻ phenotypes, including numerous mutants in PIP, sphingolipid, and sterol metabolism depicted in Fig. 5. However, unlike mutations affecting PA metabolism, the cause of the inositol auxotrophy in these mutants may not be due to a deficiency in *INO1* expression. For example, the *sac1Δ* mutant, defective in PI4P phosphatase, exhibits a strong Ino⁻ phenotype, but expresses *INO1* at wild-type levels (Rivas et al. 1999). Likewise, PI4P and $PI(4,5)P_2$ levels, which are affected in *sac1* mutants (Audhya and Emr 2002), have been implicated in PKC–CWI and TOR signaling (Fig. 4a). However, while the *slt2Δ/mpk1Δ* mutant in the PKC–CWI pathway exhibits a strong Ino⁻ phenotype, it regulates *INO1* normally (Nunez et al. 2008). Interestingly, *sac1Δ* exhibits other defects in lipid metabolism including reduced levels of PI and inositol-containing sphingolipids (Brice et al. 2009). Sphingolipids, like PIPs, are known to play complex roles in activating stress responses (Dickson 2008), and may functionally interact with sterols to generate membrane domains that function as signaling platforms (Guan et al. 2009). Thus, the root cause(s) of the Ino⁻ phenotypes observed in the mutants defective in PIP, sphingolipid, and sterol metabolism could be interrelated and may be due to their complex roles in regulating stress response pathways.

Mutations affecting membrane trafficking confer Ino⁻ phenotypes

We found that deletion of many non-essential genes affecting diverse steps in the secretory pathway confer Ino⁻ phenotypes. Significantly, Sec⁻ mutants, raised to their semi-restrictive or restrictive temperatures, and wild-type cells, deprived of inositol, exhibit UPR activation (Cox et al. 1997; Chang 2001; Chang et al. 2004; Gaspar et al. 2008). Moreover, the ER stress caused by inositol deprivation appears to be additive with the stress caused by the effect of the *sec13-1* secretory defect (Chang et al. 2004). The most extreme form of inositol deprivation occurs in *ino1* mutants, which are unable to synthesize any inositol and rapidly lose vitality after transfer to inositol free media, a phenomenon known as “inositol-less death” or “unbalanced growth” (Becker and Lester 1977; Henry et al. 1977; Keith et al. 1977). Prior to dying in the absence of inositol, *ino1Δ* cells exhibit levels of UPR induction that substantially exceed those seen in wild-type cells growing in inositol-free medium (S. Jesch, unpublished). In the absence of inositol, *ino1* mutants also stop dividing and cease

expansion of the plasma membrane, while continuing active metabolism including protein synthesis, for a period equivalent to about two doubling times before they lose viability (Atkinson et al. 1977; Henry et al. 1977). A similar phenomenon of "unbalanced growth" occurs in temperature-sensitive Sec$^-$ mutants undergoing secretory stress following a shift to their restrictive temperatures. Under these circumstances, Sec$^-$ mutants continue protein metabolism for a time after plasma membrane expansion ceases (Ramirez et al. 1983) and become dense, a property that was used as an enrichment procedure in their isolation (Novick and Schekman 1979; Novick et al. 1980). The similarity of events in Sec$^-$ mutants shifted to their restrictive temperatures and inositol starved *ino1* cells suggests that cells undergoing both types of stress experience similar uncoupling of metabolism from membrane expansion.

Growth in the absence of inositol elicits profound changes in lipid metabolism and activates numerous stress responses

The fully derepressed level of *INO1* expression in wild-type cells supports only limited synthesis of inositol which is sufficient to permit cells to survive, but not to attain the level of PI synthesis observed in cells growing in the presence of exogenous inositol (Gaspar et al. 2006). As a consequence, wild-type yeast cells growing in the absence of inositol have PI levels that are 4–5 times lower than cells supplemented with exogenous inositol and exhibit other changes in lipid metabolism (Kelley et al. 1988; Loewen et al. 2004; Gaspar et al. 2006), including changes in the levels of ceramide, precursor to the complex inositol containing sphingolipids (Fig. 5) (Alvarez-Vasquez et al. 2005). Moreover, changes in the expression of literally hundreds of genes accompany the changes in lipid composition that occur in wild-type cells in response inositol (Santiago and Mamoun 2003; Jesch et al. 2005, 2006). Many of these genes are known targets of stress response pathways (Jesch et al. 2005, 2006). While the UPR is the best studied example of a stress response that is activated in the absence of inositol (Cox et al. 1997; Chang 2001), many other stress responses are also activated (Jesch et al. 2006; Nunez et al. 2008).

In the genome-wide screening reported here, Ino$^-$ phenotypes are reported for the first time for many mutants defective in the HOG, TOR, cAMP-PKA, filamentous growth, and calcineurin stress response pathways (Figs. 4a, b, S1; Tables 3, S1). Mutants in the glucose response pathway (Hirschhorn et al. 1992; Shirra and Arndt 1999; Shirra et al. 2001), UPR (Nikawa and Yamashita 1992; Sidrauski et al. 1996; Nikawa et al. 1997) and PKC–CWI pathways (Nunez et al. 2008) were previously reported to have Ino$^-$ phenotypes. Given their diversity, it is unlikely that all of these signaling pathways mentioned above are involved directly in regulating *INO1* expression. Rather, we propose that growth in the absence of inositol is a stress-activating condition, similar to growth at elevated temperature, high or low osmolarity and/or exposure to agents such as tunicamycin, caffeine, or calcoflour white. The fact that mutations in these pathways confer an Ino$^-$ phenotype indicates that the signaling through these pathways is essential for survival in the absence of inositol.

Elevated growth temperature and the inclusion of choline in the growth medium add to the stress produced by growth in the absence of inositol

The presence of choline has the effect of enhancing the stringency of the Ino$^-$ phenotypes of a number of mutants, a phenomenon first reported by Hosaka et al. (1992) for a dominant choline sensitive mutation (*CSE1*), the gene for which was never isolated. High copy suppressors of the choline sensitivity of the *CSE1* dominant mutation include the *SCS3* and *SCS2* genes (Hosaka et al. 1994), both of which when deleted confer Ino$^-$ phenotypes that are strengthened by growth at 37°C and the presence of choline (Tables 3, S1). Interestingly, the *scs2Δ* mutant also exhibits increased PC synthesis via the CDP choline pathway (Kagiwada et al. 1998). PC turnover in wild-type cells grown at 30°C in the absence of

choline occurs via phospholipase D mediated mechanism, which generates PA and free choline (Patton-Vogt et al. 1997; Sreenivas et al. 1998). In contrast, when choline is present in the growth medium of wild-type cells, or when cells are grown at 37°C, turnover of PC shifts to a phospholipase B mediated mechanism (Dowd et al. 2001), catalyzed by Nte1p (Zaccheo et al. 2004), which generates free fatty acids and glycerol-P-choline (GroPCho) (Fig. 5). Similar to the PKC–CWI mutants (Fig. 4a), the *nte1Δ* mutant exhibits an Ino$^-$ phenotype, which is strongest at 37°C in the presence of choline (Tables 3, S1) (Nunez et al. 2008; Fernandez-Murray et al. 2009). When the CDP-choline pathway is blocked (Fig. 5), turnover of PC continues via a phospholipase D mediated route regardless of temperature or the presence of choline (Dowd et al. 2001). Significantly, mutations that block the incorporation of exogenous choline via the CDP-choline pathway (Fig. 5), not only suppress the choline sensitivity of the *scs2Δ* mutant, but also suppress its Ino$^-$ phenotype (Kagiwada and Zen 2003).

In wild-type cells, the presence of choline causes increased flow through the CDP-choline pathway, increasing utilization of DAG, which is derived from PA (Fig. 5) (Carman and Henry 2007). Thus, the presence of choline inherently affects PA metabolism, which in turn has the potential to affect *INO1* expression. The effect of choline on PA levels and *INO1* expression potentially explains both the effect of choline in strengthening of Ino$^-$ phenotypes and the ability of mutations in the CDP-choline pathway to suppress such phenotypes in certain mutants, such as *scs2Δ* (Carman and Henry 2007). The lowering of PA levels in the presence of choline may also result in further lowering of *INO1* expression in mutants in which *INO1* transcription is already impaired (Tables 3, S1).

However, the effect of choline on *INO1* expression does not explain the choline sensitivity of the Ino$^-$ phenotypes of mutants in the PCK-CWI pathway (Nunez et al. 2008) (Tables 3, S1). The *slt2Δ/mpk1Δ* mutant (Fig. 4a), exhibits no defect in *INO1* transcription, even in the presence of choline at 37°C despite its strong Ino$^-$ phenotype under these conditions (Nunez et al. 2008) (Tables 3, S1). However, in comparison with wild-type cells, the *slt2Δ/mpk1Δ* mutant exhibits higher levels of PC synthesis and turnover via a phospholipase B mediated route, when grown in I$^-$C$^+$ medium at 37° (Nunez et al. 2008). The Ino$^-$ phenotype of *slt2Δ/mpk1Δ* is also suppressed by overexpression of the *NTE1* gene, suggesting that altered PC metabolism plays a significant role in its phenotype (Nunez et al. 2008; Fernandez-Murray et al. 2009). Thus, while the PKC–CWI pathway does not regulate *INO1* expression in response to inositol supplementation, it does appear to be essential for maintaining lipid homeostasis in the absence of exogenous inositol, especially at high temperature in the presence of choline. We propose that the strengthening of the Ino$^-$ phenotype of *slt2Δ/mpk1Δ* and other PKC–CWI mutants is due to the additive effects of high temperature, lack of inositol, and the presence of choline on the underlying stress responsible for activating PKC–CWI signaling. Significantly, all of these environmental factors have been shown individually to perturb lipid metabolism in wild-type cells (Gaspar et al. 2006, 2008) and may contribute to the Ino$^-$ phenotypes observed in mutants in other stress response pathways as discussed earlier.

Many of the mutants identified here as showing Ino$^-$ phenotypes that are strengthened by growth at 37°C in the presence of choline are defective in a stress response, or other cellular function, such as lipid metabolism or membrane trafficking, that are predicted to contribute to cellular stress when partially impaired. However, some mutants with defects related to partial impairment of *INO1* expression due to defects in RNA-Pol II transcription, also show strengthening of their Ino$^-$ phenotypes in response to choline and temperature. In such mutants, *INO1* expression maybe at a level that is barely sufficient for survival in the absence of inositol. Since higher growth temperature increases the demand for PI synthesis (Gaspar et al. 2008), while the presence of choline reduces in *INO1* expression (Hirsch and

Henry 1986), growth of strains with marginal *INO1* expression may not be possible under these conditions. Overall, the results of our genome-wide screen for Ino$^-$ phenotypes strongly suggest a role for lipid metabolism in stress response signaling. Future work will focus on identifying relevant lipid signals that mediate between inositol-dependent lipid metabolism and stress response signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

Ino$^-$	Inositol auxotrophy
RNA-Pol II	RNA polymerase II
PI	Phosphatidylinositol
PA	Phosphatidic acid
ER	Endoplasmic reticulum
DAG	Diacylglycerol
PC	Phosphatidylcholine
PKC–CWI	Protein kinase C–cell wall integrity
UPR	Unfolded protein response
AMPK	AMP-dependant kinase
HOG	High osmolarity glycerol
ERAD	ER-associated protein degradation
TOR	Target of rapamycin
PKA	cAMP-protein kinase A
MAPK	Mitogen activated protein kinase
ORF	Open reading frame
GO	Gene Ontology
SGD	*Saccharomyces* genome database
YPD	1% yeast extract, 2% bactopeptone, 2% glucose
I	Inositol
C	Choline
GroPCho	Glycerol-phospho-choline
GPI	Glycosylphosphatidylinositol
Gro-3-P	Glycerol-3-phosphate
lyso-PA	Lyso-phosphatidic acid
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
PIP	Phosphoinositides

IP_3	Inositol 1,4,5-triphosphate
IPC	Inositol-phosphorylceramide
MIPC	Mannosyl-inositol-phosphorylceramide
$M(IP)_2C$	Mannosyl-diinositol-phosphorylceramide

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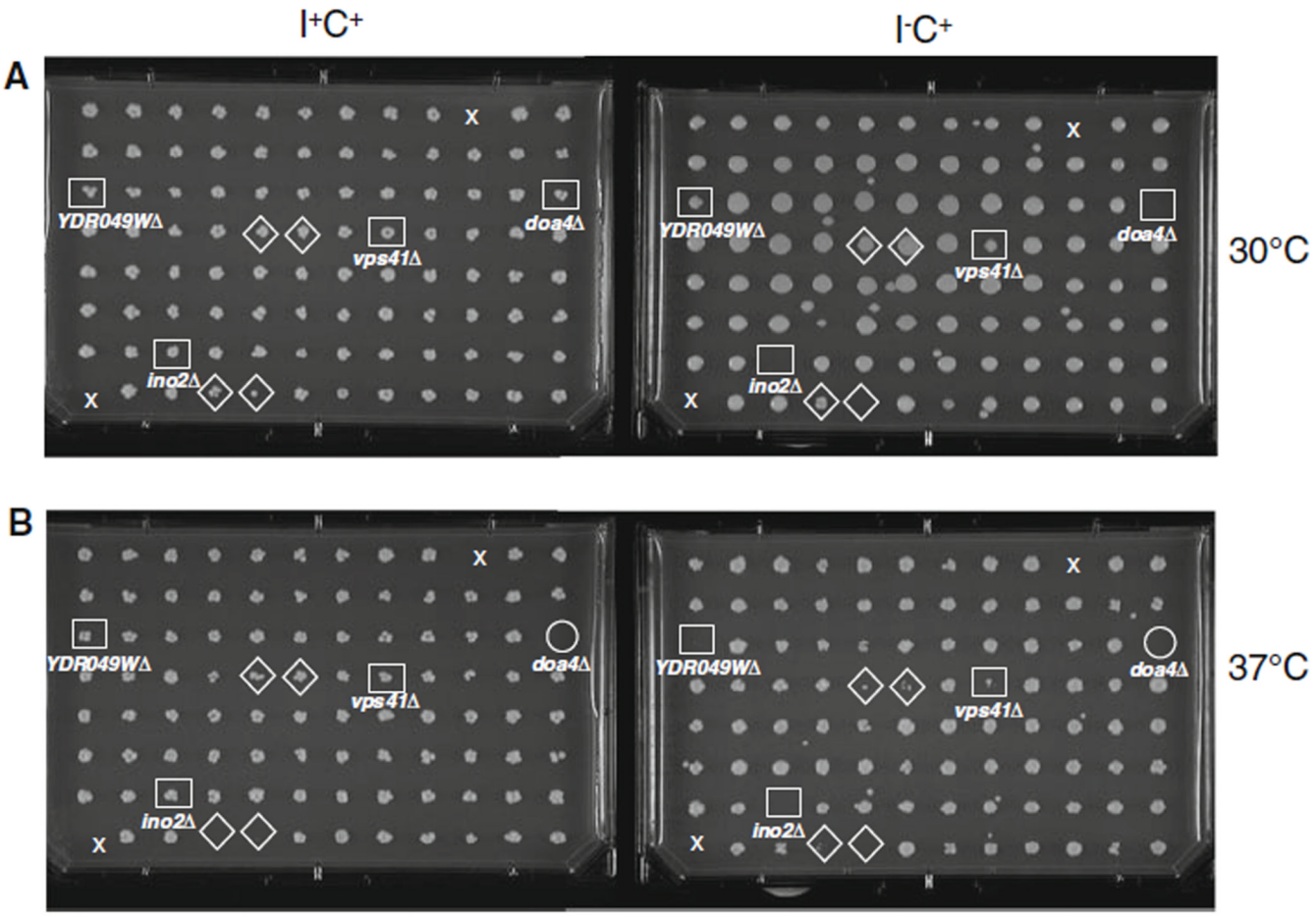

Fig. 1.

Genome-wide screen for the Ino^{-} phenotype. Representative images of 1 out of a total of 54 96-well microtiter plates from the screen. **a** A set of mutant strains from the primary screening that includes the *doa4Δ*, *ino2Δ*, *vps41Δ*, *ydr049wΔ*, mutants on I^{+}C^{+}, and I^{-}C^{+} media at 30°C. **b** Same set of mutant strains grown on I^{+}C^{+} and I^{-}C^{+} media at 37°C. The detailed screening protocol is described in "Materials and methods" and complete description of the phenotypes of these mutants is presented in Table S1. The position of each mutant colony, which confers an Ino^{-} phenotype is surrounded by a *square*. Colony positions surrounded by a *circle* indicate lack of growth in I^{+}C^{+} medium, scored as no growth (*NG*) at 37°C in Tables 3, S1. Positions surrounded by a *diamond* indicates a growth defect on I^{-}C^{+} medium that were not validated on a duplicate plate or in a subsequent spotting assay

	GO Biol Process Category
1	response to stress
2	protein modification process
3	signaling process
4	biological process unknown
5	chromosome organization
6	response to chemical stimulus
7	cellular carbohydrate metabolic process
8	pseudohyphal growth
9	transcription
10	mitochondrion organization
11	cellular homeostasis
12	cell cycle
13	vesicle-mediated transport
14	DNA metabolic process
15	translation
16	fungal-type cell wall organization
17	meiosis
18	transposition
19	sporulation resulting in formation of a cellular spore
20	cellular protein catabolic process
21	cellular component morphogenesis
22	peroxisome organization
23	transport
24	ribosome biogenesis
25	cellular lipid metabolic process
26	conjugation
27	cellular membrane organization
28	vacuole organization
29	RNA metabolic process
30	cellular amino acid and derivative metabolic process
31	protein complex biogenesis
32	generation of precursor metabolites and energy
33	cellular respiration
34	protein folding
35	cell budding
36	cytoskeleton organization
37	vesicle organization
38	chromosome segregation
39	cytokinesis
40	heterocycle metabolic process
41	cofactor metabolic process
42	nucleus organization
43	cellular aromatic compound metabolic process
44	vitamin metabolic process

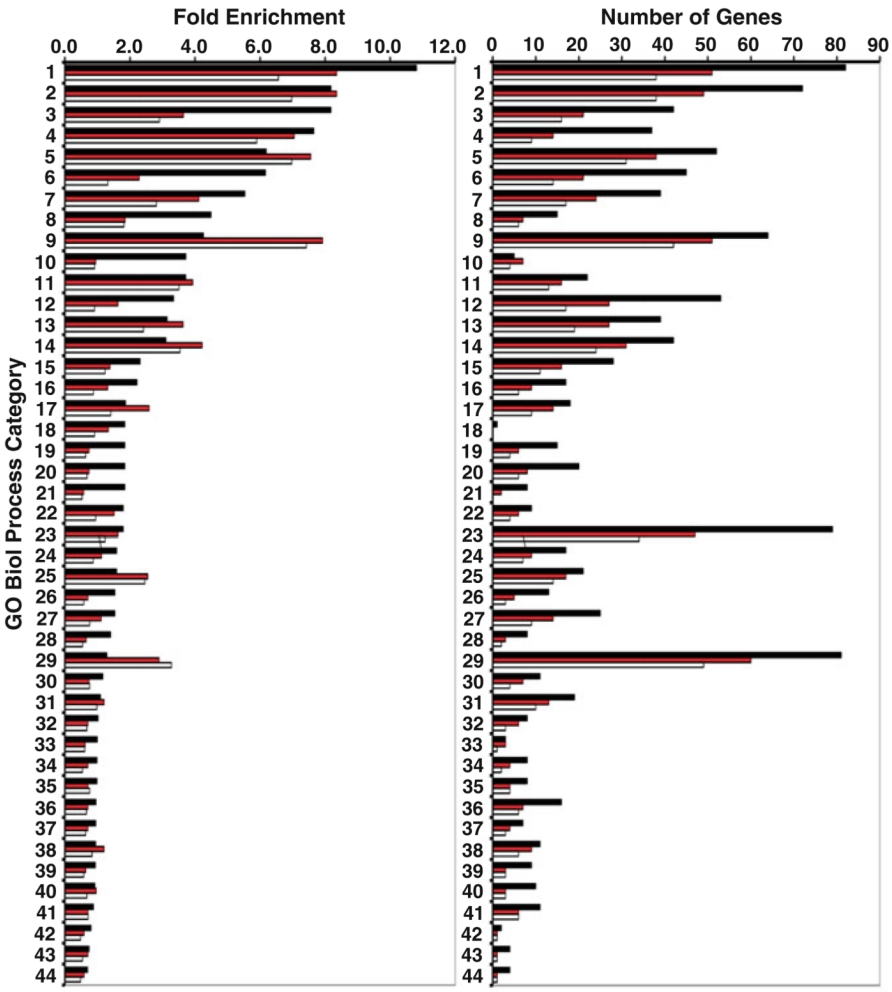

Fig. 2.

Clustering of non-essential genes that confer Ino$^-$ phenotypes by enrichment in GO biological process categories. Ino$^-$ mutants were classified according to the GO Slim classification as described in the “Materials and methods”. In each category, *color bars* indicate the fold enrichment or number of mutant strains conferring Ino$^-$ phenotypes that were detected under each growth condition: I$^-$C$^-$ medium at 30°C (*white bars*), I$^-$C$^+$ medium at 30°C (*red bars*) and I$^-$C$^+$ medium at 37°C (*black bars*). Fold enrichment is defined as $-\text{Log}_{10}$ (adjusted *P* value). The *P* value was calculated by performing a hypergeometric test followed by Benjamini and Hochberg false discovery rate correction (Benjamini and Yekutieli 2001; Maere et al. 2005) as described in “Materials and methods”. A complete list of non-essential genes in each GO category is provided in Table S2

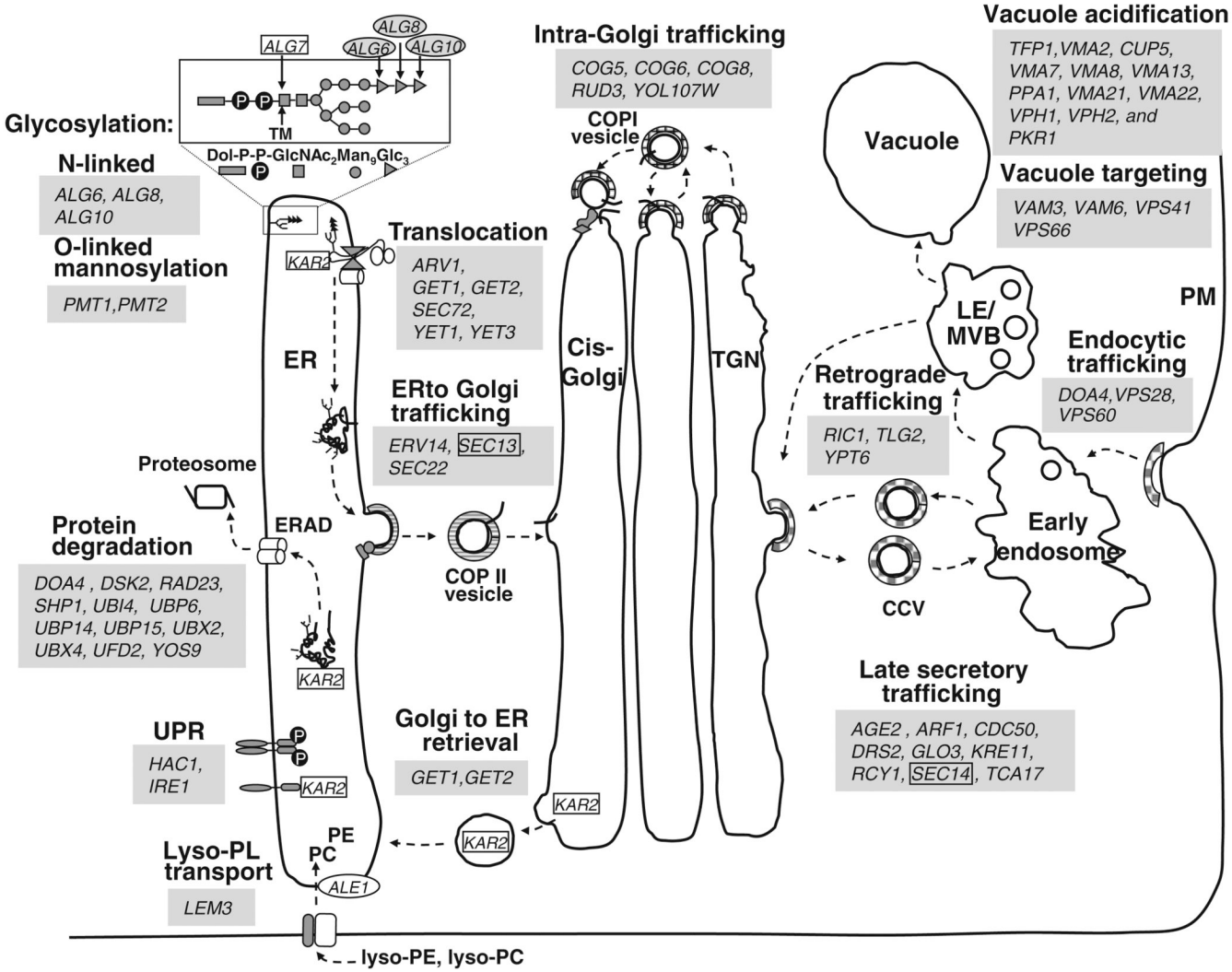

Fig. 3.

Mutations in non-essential genes involved in membrane trafficking result in Ino$^-$ phenotypes. Mutations in genes enclosed in *gray boxes* confer Ino$^-$ phenotypes. Essential genes are enclosed by a *rectangle*, for example, *KAR2*. Conditional alleles that confer Ino$^-$ phenotypes have been reported for essential genes in *gray boxes*. *ER* endoplasmic reticulum, *UPR* unfolded protein response, *ERAD* ER-associated degradation, *Lyso-PL* lyso-phospholipids, *TGN* trans golgi network, *PM* plasma membrane, *TM* tunicamycin, *LE/MVB* late endosome/multivesicular bodies

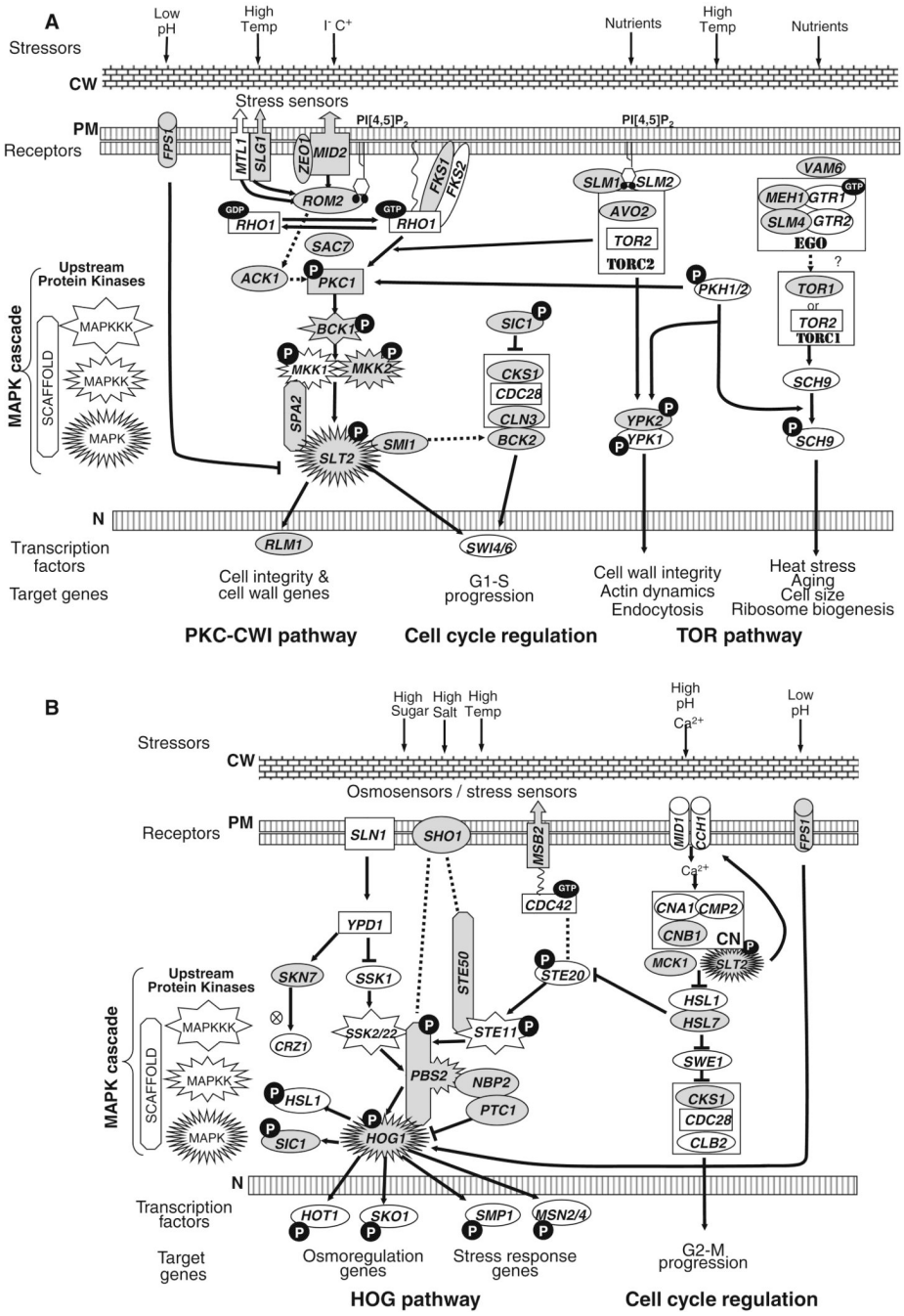

Fig. 4.

Mutations in non-essential genes affecting stress response pathways confer Ino⁻ phenotypes. **a** Protein kinase C–Cell Wall integrity (PKC–CWI) pathway, signaling required for G1-S progression and Target of Rapamycin (*TOR*) pathway. **b** High osmolarity glycerol (HOG) pathway and signaling required for G2-M progression. Mutations in genes shaded in gray confer an Ino⁻ phenotype. Genes enclosed in a *rectangle* are essential. Genes enclosed in a *cylinder* encode pore-forming proteins. Genes enclosed in a *box* with an *upward arrow* encode glycosylated transmembrane sensors. A *black circle* with a *white* letter "*P*" represents a phosphorylated target. A *black oval* with *white* letters "*GTP*" indicates a GTP-binding protein. Genes encoding mitogen activated protein kinase (*MAPK*) cascade

components (kinases and scaffold proteins) are indicated with *star symbols* and elongated octagons according to the key on the *left side* of the figure. *CW* cell wall, *PM* plasma membrane, *N* nucleus

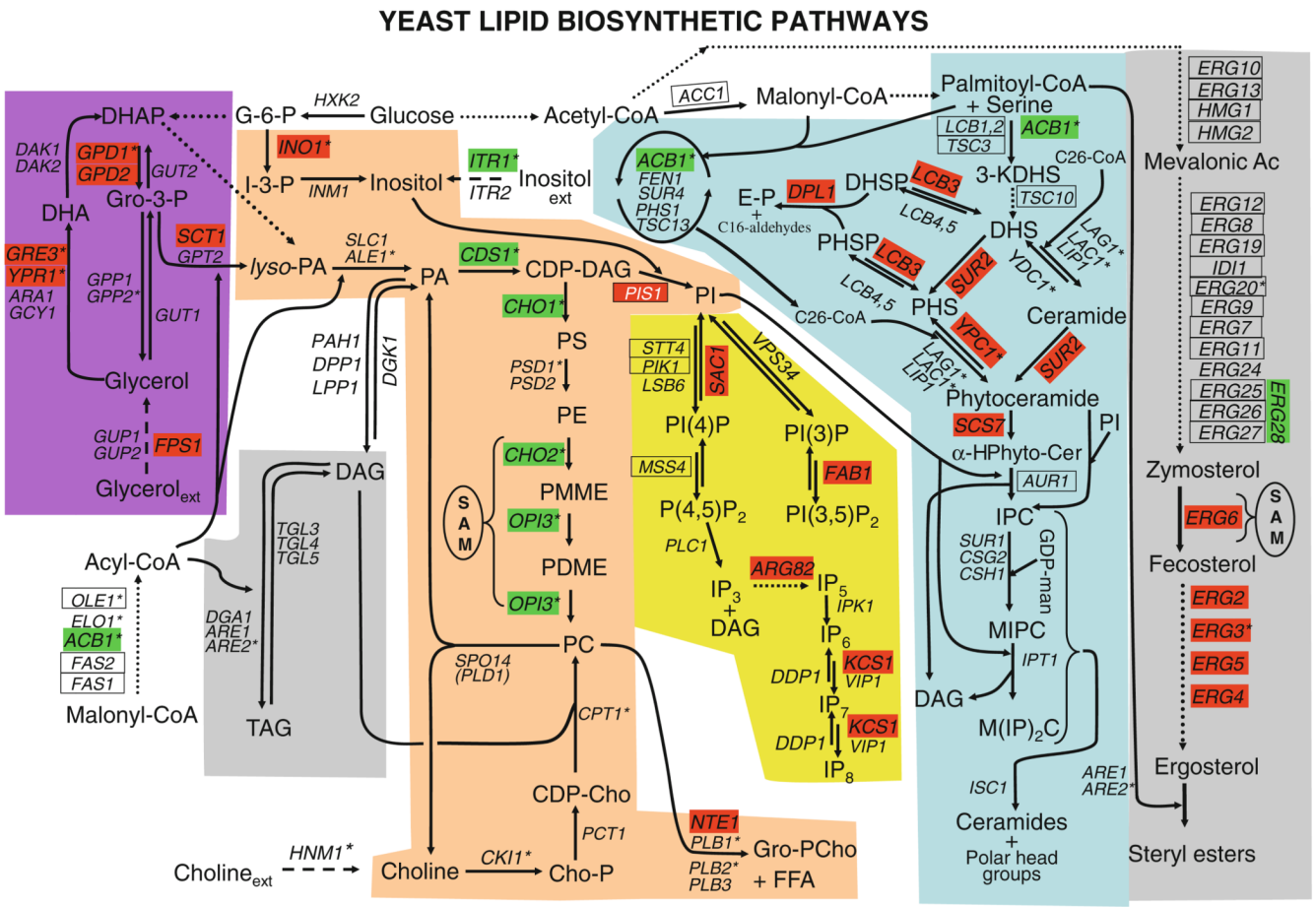

Fig. 5.

Major pathways of lipid metabolism in yeast. The pathways are *color* coded: (*tan*) glycerophospholipid pathway (*violet*) glycerol pathway, (*yellow*) phosphoinositides and inositol phosphates pathway, (*blue*) ceramide and sphingolipid pathway and (*gray*) sterol and TAG pathways. *Solid arrows* represent routes of metabolic conversion. *Dotted lines* represent multistep metabolic conversions. The names of structural genes (*italicized*) encoding enzymes catalyzing specific metabolic conversions are shown adjacent to *arrows*. An *asterisk* next to the gene name indicates genes regulated by inositol. Mutations in genes *colored* in green confer an overproduction of inositol (Opi$^-$) phenotype due to elevated PA levels (Henry and Patton-Vogt 1998). Mutations in genes *colored* in *red* confer an Ino$^-$ phenotype. An allele of the essential *PIS1* gene (*white letters* on *red* background) has an Ino$^-$ phenotype. Genes enclosed in a *rectangle* are essential. Metabolites are indicated in plain text: *DHAP* dihydroxyacetone phosphate, *DHA* dihydroxyacetone, *Gro-3-P* glycerol 3-phosphate, *G-6-P* glucose 6-phosphate, *I-3-P* inositol 3-phosphate, *lyso-PA* lyso-phosphatidic acid, *PA* phosphatidic acid, *DAG* diacylglycerol, *TAG* triacylglycerol, *CDP-DAG* cytidine-diphosphate diacylglycerol, *PS* phosphatidylserine, *PE* phosphatidylethanolamine, *SAM* S-adenosyl methionine, *PMME* phosphatidyl-*N*-monomethylethanolamine, *PDME* phosphatidyl-*N*,*N*-dimethylethanolamine, *PC* phosphatidylcholine, *Gro-PCho* glycerophosphocholine, *FFA* free fatty acids, *CDP-Cho* cytidine-diphosphate-choline, *Cho-P* choline-phosphate, *PI* phosphatidylinositol, *PI3P* phosphatidylinositol 3-phosphate, *PI4P* phosphatidylinositol 4-phosphate, *PI(3,5)*P_2, phosphatidylinositol 3,5-bisphosphate, *PI(4,5)*P_2, phosphatidylinositol 4,5-bisphosphate, *IP*$_3$ inositol 1,4,5-triphosphate, *IP*$_5$ inositol 1,3,4,5,6-pentakisphosphate, *IP*$_6$ inositol 1,2,3,4,5,6-

hexakisphosphate, IP_7 diphosphoinositol pentakisphosphate, IP_8 bis-diphosphoinositol tetrakisphosphate, *3-KDHS* 3-ketodihydrosphingosine, *DHS* dihydrosphingosine, *PHS* phytosphingosine, *DHSP* dihydrosphingosine 1-phosphate, *PHSP* phytosphingosine 1-phosphate, *E-P* ethanolamine-phosphate, α-*HPhyto-Cer* alpha-hydroxyphytoceramide, *IPC* inositol-phosphoceramide, *MIPC* mannose-inositol-phosphoceramide, $M(IP)_2C$ mannosyl-diinositol-phosphorylceramide

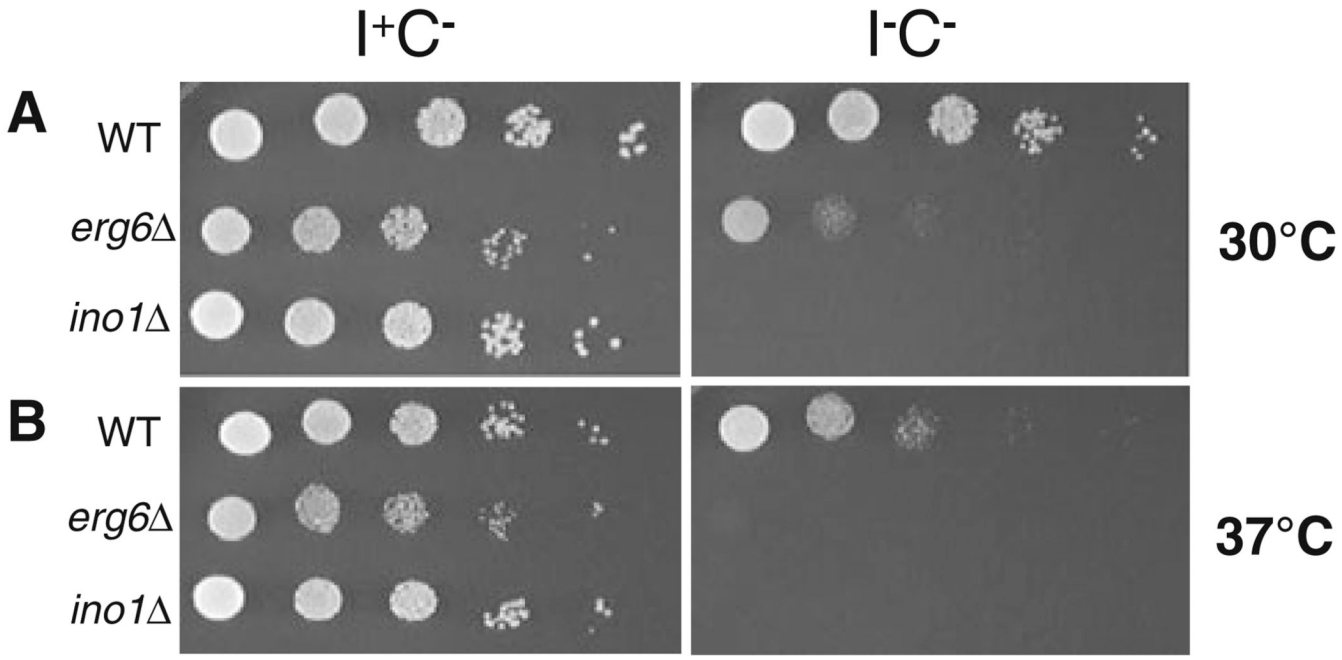

Fig. 6.

Inositol auxotrophy phenotype of erg6Δ. Analysis of the inositol auxotrophy (Ino^- phenotype). Spotting assays were performed as described in the "Materials and methods". 5-μl aliquots of a series of tenfold dilutions were spotted onto I^+C^- and I^-C^- media containing 2% agar and incubated at 30°C (**a**) and at 37°C (**b**). The *ino1Δ* strain is provided as an example of a strong Ino^- mutant

Table 1

Number of mutations in yeast reported to confer an Ino$^-$ phenotype

	Non-essential genes[a]	Essential genes[b]	Total
This study	385	0	385
Previously reported	34[c]	20[c]	54
Total	419	20	439

[a] Mutations in non-essential genes found in this study to confer an Ino$^-$ phenotype

[b] Conditional mutants reported to have Ino$^-$ phenotypes under one or more condition of growth

[c] Reference sources for previously reported Ino$^-$ phenotypes are listed in Tables 3, S1

Table 2

Sumary of Ino^{-} phenotypes of mutants carrying deletions in non-essential genes described in this study

	I^{-}C^{-} at 30°C	**I^{-}C^{+} at 30°C**	**I^{-}C^{+} at 37°C**
Strong	31	45	243
Weak	74	106	134
Very weak	57	68	22
Total	162	219	399[a]

[a] Of the 419 mutants, 20 which had Ino^{-} phenotypes at 30°C did not grow in either I^{+}C^{+}, I^{+}C^{-} or YPD medium at 37°C

Table 3

Mutations that confer Ino$^-$ phenotypes

ORF	Gene	I$^-$ C$^-$ 30°C	I$^-$C$^+$ 30°C	I$^-$C$^+$ 37°C	References
A. Protein complexes involved in transcription regulation					
A1. Subunits of the SWI/SNF chromatin remodeling complex					
YOR290C	*SNF2*	+	VW	VW	(Peterson and Herskowitz 1992)
YBR289W	*SNF5*	S	S	S	(Peterson and Herskowitz 1992)
YHL025W	*SNF6*	S	S	S	(Peterson and Herskowitz 1992)
YPL016W	*SWI1*[a]	NS	NS	NS	(Peterson and Herskowitz 1992)
YJL176C	*SWI3*	W	S	S	(Peterson and Herskowitz 1992)
A2. Subunits of the remodel the structure of chromatin (RSC) complex					
YGR056W	*RSC1*	VW	VW	S	This study
YLR357W	*RSC2*	VW	W	S	This study
A3. Subunits and proteins associated with the Ino80 chromatin remodeling complex					
YNL059C	*ARP5*[a]	NG	NG	NG	(Shen et al. 2003a)
YOR141C	*ARP8*	W	S	NG	(Shen et al. 2003a)
YFL013C	*IES1*	+	+	S	This study
YNL215W	*IES2*	W	W	NG	This study
YOR189W	*IES4*	VW	W	S	This study
YER092W	*IES5*	+	+	S	This study
YEL044W	*IES6*	S	S	NG	(Fernandez-Murray et al. 2009)
YGL150C	*INO80*[a]	NS	NS	NS	(Ebbert et al. 1999)
YDL002C	*NHP10*	+	+	S	This study
A4. Subunits and proteins associated with the ADA and SAGA complexes					
YDR448W	*ADA2*	S	W	W	This study
YGR252W	*GCN5*	VW	VW	VW	This study
YPL254W	*HFI1*[a]	NG	NG	NG	(Horiuchi et al. 1997)
YDR176W	*NGG1*	VW	W	W	This study
YGL066W	*SGF73*	W	W	S	This study
YOL148C	*SPT20*[a]	NG	NG	NG	(Roberts and Winston 1996)

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
YDR392W	*SPT3*	VW	W	S	This study
YBR081C	*SPT7*[a]	NS	NS	NS	(Gansheroff et al. 1995)
YLR055C	*SPT8*	+	+	S	This study
A5. Subunit of a histone deacetylase (HAD1) complex					
YPR179C	*HAD3*	+	+	S	This study
A6. Subunits of the Set3 deacetylase complex					
YGL194C	*HOS2*	W	W	S	(Cohen et al. 2008)
YOL068C	*HST1*	VW	VW	VW	This study
YKR029C	*SET3*	+	W	S	(Cohen et al. 2008)
YBR103W	*SIF2*	W	W	S	(Cohen et al. 2008)
YCR033W	*SNT1*	W	S	S	(Cohen et al. 2008)
A7. Subunits of the RNA polymerase II SRB/mediator complex					
YHR041C	*SRB2*	S	S	S	(Koleske et al. 1992)
YNL236W	*SIN4*	S	S	NG	This study
YGR104C	*SRB5*	VW	VW	NG	(Betz et al. 2002)
YGL127C	*SOH1*	VW	W	S	This study
YGL025C	*PGD1*	S	S	S	This study
A8. Subunits and proteins associated with the Paf1 complex					
YLR418C	*CDC73*	S	S	S	This study
YOL145C	*CTR9*[a]	NS	NS	NS	(Betz et al. 2002)
YOR123C	*LEO1*	W	W	S	This study
YBR279W	*PAF1*	S	S	NG	(Betz et al. 2002)
YGL244W	*RTF1*	VW	W	S	(Betz et al. 2002)
A9. Subunits and proteins associated with the CCR4-NOT complex					
YKR036C	*CAF4*	+	VW	W	This study
YAL021C	*CCR4*	+	+	W	(Betz et al. 2002)
YGR092W	*DBF2*	W	S	S	This study
A10. Subunits of the COMPASS (Set1C) complex					
YLR015W	*BRE2*	+	+	VW	This study
YDR469W	*SDC1*	VW	VW	W	This study

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
YPL138C	*SPP1*	+	+	S	This study
YAR003W	*SWD1*	+	+	S	This study
YBR175W	*SWD3*	VW	+	S	This study
A11. Subunits of the H2B ubiquitination complex					
YDL074C	*BRE1*	W	W	S	This study
YPL055C	*LGE1*	+	W	S	This study
YGL058W	*RAD6*	VW	W	S	This study
B. Ribosomal biogenesis pathway					
B1. Components of the small (40S) ribosomal subunit					
YLR048W	*RPS0B*	W	W	W	This study
YOR096W	*RPS7A*	VW	VW	W	This study
YBR189W	*RPS9B*	+	W	W	This study
YDL083C	*RPS16B*	+	+	S	This study
YOL121C	*RPS19A*	+	+	S	This study
B2. Components of the large (60S) ribosomal subunit					
YKL006W	*RPL14A*	VW	W	S	This study
YBL027W	*RPL19B*	+	VW	W	This study
YMR242C	*RPL20A*	+	VW	W	This study
YBR191W	*RPL21A*	+	VW	W	This study
YLR061W	*RPL22A*	W	W	VW	This study
YGL031C	*RPL24A*	+	+	VW	This study
YHR010W	*RPL27A*	W	S	S	This study
YDL191W	*RPL35A*	W	W	S	This study
YPR043W	*RPL43A*	VW	VW	VW	This study
B3. Component of small ribosomal subunit (SSU) processosome					
YOR078W	*BUD21*	+	VW	VW	This study
B4. Methyltransferase required for rRNA processing and nuclear export of 40S ribosomal subunits					
YCR047C	*BUD23*	+	+	S	This study
B5. Components of the ribosomal stalk					
YDL081C	*RPP1A*	+	+	S	This study
YDL130W	*RPP1B*	+	+	S	This study

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
B6. Component of eukaryotic eIF3 and required for processing of 20S pre-rRNA					
YLR192C	*HCR1*	+	+	S	This study
B7. Elongation factor 2 (EF-2), also encoded by EFT1					
YDR385W	*EFT2*	+	+	S	This study
C. Protein modification pathways					
C1. Glycosyltransferases of the ER, involved in N-linked protein glycosylation					
YOR002W	*ALG6*	+	S	This study	
YOR067C	*ALG8*	+	S	This study	
YNL219C	*ALG9*	+	+	W	This study
YGR227W	*DIE2*	+	+	S	This study
C2. Glycosyltransferases of the Golgi involved in branched glycosylation					
YJR075W	*HOC1*	+	+	W	This study
YBR015C	*MNN2*	+	+	W	This study
C3. Glycosyltransferases of the ER, involved in *O*-linked protein mannosylation					
YDL095W	*PMT1*	+	VW	S	This study
YAL023C	*PMT2*	W	W	S	This study
C4. GPI-anchored glycoproteins					
YCR089W	*FIG 2*	+	+	W	This study
YMR307W	*GAS1*	+	VW	S	This study
YNL322C	*KRE1*	+	+	S	This study
YER150W	*SPI1*	+	+	W	This study
YEL040W	*UTR2*	+	+	W	This study
C5. Subunits of the N-terminal acetyltransferase NatA (Nat1p, Ard1p, Nat5p)					
YHR013C	*ARD1*	W	S	S	This study
YDL040C	*NAT1*	VW	W	S	This study
C6. Subunit of a palmitoyltransferase complex composed of Shr5p and Erf2p					
YOL110W	*SHR5*	+	+	S	This study
D. Membrane trafficking pathways					
D1. Proteins involved in translocation into the ER (Translocon)					
YLR292C	*SEC72*	+	+	S	This study
YKL065C	*YET1*	W	S	S	(Wilson and Barlowe 2010)

Mol Genet Genomics. Author manuscript; available in PMC 2012 February 1.

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
YDL072C	*YET3*	S	S	S	(Wilson and Barlowe 2010)
YLR242C	*ARV1*	W	W	S	This study
D2. Proteins involved in COPII vesicle trafficking from the ER to the Golgi					
YGL054C	*ERV14*	W	W	S	This study
YLR208W	*SEC13*[a]	NS	NS	NS	(Gilstring et al. 1999)
YLR268W	*SEC22*	W	W	S	This study
D3. Proteins involved in Golgi to ER retrival					
YGL020C	*GET1*	+	W	S	This study
YER083C	*GET2*	W	W	NG	This study
D4. Proteins involved in protein ubiquitination/deubiquitination and degradation via proteosome and vacuole					
YDR069C	*DOA4*	S	S	NG	(Henry and Patton-Vogt 1998)
YMR276W	*DSK2*	+	VW	S	This study
YEL037C	*RAD23*	+	+	W	This study
YBL058W	*SHP1*	+	+	W	This study
YLL039C	*UBI4*	+	+	S	This study
YBR058C	*UBP14*	+	VW	S	This study
YMR304W	*UBP15*	+	+	W	This study
YFR010W	*UBP6*	W	W	S	This study
YML013W	*UBX2*	VW	VW	S	This study
YMR067C	*UBX4*	+	+	S	This study
YDL190C	*UFD2*	+	+	S	This study
YDR057W	*YOS9*	+	+	W	This study
D5. Proteins involved in COPI vesicle intra-Golgi trafficking and the COG complex					
YNL051W	*COG5*	VW	VW	VW	This study
YNL041C	*COG6*	W	W	W	This study
YML071C	*COG8*	W	W	W	This study
YOR216C	*RUD3*	VW	W	W	This study
YOL107W	*YOL107 W*	+	+	S	This study
D6. Proteins involved in late secretory trafficking					
YIL044C	*AGE2*	+	+	S	This study

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
YDL192W	*ARF1*	+	W	S	This study
YCR094W	*CDC50*	+	+	W	This study
YAL026C	*DRS2*	+	+	S	This study
YER122C	*GLO3*	W	VW	W	This study
YGR166W	*KRE11*	+	+	S	This study
YNL323W	*LEM3*	+	VW	W	This study
YJL204C	*RCY1*	+	VW	S	This study
YMR079W	*SEC14*[a]	NS	NS	NS	(Kearns et al. 1997)
YEL048C	*TCA17*	+	+	S	This study
D7. Proteins involved in vacuole targeting					
YOR106W	*VAM3*	+	+	S	This study
YDL077C	*VAM6*	+	+	W	This study
YDR080W	*VPS41*	+	VW	W	This study
YPR139C	*VPS66*	+	W	S	This study
D8. Proteins involved in retrograde trafficking, from early and late endosomes, to the TGN					
YLR039C	*RIC1*	VW	W	NG	(Kodaki et al. 1995)
YOL018C	*TLG2*	VW	VW	VW	This study
YLR262C	*YPT6*	+	+	VW	This study
D9. Proteins involved in endocytic trafficking, from plasma membrane to the MVB and vacuole					
YPL065W	*VPS28*	+	+	S	This study
YDR486C	*VPS60*	+	+	S	This study
D10. Subunits of the vacuolar H(+)-ATPase complex and associated proteins with vacuolar acidification					
YEL027W	*CUP5*	S	S	S	This study
YMR123W	*PKR1*	+	VW	W	This study
YHR026W	*PPA1*	S	S	S	This study
YDL185W	*TFP1*	W	S	S	This study
YPR036W	*VMA13*	S	S	S	This study
YBR127C	*VMA2*	S	S	S	This study
YGR105W	*VMA21*	VW	W	S	This study
YHR060W	*VMA22*	W	W	S	This study

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
YGR020C	*VMA7*	VW	W	S	This study
YEL051W	*VMA8*	S	S	S	This study
YOR270C	*VPH1*	+	+	W	This study
YKL119C	*VPH2*	W	S	S	This study
E. Stress response pathways					
E1. Components and associated proteins involved in the PKC–CWI signaling pathway					
YDL203C	*ACK1*	+	+	S	This study
YJL095W	*BCK1*	S	S	S	(Nunez et al. 2008)
YER167W	*BCK2*	+	+	W	This study
YLR342W	*FKS1*	VW	VW	S	This study
YLR332W	*MID2*	+	VW	W	This study
YPL140C	MKK2	+	+	W	This study
YBL105C	*PKC1*[a]	NS	NS	NS	(Nunez et al. 2008)
YPL089C	*RLM1*	+	VW	S	This study
YLR371W	*ROM2*	VW	W	S	This study
YDR389W	*SAC7*	W	W	S	This study
YOR008C	*SLG1*	VW	W	S	This study
YHR030C	*SLT2*	VW	S	S	(Nunez et al. 2008)
YGR229C	*SMI1*	+	+	S	This study
YLL021W	*SPA2*	+	+	S	This study
YOL109W	*ZEO1*	+	+	S	This study
E2. Components and associated proteins involved in the HOG signaling pathway					
YLR113W	*HOG1*	W	W	W	This study
YGR014W	*MSB2*	+	VW	W	This study
YDR162C	*NBP2*	+	+	S	This study
YDL006W	*PTC1*	W	W	NG	This study
YER118C	*SHO1*	+	+	W	This study
YCL032W	*STE50*	+	+	S	This study
E3. Components and associated proteins involved in the cell cycle progression					
YBR135W	*CKS1*[a]	NS	NS	NS	(Yu and Reed 2004)

ORF	Gene	I⁻ C⁻ 30°C	I⁻C⁺ 30°C	I⁻C⁺ 37°C	References
YAL040C	*CLN3*	+	+	W	This study
YBR133C	*HSL7*	+	+	S	This study
YLR079W	*SIC1*	+	W	S	This study
E4. Components and associated proteins involved in the TOR signaling pathway					
YMR068W	*AVO2*	+	+	S	This study
YKR007W	*MEH1*	+	+	S	This study
YIL105C	*SLM1*	+	+	S	This study
YBR077C	*SLM4*	+	+	W	This study
YJR066W	*TOR1*	+	+	S	This study
YDL077C	*VAM6*	+	+	W	This study
YMR104C	*YPK2*	W	W	W	This study
E5. Components and associated proteins involved in the calcineurin signaling pathway					
YKL190W	*CNB1*	VW	VW	W	This study
YNL307C	*MCK1*	+	VW	W	This study
YHR206W	*SKN7*	+	+	S	This study
E6. Components and associated proteins involved in the cAMP-PKA signaling pathway					
YER177W	*BMH1*	+	VW	S	This study
YOR371C	*GPB1*	+	+	W	This study
YAL056W	*GPB2*	+	+	S	This study
YMR016C	*SOK2*	+	+	S	This study
F. Lipid and glycerol metabolic pathways					
F1. Proteins involved in phospholipid metabolism					
YJL153C	*INO1*	S	S	S	(Culbertson and Henry 1975)
YDR123C	*INO2*	S	S	S	(Donahue and Henry 1981)
YOL108C	*INO4*	S	S	S	(Donahue and Henry 1981)
YML059C	*NTE1*	+	W	S	(Nunez et al. 2008)
YPR113W	*PIS1*[a]	NS	NS	NS	(Nikawa et al. 1987)
YER120W	*SCS2*	W	W	S	(Kagiwada et al. 1998)
YGL126W	*SCS3*	VW	S	S	(Hosaka et al. 1994)
F2. Proteins involved in glycerol metabolism					
YLL043W	*FPS1*	+	VW	NG	This study

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ORF	Gene	I^-C^- 30°C	I^-C^+ 30°C	I^-C^+ 37°C	References
YDL022W	*GPD1*	+	+	W	This study
YOL059W	*GPD2*	W	W	W	This study
YHR104W	*GRE3*	VW	W	S	This study
YBL011W	*SCT1*	VW	W	S	This study
YDR368W	*YPR1*	+	+	W	This study
F3. Proteins involved in phosphoinositides and inositol polyphosphates metabolism					
YDR173C	*ARG82*	S	S	NG	This study
YFR019W	*FAB1*	S	S	NG	This study
YDR017C	*KCS1*	W	W	S	This study
YKL212W	*SAC1*	S	S	S	(Whitters et al. 1993)
F4. Proteins involved in sphingolipids and ceramides metabolism					
YBR183W	*YPC1*	VW	+	W	This study
YDR297W	*SUR2*	+	+	W	This study
YJL134W	*LCB3*	VW	W	S	This study
YDR294C	*DPL1*	+	+	W	This study
YMR272C	*SCS7*	VW	W	VW	This study
F5. Proteins involved in sterols metabolism					
YMR202W	*ERG2*	W	W	S	This study
YLR056W	*ERG3*	W	W	S	This study
YGL012W	*ERG4*	+	W	S	This study
YMR015C	*ERG5*	+	+	S	This study
YML008C	*ERG6*	W	W	S	This study

Table 3 contains only the mutations conferring an Ino^- phenotype that were mentioned in the "Introduction", "Results" or "Discussion" sections, in the approximate order in which they are described in the text. A complete list of all mutants exhibiting inositol auxotrphy detected in this study or previously reported is contained in Table S1. Phenotypes were scored as follows: mutants that grew well on I^+C^- medium at a given temperature, but whose growth was visibly weaker on either I^-C^- or I^-C^+ media, were scored as very weak (*VW*). Mutants exhibiting very reduced but still detectable growth in I^-C^- or I^-C^+ media were scored as weak (*W*). Mutants exhibiting no visible growth on I^-C^- or I^-C^+ media were scored as strong (S) (See Fig. 1). A score of "+" indicates no growth reduction in any inositol lacking media, and compared to growth on I^+C^- medium at the corresponding temperature. A score of "NS" indicates that the mutant conferring the Ino^- phenotype was not present in the homozygous diploid strain collection, and hence was not screened. A score of "NG" (no growth) indicates that the deletion mutant from the homozygous diploid collection failed to grow in either I^+C^+, I^+C^- and/or YPD medium at a given temperature. *Underlined* gene names indicate deletions of essential genes for which conditional alleles have been reported to confer an Ino^- phenotype. References are provided for those mutations previously shown to confer an Ino^- phenotype

[a]Mutations previously shown to confer an Ino^- phenotype that were not confirmed in this study