DOI: 10.1534/genetics.106.057489

Genomic Analysis of the Opi⁻ Phenotype

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Manuscript received February 21, 2006
Accepted for publication March 28, 2006

ABSTRACT

Most of the phospholipid biosynthetic genes of *Saccharomyces cerevisiae* are coordinately regulated in response to inositol and choline. Inositol affects the intracellular levels of phosphatidic acid (PA). Opi1p is a repressor of the phospholipid biosynthetic genes and specifically binds PA in the endoplasmic reticulum. In the presence of inositol, PA levels decrease, releasing Opi1p into the nucleus where it represses transcription. The *opi1* mutant *o*ver*p*roduces and excretes *i*nositol into the growth medium in the absence of inositol and choline (Opi⁻ phenotype). To better understand the mechanism of Opi1p repression, the viable yeast deletion set was screened to identify Opi⁻ mutants. In total, 89 Opi⁻ mutants were identified, of which 7 were previously known to have the Opi⁻ phenotype. The Opi⁻ mutant collection included genes with roles in phospholipid biosynthesis, transcription, protein processing/synthesis, and protein trafficking. Included in this set were all nonessential components of the NuA4 HAT complex and six proteins in the Rpd3p–Sin3p HDAC complex. It has previously been shown that defects in phosphatidylcholine synthesis (*cho2* and *opi3*) yield the Opi⁻ phenotype because of a buildup of PA. However, in this case the Opi⁻ phenotype is conditional because PA can be shuttled through a salvage pathway (Kennedy pathway) by adding choline to the growth medium. Seven new mutants present in the Opi⁻ collection (*fun26*, *kex1*, *nup84*, *tps1*, *mrpl38*, *mrpl49*, and *opi10/yol032w*) were also suppressed by choline, suggesting that these affect PC synthesis. Regulation in response to inositol is also coordinated with the unfolded protein response (UPR). Consistent with this, several Opi⁻ mutants were found to affect the UPR (*yhi9*, *ede1*, and *vps74*).

TRANSCRIPTION of the phospholipid biosynthetic structural genes in *Saccharomyces cerevisiae* is regulated by inositol and choline (Paltauf *et al.* 1992; Greenberg and Lopes 1996; Henry and Patton-Vogt 1998; Carman and Henry 1999; Santiago and Mamoun 2003; Jesch *et al.* 2005). Gene expression is maximally repressed in the presence of inositol and choline and derepressed when they become limiting. This regulation requires the concerted effort of several transcription factors, including Ino2p, Ino4p, Opi1p, the Ume6p–Sin3p–Rpd3p histone deacetylase (HDAC) complex, the ISW2 and INO80 chromatin-remodeling complexes, and Mot1p (White *et al.* 1991; Ambroziak and Henry 1994; Nikoloff and Henry 1994; Jackson and Lopes 1996; Rundlett *et al.* 1996, 1998; Kadosh and Struhl 1997, 1998; Elkhaimi *et al.* 2000; Shen *et al.* 2000; Fazzio *et al.* 2001; Dasgupta *et al.* 2005). Ino2p and Ino4p belong to a family of bHLH regulatory proteins, which form a heterodimer that binds to a UAS_{INO} sequence to activate transcription of most phospholipid biosynthetic genes (*e.g.*, *INO1*, *CHO1*, *CHO2*, and *OPI3* in Figure 1) (Santiago and Mamoun 2003; Jesch *et al.* 2005). The Ume6p–Sin3p–Rpd3p HDAC complex, the ISW2 and INO80 chromatin-remodeling complexes, and Mot1p are global regulators that play a negative role in phospholipid biosynthetic gene expression (Jackson and Lopes 1996; Rundlett *et al.* 1996, 1998; Kadosh and Struhl 1997, 1998; Elkhaimi *et al.* 2000; Shen *et al.* 2000; Fazzio *et al.* 2001; Dasgupta *et al.* 2005). Opi1p was the first repressor found to specifically regulate the phospholipid biosynthetic pathway.

The *OPI1* locus was identified in a screen for mutants that *o*ver*p*roduce and excrete *i*nositol (Opi) into the growth medium in the absence of inositol and choline (Opi⁻ phenotype) (Greenberg *et al.* 1982). The Opi⁻ phenotype correlates with constitutive overexpression of the *INO1* gene (Hirsch and Henry 1986), which is responsible for *de novo* synthesis of inositol (Figure 1) (Culbertson and Henry 1975). Therefore it is not surprising that several other mutants defective in repression of *INO1* also have an Opi⁻ phenotype. These include mutations in the *UME6*, *SIN3*, and *RPD3* global repressor genes (Elkhaimi *et al.* 2000). The link between regulation of *INO1* gene expression and the Opi⁻ phenotype is further supported by the existence of the dominant *OPI5+* mutant allele (Swift and McGraw 1995). This mutant is a deletion of the *INO1* promoter (renamed the *INO1-100* allele), which yields constitutive derepressed expression of the *INO1* gene (Swift and McGraw 1995).

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Recently, our understanding of the role of Opi1p in the repression of phospholipid biosynthetic gene expression has improved dramatically. Opi1p is tethered in the endoplasmic reticulum (ER) by Scs2p, an integral membrane protein (Gavin *et al.* 2002; Kagiwada and Zen 2003; Loewen *et al.* 2003, 2004; Loewen and Levine 2005). Repression in response to inositol and choline is mediated by the level of phosphatidic acid (PA) (Figure 1). In the absence of inositol, PA levels are elevated and Opi1p binds PA (Loewen *et al.* 2004). When inositol is added, PA levels decrease, releasing Opi1p from the ER. Opi1p then translocates to the nucleus where it inhibits transcription by presumably interacting with the Ino2p transcriptional activator (Wagner *et al.* 2001; Gardenour *et al.* 2004; Heyken *et al.* 2005). The addition of choline by itself has little effect on PA levels. However, in combination with inositol, choline further reduces PA levels, resulting in additional repression (Henry and Patton-Vogt 1998). Not surprisingly, blocks in *de novo* phosphatidylcholine (PC) biosynthesis that elevate PA levels also yield an Opi$^-$ phenotype (Klig *et al.* 1988; Summers *et al.* 1988; McGraw and Henry 1989; Shen and Dowhan 1996). Thus, *cds1*, *cho2*, and *opi3* mutants all have the Opi$^-$ phenotype (Figure 1). However, the Opi$^-$ phenotype of these mutants is conditional because it can be suppressed by adding choline (C) to the medium. Choline restores PC synthesis through a salvage pathway (Kennedy pathway), thereby alleviating the accumulation of PA caused by the block in the *de novo* PC pathway (Figure 1) (Henry and Patton-Vogt 1998).

Opi1p function is also regulated by post-translational modifications. Opi1p is phosphorylated at Ser26 by protein kinase C (Sreenivas *et al.* 2001), at Ser31 and Ser251 by protein kinase A (Sreenivas and Carman 2003), and at Ser10 by casein kinase II (Chang and Carman 2006). Mutations at each of these phosphorylation sites show that phosphorylation of Opi1p plays a role in regulation of target gene expression in response to inositol.

Recently, it has been shown that under derepressing conditions the *INO1* promoter is recruited to the nuclear membrane where Ino4p and Ino2p activate its transcription (Brickner and Walter 2004). Under repressing conditions, Opi1p translocates to the nucleus and presumably interacts with Ino2p (Wagner *et al.* 2001; Gardenour *et al.* 2004; Heyken *et al.* 2005). The interaction with Ino2p is suggested by *in vitro* experiments (Wagner *et al.* 2001; Heyken *et al.* 2005) and the existence of dominant *INO2*^{c} mutations that yield an Opi$^-$ phenotype (Gardenour *et al.* 2004; Heyken *et al.* 2005).

Phospholipid biosynthesis is coordinated with the unfolded protein response (UPR) (Cox *et al.* 1997; Chang *et al.* 2002; Jesch *et al.* 2005). The UPR is initiated in the ER in response to the accumulation of unfolded proteins (Patil and Walter 2001; Schroder and Kaufman 2005). The UPR can also be induced by depletion of inositol (Cox *et al.* 1997; Chang *et al.* 2002). Upon UPR induction, Ire1p is activated, initiating splicing of *HAC1* mRNA (Sidrauski and Walter 1997). The properly spliced *HAC1* transcript produces the Hac1p basic leucine zipper transcription factor that binds to the UPR element (UPRE) of target genes such as *KAR2* but also regulates UAS$_{INO}$-containing promoters by counteracting the function of Opi1p (Cox and Walter 1996).

Recent studies show that screening the viable yeast deletion set (VYDS) (Winzeler *et al.* 1999; Giaever *et al.* 2002) can yield valuable insight into well-studied processes such as regulation in response to phosphate concentration (Huang and O'Shea 2005). To further understand the repression of phospholipid biosynthesis, the VYDS was screened to identify mutants that display the Opi$^-$ phenotype. These mutants were characterized with respect to the severity of the phenotype, dependence on *de novo* PC biosynthesis, and defects in the UPR.

MATERIALS AND METHODS

Yeast strains, media, and growth conditions: Yeast strains used in this study include the VYDS in the BY4742 background (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) (Winzeler *et al.* 1999; Giaever *et al.* 2002) and BRS1005 (AID-1) (*MAT***a**/*MAT*α, *ade1/ade1, ino1-13/ino1-13*) (White *et al.* 1991). Yeast cultures were grown at 30° in complete synthetic medium (Kelly and Greenberg 1990) containing 2% glucose (w/v) but lacking inositol and choline (I−C−). Where indicated, I−C− medium was supplemented with 75 μM inositol (I+) ± 1 mM choline (C+), 1 mM ethanolamine (E+), 1 mM monomethylethanolamine (MME+), or 1 mM dimethylethanolamine (DME+). X-gal medium was prepared as previously described (Hudak *et al.* 1994) and supplemented with tunicamycin (Tm) (1 μg/ml) to induce the UPR (Cox and Walter 1996).

Plasmids: Plasmid pJC104 containing four copies of the UPRE upstream of the *CYC1-lacZ* reporter was a gift from Peter Walter (Howard Hughes Medical Institute, University of California, San Francisco) (Cox and Walter 1996). Plasmid pJH330 has been previously described (Elkhaimi *et al.* 2000) and contains 543 bp of the *INO1* promoter and 132 codons of the *INO1* ORF fused in frame to the *lacZ* reporter in YEp357R (Myers *et al.* 1986). The *OPI1-cat* reporter plasmid (pMK162) has been described (Kaadige and Lopes 2003). A 7.2-kb *Sst*II–*Cla*I fragment containing the *OPI1-cat* reporter (525 bp of the *OPI1* promoter) was excised from pMK162 and used to transform relevant yeast strains. This results in single-copy integration at the *GAL4* locus. Yeast transformations were performed using the YEASTMAKER transformation system 2 (BD Biosciences Clontech, Mountain View, CA).

Opi$^-$ phenotype plate assay: The Opi$^-$ phenotype was assessed by a cross-feeding assay using an inositol auxotrophic strain. The original assay (Greenberg *et al.* 1982; McGee *et al.* 1994) was used to screen the VYDS. Briefly, the *ino1* homozygous diploid tester strain BRS1005 was spread on I−C− medium. Strains from the VYDS were pinned at a density of 48 strains per plate. Inositol excretion by the VYDS strains results in a ring of growth of the BRS1005 tester strain.

A second round of screening tested each of the Opi$^-$ mutants individually. Each Opi$^-$ mutant strain was streaked horizontally on a plate containing I−C− medium. After 3 days of growth at 30°, the tester strain BRS1005 was streaked perpendicularly to the Opi$^-$ strain. Inositol excretion results in growth of the BRS1005 tester strain (McGee *et al.* 1994).

TABLE 1

Yeast mutants with an Opi⁻ phenotype

Biological process/function (no. of mutants)	Mutants
Phospholipid biosynthesis (6)	*cho2*, *erg28*, *itr1*, *lem3*, *opi3*, *opi1*
Transcription: NuA4 HAT (5)	*eaf3*, *eaf5*, *eaf7*, *vid21*, *yaf9*
Transcription: HDAC (6)	*dep1*, *pho23*, *rpd3*, *rxt2*, *sin3*, *ume6*
Transcription (1)	*reg1*
Protein processing/synthesis (7)	*alg9*, *kex1*, *lip2*, *mrpl38*, *mrpl49*, *rts1*, *ysp3*
Trafficking: AP-3 complex (5)	*apl5*, *apl6*, *apm3*, *aps3*, *vps41*
Trafficking (38)	*age2*, *bsd2*, *did4*, *ede1*, *pep3*, *pep5*, *pep7*, *pep8*, *snf7*, *snf8*, *srn2*, *stp22*, *vam6*, *vps3*, *vps4*, *vps5*, *vps8*, *vps9*, *vps13*, *vps16*, *vps17*, *vps21*, *vps24*, *vps25*, *vps27*, *vps28*, *vps29*, *vps30*, *vps34*, *vps35*, *vps36*, *vps38*, *vps51*, *vps61*, *vps66*, *vps74*, *ypt6*, *ypt7*
Miscellaneous (12)	*arc18*, *ckb2*, *mtq1*, *mub1*, *mud1*, *nup84*, *ras2*, *rgp1*, *tna1*, *tps1*, *ubc13*, *yhi9*
Unknown (9)	*fun26*, *ldb16*, *ycl006c*, *ydl096c*, *ydr360w*, *ykr035c*, *ylr338w*, *yol032w*, *ypr044c*

Enzyme assays: β-Galactosidase and chloramphenicol acetyltransferase (Cat) assays were performed as described previously (Lopes *et al.* 1991; Kaadige and Lopes 2006). Units of β-galactosidase activity = (OD_{420}/minute/milligram total protein) × 1000. Units of Cat activity = percentage of conversion of ^{14}C-chloramphenicol/microgram total protein/time (in hours) (Griggs and Johnston 1991).

RESULTS

A genomewide screen identified 89 Opi⁻ mutants: Three versions of the Opi test have been reported (Greenberg *et al.* 1982; Klig *et al.* 1988; McGee *et al.* 1994). The original Opi⁻ screen was performed by replica plating yeast, mutagenized with ethyl methane sulfonate, onto synthetic medium lacking inositol that had been spread with a lawn of the AID-1 indicator strain. AID-1 is an inositol auxotroph (*ino1-13*) that also harbors the *ade1* marker, which allows identification of inositol-excreting mutants by a red halo of growth around the mutant strain. Four complementation groups were originally identified, *OPI1–OPI4* (Greenberg *et al.* 1982). While the *OPI1* and *OPI3* genes have been studied extensively, the *opi2* and *opi4* mutants were not further investigated. Subsequent genetic screens identified several other Opi⁻ mutants, including *cdg1* (*CDS1* gene) (Klig *et al.* 1988), *cho2* (Summers *et al.* 1988), *ume6*, *rpd3*, *sin3* (Elkhaimi *et al.* 2000), and *reg1* (Ouyang *et al.* 1999).

The VYDS mutant collection was screened for the Opi⁻ phenotype using the original version of the Opi⁻ test. The VYDS collection was pinned onto plates lacking inositol and choline that were spread with a suspension of the BRS1005 (AID-1) indicator strain. It was necessary to pin the collection at a density of 48 mutants per plate to create sufficient separation between strains. This screen identified 218 Opi⁻ mutants. However, in some cases, the red halo of growth covered several adjacent mutant strains, obscuring which strains actually displayed the Opi⁻ phenotype. Thus, a second screen was conducted using a more recent adaptation of the Opi test (McGee *et al.* 1994). Each of the 218 Opi⁻ mutants were individually streaked onto plates with medium lacking inositol and choline and incubated at 30° for 3 days. A suspension of the BRS1005 tester strain was streaked perpendicularly to the mutant and incubated for an additional 3 days. This second screen reduced the number of Opi⁻ mutants to 89 (Table 1).

It is important to note that inositol auxotrophic strains starved for inositol die, a phenomenon termed "inositol-less death" (Henry *et al.* 1977). Consequently, spreading the BRS1005 strain on medium lacking inositol as was done here, and in the original screen (Greenberg *et al.* 1982), could have affected its viability. However, there is a window of opportunity, lasting approximately one generation in liquid medium, where cells survive and continue to divide normally (Henry *et al.* 1977). Nevertheless, reduced viability of the BRS1005 indicator strain may have precluded identification of mutants with a weak Opi⁻ phenotype.

As expected, the VYDS collection screen identified *opi1*, *cho2*, *opi3*, *ume6*, *rpd3*, *sin3*, and *reg1* (Table 1). Several previously isolated Opi⁻ mutants were not identified for predictable reasons. *CDS1* is involved in synthesizing cytidine diphosphate–diacylglycerol (CDP–DAG) from PA (Figure 1) and was originally defined by a point mutation (*cdg1*) that displayed the Opi⁻ phenotype (Klig *et al.* 1988; Shen and Dowhan 1996). However, the *cds1* null allele is inviable (Shen *et al.* 1996) and therefore not present in the VYDS. *CHO1* converts CDP–DAG and serine to phosphatidylserine (PS) (Figure 1) and displays the Opi⁻ phenotype under the conditions tested here, but the *cho1* mutant was not present in the VYDS. *PSD1* (Clancey *et al.* 1993; Trotter *et al.* 1993) and *PSD2* (Trotter *et al.* 1995) are involved with decarboxylating PS to phosphatidylethanolamine (PE) (Figure 1). These genes encode redundant functions and therefore only the double mutant would be expected to yield the Opi⁻ phenotype. *MOT1* and *SMP2* have also recently been shown to be negative regulators of

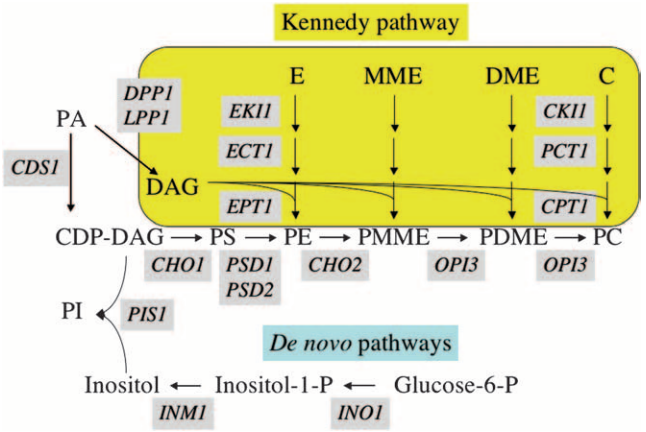

Figure 1.—*S. cerevisiae* phospholipid biosynthetic pathway. CDP–DAG is the common precursor for the synthesis of phosphatidylinositol (PI) and PC. PI is made *de novo* from glucose-6-P but can also be synthesized from exogenously supplied inositol. PC is synthesized *de novo* from CDP–DAG via a series of methylation reactions carried out by the products of the *CHO2* and *OPI3* genes. Alternatively, PC can be made via a salvage pathway (Kennedy pathway; highlighted in yellow to facilitate discussion) when E, MME, DME, or C is supplied exogenously. Genes encoding the biosynthetic enzymes are depicted in gray boxes.

INO1 expression (Dasgupta *et al.* 2005; Santos-Rosa *et al.* 2005), suggesting that they may have an Opi$^-$ phenotype. However, *MOT1* is an essential gene and the *smp2* mutant was not present in the VYDS.

The Opi$^-$ mutant collection identified several biological functions: The VYDS contains 4827 strains. Of the 4827 deletion strains, 89 (~1.8%) displayed the Opi$^-$ phenotype (Table 1). Of the 89 Opi$^-$ phenotype mutants, ~90% of the genes have been assigned to a particular biological process (http://db.yeastgenome.org/cgi-bin/go/gotermmapper) (Figure 2). We used the GoTermMapper program to determine if any of the functional categories of genes were overrepresented in the Opi$^-$ mutant set. There was an overrepresentation of Opi$^-$ mutants in vesicle-mediated transport (30% observed, 4% expected), transport (53% observed, 15% expected), transcription (15% observed, 7% expected), protein modification (11% observed, 7% expected), protein catabolism (12% observed, 3% expected), organelle organization and biogenesis (37% observed, 14% expected), and lipid metabolism (7% observed, 3% expected) (Figure 2).

The VYDS screen also identified two transcription factor complexes. The screen identified seven components of the Rpd3p–Sin3p HDAC. Deletions of *RPD3*, *SIN3*, and *UME6* genes have been previously shown to yield an Opi$^-$ phenotype (Elkhaimi *et al.* 2000). The VYDS screen identified four more components of the HDAC complex: Pho23p (Loewith *et al.* 2001), Eaf3p (Reid *et al.* 2004; Gavin *et al.* 2006), Dep1p, and Rxt2p gene (Carrozza *et al.* 2005) (Table 1, Figure 3A). However, the HDAC complex has recently been shown to include six other factors not uncovered in the Opi$^-$ screen. These include Ash1p, Ume1p, Sap30p, Cti6p, Sds3p, and Rxt3p (Ho *et al.* 2002; Reid *et al.* 2004; Carrozza *et al.* 2005; Gavin *et al.* 2006). Thus, we tested mutants in the genes that encode each of these factors independently. This revealed that the *ume1* and *sap30* mutant strains also yielded the Opi$^-$ phenotype (Figure 3B) while the remaining four mutant strains did not (data not shown). The weak Opi$^-$ phenotype of the *ume1* and *sap30* mutant strains may have been the reason that these were not identified originally (Figure 3B). These two additional Opi$^-$ mutants increased the total number of Opi$^-$ mutants to 91.

In addition to the HDAC complex mutants, the screen also identified all of the nonessential components of the NuA4 histone acetyltransferase (HAT)

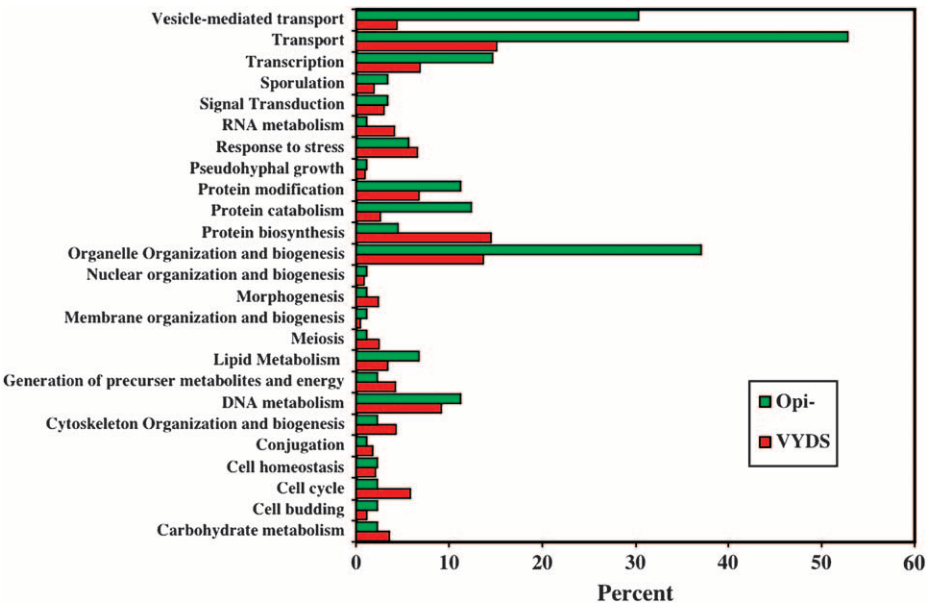

Figure 2.—Comparison of the relative percentage of genes in functional categories for the VYDS and the Opi$^-$ screen. The data were obtained using the GoTermMapper program (http://db.yeastgenome.org/cgi-bin/GO/goTermMapper). Green bars reflect the percentage of genes in the Opi$^-$ mutant set in each of the functional categories while red bars reflect the expected percentages of genes in the VYDS in each of the functional categories. Note that the percentage of genes in the VYDS category was modified from the on-line data set to include only nonessential genes.

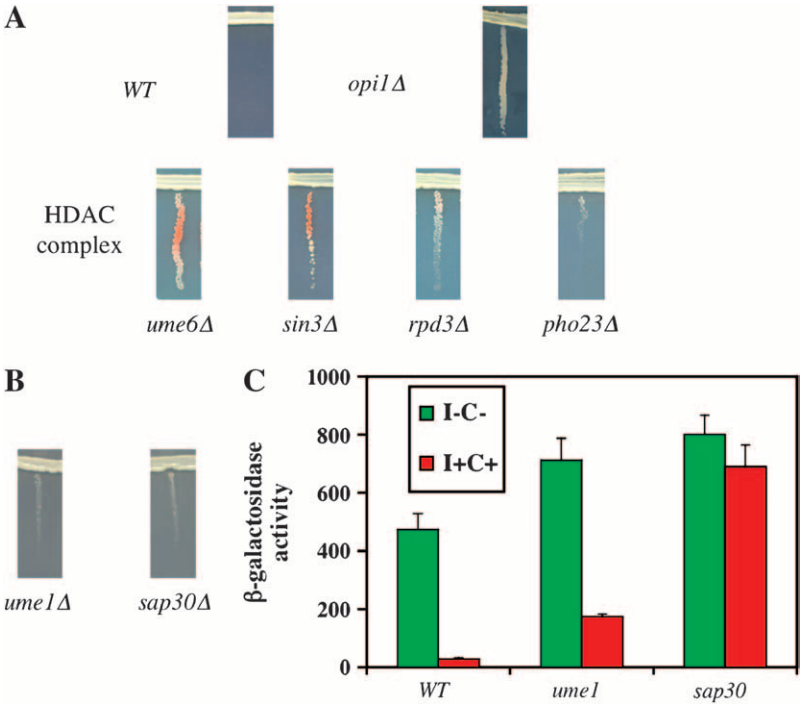

FIGURE 3.—Opi⁻ phenotype of mutants in a HDAC chromatin modification complex. (A) A wild-type strain (BY4742) and strains containing mutations in the *OPI1* gene and members of the Rpd3 HDAC (*UME6*, *SIN3*, *RPD3*, and *PHO23*) complex were grown on medium lacking inositol and choline for 72 hr at 30°. The tester strain BRS1005 was streaked perpendicularly to mutant strains and incubated at 30° for an additional 72 hr. (B) The *ume1*Δ and *sap30*Δ mutants were also tested for the Opi⁻ phenotype because these were recently found to be members of the same HDAC complex (CARROZZA *et al.* 2005; GAVIN *et al.* 2006). (C) The *ume1*Δ and *sap30*Δ mutants altered regulation of the *INO1-lacZ* reporter. Strains were grown in I−C− (derepressing; green bars) and I+C+ (repressing; red bars) conditions. Each bar is the mean of at least three independent transformants.

complex: *EAF3*, *EAF5*, *EAF7*, *VID21*, and *YAF9* (Table 1; Figure 4, A and B). This is curious since the NuA4 complex has HAT activity, which is responsible for increased gene expression. Thus, mutations in this complex should yield reduced expression of the target genes. One possible explanation for the Opi1 phenotype is that the NuA4 complex may act indirectly on *INO1* expression by increasing *OPI1* expression. This was a reasonable assumption since altered expression of *OPI1* has been shown to affect target gene expression (WAGNER *et al.* 1999). To test this possibility, we assayed the effect of several NuA4 component mutants on expression of an *OPI1-cat* reporter (Figure 4C). As reported, the expression of the *OPI1-cat* reporter was repressed twofold by the addition of inositol and choline (JIRANEK *et al.* 1998; WAGNER *et al.* 1999; KAADIGE and LOPES 2003, 2006). However, the NuA4 component mutants did not yield reduced expression of the *OPI1-cat* reporter. Instead, they yielded elevated expression under repressing conditions (Figure 4C). That is, they have the same effect on *OPI1-cat* as they have on *INO1* expression. Thus, the phenotype of the NuA4 mutants is not due to an indirect effect of reduced *OPI1* expression.

The trafficking category included a large number of *v*acuolar *p*rotein *s*orting (*vps*) mutants, including the four components of the AP-3 adaptor protein complex, encoded by the *APL5*, *APL6*, *APM3*, and *APS3* genes (ODORIZZI *et al.* 1998; BOWERS and STEVENS 2005) (Table 1). The AP-3 adaptor complex is involved in

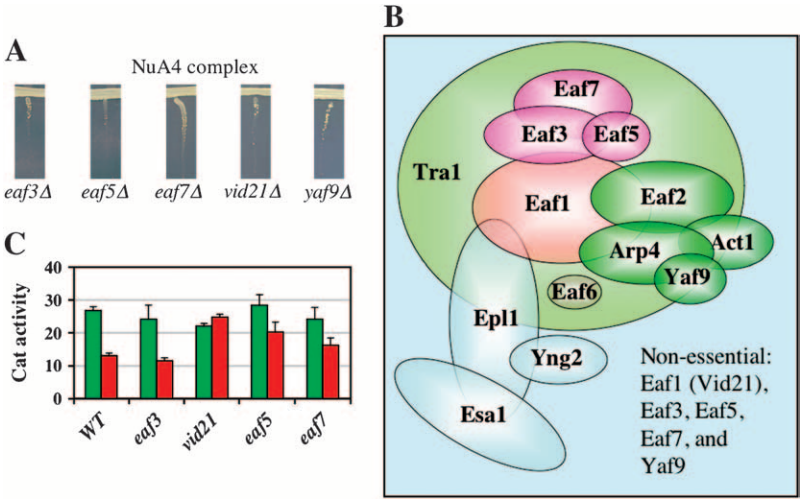

FIGURE 4.—Opi⁻ phenotype of mutants in the NuA4 chromatin-remodeling complexes. (A) Strains containing mutations in members of the NuA4 HAT complex (*EAF3*, *EAF5*, *EAF7*, *VID21*, and *YAF9*) were grown on medium lacking inositol and choline for 72 hr at 30°. The tester strain BRS1005 was streaked perpendicularly to mutant strains and incubated at 30° for an additional 72 hr. Refer to Figure 3A for wild-type strain control. (B) Depiction of the NuA4 complex (reproduced from DOYON and COTÉ 2004) listing the nonessential components. (C) Effect of the NuA4 Opi⁻ mutant on expression of an *OPI1-cat* reporter. Strains were grown in I−C− (derepressing; green bars) and I+C+ (repressing red bars) conditions. Each bar is the mean of at least three independent transformants.

TABLE 2

X-gal I+C+ (repressing) plate phenotype of Opi⁻ mutants containing the *INO1-lacZ* reporter

Dark blue (24)	*dep1, ldb16, opi1, pep7, pho23, reg1, rxt2, sin3, snf7, srn2, tna1, ume6, vps4, vps21, vps25, vps27, vps28, vps30, vps36, vps41, yhi9, ypt7, ycl006c, ylr338w*
Medium blue (35)	*alg9, apl5, apl6, aps3, bsd2, did4, eaf5, eaf7, ede1, itr1, mub1, mud1, pep3, pep5, pep8, rgp1, rpd3, rts1, stp22, vam6, vid21, vps3, vps5, vps8, vps9, vps16, vps17, vps29, vps34, vps35, vps38, vps51, vps61, vps66, yaf9*
Light blue/white (30)	*age2, apm3, arc18, cho2, ckb2, eaf3, erg28, fun26, kex1, lem3, lip2, mrpl38, mrpl49, mtq1, nup84, opi3, ras2, snf8, tps1, ubc13, vps13, vps24, vps74, ypt6, ysp3, ydl096c, ydr360w, ykr035c, yol032w, ypr044c*

clathrin-independent transport of proteins (such as alkaline phosphatase, Vam3p, and Yck3p) from the late Golgi to the vacuole (Odorizzi *et al.* 1998; Sun *et al.* 2004; Bowers and Stevens 2005). The product of the *VPS41* gene associates with the AP-3 complex and is required for the formation of AP-3 vesicles from the late Golgi (Darsow *et al.* 2001; Bowers and Stevens 2005). The *vps41* mutant was also identified in the VYDS screen (Table 1).

The Opi⁻ mutants affected regulation of *INO1-lacZ* expression: The Opi⁻ phenotype of previously defined Opi⁻ mutants correlates with a defect in the repression of *INO1* or *INO1-lacZ* expression (Summers *et al.* 1988; McGraw and Henry 1989; White *et al.* 1991; Shen and Dowhan 1997; Ouyang *et al.* 1999; Elkhaimi *et al.* 2000). However, the mechanism for inositol excretion remains unknown; thus it was reasonable to predict that some mutants from the VYDS screen might affect inositol excretion but not repression of *INO1* expression. To test for this, an *INO1-lacZ* reporter (pJH330) was transformed into the Opi⁻ mutant strains. Transformants were assayed on X-gal plates under derepressing (I−C−) and repressing (I+C+) conditions.

Indeed, the results revealed that a significant number of the Opi⁻ mutants did not affect repression of *INO1-lacZ* expression (Table 2). As expected, the wild-type transformants grew as white colonies on X-gal I+C+ medium (repressing). In contrast, the *opi1* strain grew as dark-blue colonies, indicating that *INO1-lacZ* was constitutively expressed. The 89 Opi⁻ mutants were plated in triplicate on X-gal I+C+ medium and categorized according to severity of the phenotype (Table 2). Dark blue indicates a strong phenotype similar to that of an *opi1* mutant strain. Medium blue suggests an intermediate effect on the *INO1-lacZ* reporter, and light blue/white indicates weak or no effect on repression of *INO1-lacZ*. The assay identified 24 Opi⁻ mutants that grew as dark blue colonies, 35 with a medium-blue phenotype, and 30 displayed a light-blue/white phenotype (Table 2).

To quantify the effect on *INO1-lacZ* expression, the *INO1-lacZ* transformants were grown in I−C− (derepressing) and I+C+ (repressing) media and assayed for β-galactosidase activity (Figure 5, A and B, respectively; see also supplemental data at http://www.genetics.org/supplemental/). In I−C− conditions, the wild-type strain yielded 475 units of β-gal activity (yellow bar in Figure 5A). Most of the Opi⁻ mutants displayed *INO1-lacZ* expression levels comparable to the wild-type strain (Figure 5A). Thirteen mutants yielded levels of expression that were more than twofold the level of the wild-type strain while 17 mutant strains yielded levels of expression that were <50% of wild type. In I+C+ conditions, the wild-type strain yielded 28 units of β-gal activity (yellow bar in Figure 5B). The strains that grew as dark-blue colonies on X-gal I+C+ medium (Table 2) generally yielded more β-gal activity than the wild-type strain (red bars in Figure 5B). The Opi⁻ mutants that grew as medium-blue colonies on X-gal I+C+ medium (Table 2) yielded intermediate β-gal activity levels (blue bars in Figure 5B). Likewise, the Opi⁻ mutants that grew as light-blue/white colonies on X-gal I+C+ medium (Table 2) tended to yield decreased β-gal activity levels (open bars in Figure 5B). In addition, the two HDAC mutants not identified in the VYDS screen were also assayed and found to be defective in repression (Figure 3C).

In general, there was a good correlation between the plate phenotype and the quantitative β-gal assay. However, this need not have been the case since *INO1* expression is also growth phase regulated (Lamping *et al.* 1995; Jiranek *et al.* 1998; Robinson *et al.* 2000). Thus, those mutants whose X-gal plate and β-gal activity phenotypes do not correlate may reflect altered growth-phase regulation. Nevertheless, the data clearly showed that some of the Opi⁻ mutants did not appear to affect *INO1-lacZ* repression. In some cases (*cho2* and *opi3*), the absence of a defect in *INO1-lacZ* repression was predictable because defects in PC biosynthesis yield an Opi⁻ phenotype that can be suppressed by the addition of choline (Summers *et al.* 1988; McGraw and Henry 1989). Thus, some of the other Opi⁻ mutants may also affect PC biosynthesis and be similarly suppressed by choline. This was found to be the case and is described below.

Seven new Opi⁻ mutants were suppressed by growth in choline: PC synthesis *de novo* proceeds from PA to CDP–DAG to PC and requires three sequential methylation reactions (Figure 1). As stated above, PA levels control the translocation of Opi1p from the ER into the nucleus (Loewen *et al.* 2004). Thus, blocks in *de novo*

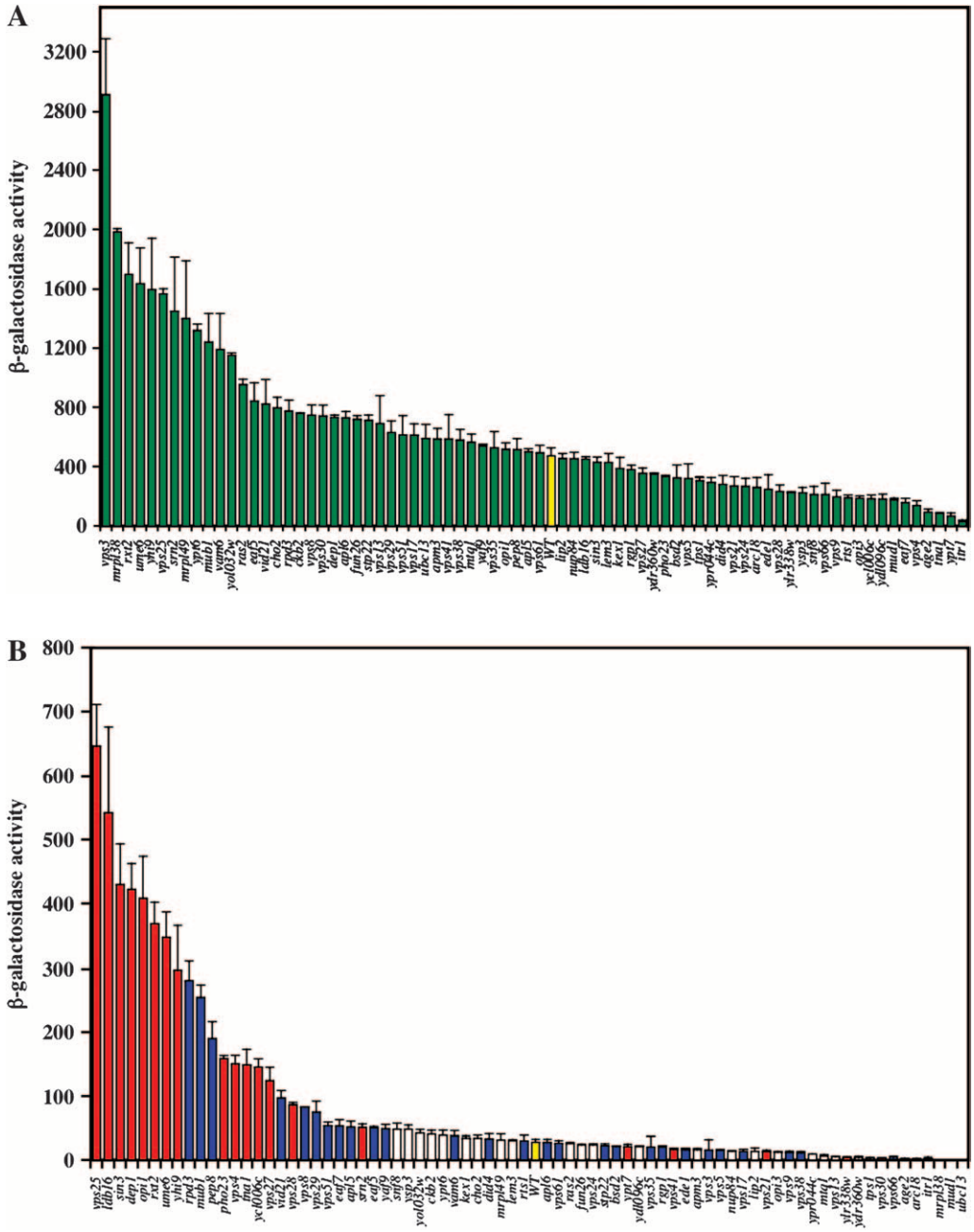

FIGURE 5.—Opi⁻ mutants altered regulation of the *INO1-lacZ* reporter. (A) Opi⁻ mutants grown in I−C− (derepressing) conditions. The yellow bar highlights the wild-type strain. (B) Opi⁻ mutants grown in I+C+ (repressing) conditions. The yellow bar again highlights the wild-type strain. Red bars represent mutants that grew as dark blue on the X-gal plate assay (Table 2). Blue bars and open bars represent mutants that grew as medium blue and white/light blue, respectively, on the X-gal plate assay (Table 2). Each bar is the mean of at least three independent transformants. Note that 75 mutants were assayed. The remaining 14 mutants either did not grow or grew poorly under the conditions tested here. These are listed in the supplemental data at http://www.genetics.org/supplemental/.

synthesis of PC yield an Opi⁻ phenotype due to elevated PA levels. However, the Opi⁻ phenotype of these mutants can be suppressed by adding the appropriate supplement (E, MME, DME, or C) that restores PC synthesis through the Kennedy (salvage) pathway (SUMMERS *et al.* 1988; MCGRAW and HENRY 1989) (Figure 1). Thus, a *cho2* mutant has an Opi⁻ phenotype in I− medium and the phenotype is retained in the presence of E but suppressed by the addition of MME, DME, and C (Table 3 and Figure 6) (SUMMERS *et al.* 1988).

To determine if any of the 89 Opi⁻ mutants might also affect PC biosynthesis, the Opi⁻ test was performed on medium lacking inositol but containing choline. The Opi⁻ phenotype of 9 mutants was suppressed by the addition of choline (Table 3). These mutants were retested on medium lacking inositol but supplemented with E, MME, or DME (Figure 6) to determine if they affected a specific biochemical step in *de novo* PC biosynthesis. The addition of E, MME, and DME suppressed the Opi⁻ phenotype of the *mrpl38*, *mrpl49*, and *yol032w* mutant strains (Table 3). As expected, MME and DME suppressed the Opi⁻ phenotype of the *cho2* mutant strain (Table 3 and Figure 6) (SUMMERS *et al.* 1988) but also suppressed the *fun26*, *kex1*, and *nup84* mutant strains (Table 3 and Figure 6). As previously reported, the *opi3* mutant was suppressed by the addition of DME (MCGRAW and HENRY 1989) (Table 3 and Figure 6). The addition of DME to an *opi3* mutant suppresses the

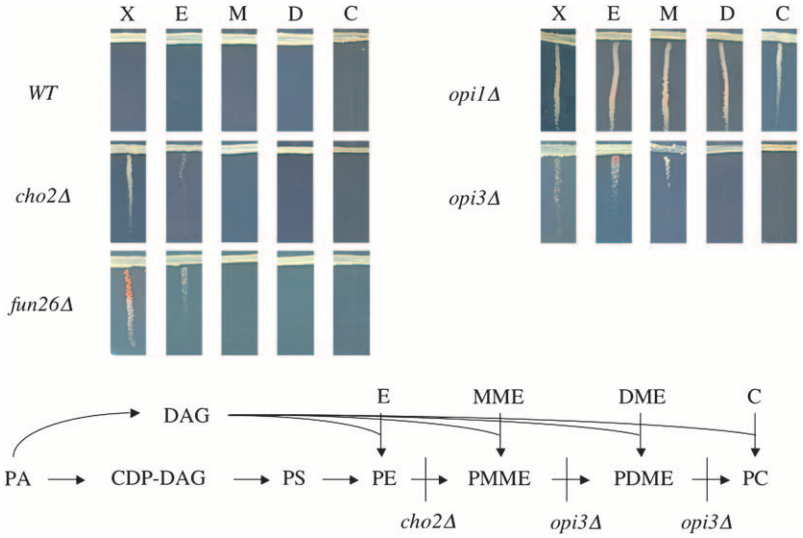

FIGURE 6.—Kennedy pathway utilization suppressed Opi⁻ mutants defective in *de novo* synthesis of PC. The Kennedy pathway utilizes E, MME (or M), DME (or D), and C to synthesize PE, PMME, PDME, and PC, respectively. The Opi⁻ phenotype assay was performed on medium lacking inositol and supplemented with E, M, D, or C. A wild-type strain (BY4742) and an *opi1*Δ strain were used as controls for absence of the Opi⁻ phenotype and an unconditional Opi⁻ phenotype.

Opi⁻ phenotype because it allows for the synthesis of high levels of phosphatidyldimethylethanolamine (PDME) (McGraw and Henry 1989). This is apparently sufficient to relieve the buildup of PA in the *opi3* mutant. The *tps1* mutant was also suppressed by the addition of DME (Table 3). Thus, seven new Opi⁻ mutants likely affect *de novo* synthesis of PC.

The Opi⁻ plate assay is obligatorily performed in medium lacking inositol. However, the ability of PC branch precursors (E, MME, DME, and C) to suppress the Opi⁻ phenotype can also be observed in repressing conditions. For example, blocks in *de novo* PC biosynthesis are defective in inositol-mediated repression of the *INO1* gene and its protein product; however, this defect is suppressed by addition of PC branch precursors (Letts and Henry 1985; Summers *et al.* 1988; McGraw and Henry 1989; Shen and Dowhan 1997). To determine if this property applied to any of the new Opi⁻ mutants, we chose two mutants that affect PC biosynthesis (*fun26 and yol032w*) and tested them for defects in *INO1-lacZ* regulation (Figure 7). A wild-type strain and *cho2*, *opi3*, *fun26*, *and yol032w* mutant strains containing an *INO1-lacZ* reporter (pJH330) were assayed for β-galactosidase activity. Strains were grown in the absence (I−) and presence (I+) of inositol ± E (E+), MME (MME+), DME (DME+), or C (C+). As expected, in the wild-type strain, *INO1-lacZ* expression was derepressed in all five media lacking inositol (Figure 7A). The presence of inositol alone reduced *INO1-lacZ* expression, which was further repressed when supplemented with E, MME, DME, or C (Figure 7A). These results were consistent with those previously reported for regulation of *INO1*, *INO1-lacZ*, and Ino1p levels (Hirsch and Henry 1986; Summers *et al.* 1988; McGraw and Henry 1989; Lopes *et al.* 1991).

TABLE 3

Opi⁻ phenotype of mutants affected by the Kennedy pathway

Genotype	I−[a]	E+	MME+	DME+	C+
Wild type	−[b]	−	−	−	−
opi1	+	+	+	+	+
mrpl38	+	−	−	−	−
mrpl49	+	−	−	−	−
yol032w	+	−	−	−	−
cho2	+	+	−	−	−
fun26	+	+	−	−	−
kex1	+	+	−	−	−
nup84	+	+	−	−	−
opi3	+	+	+	−	−
tps1	+	+	+	−	−

[a] All five growth conditions include complete synthetic medium lacking inositol.

[b] "+" indicates inositol excretion. "−" indicates no inositol excretion, *i.e.*, a wild-type phenotype.

The Opi⁻ phenotype of the *cho2* and *fun26* mutants was suppressed by addition of MME, DME, and C (Figure 6 and Table 3). Consistent with this plate phenotype, expression of *INO1-lacZ* was derepressed in all five I− media as well as in I+ and I+E+ media (Figure 7, B and D). As predicted from the plate phenotype, repression of *INO1-lacZ* expression was restored in I+ media supplemented with MME, DME, and C (Figure 7, B and D). The Opi⁻ phenotype of the *opi3* mutant was suppressed by the addition of DME and C (Figure 6 and Table 2). Consistent with this pattern, *INO1-lacZ* expression was derepressed in all five I− media as well as in I+, I+E+, and I+MME+ media (Figure 7C). As expected, repression of *INO1-lacZ* was restored in I+DME+ and I+C+ media (Figure 7C). The Opi⁻ phenotype of *yol032w* mutant was suppressed by the addition of E (Table 3). *INO1-lacZ* expression was derepressed in all five I− media at a level approximately twofold greater than that observed in the wild-type strain (compare Figure 7E to 7A) and partially repressed in I+ media. Complete repression was restored in I+E+, I+MME+,

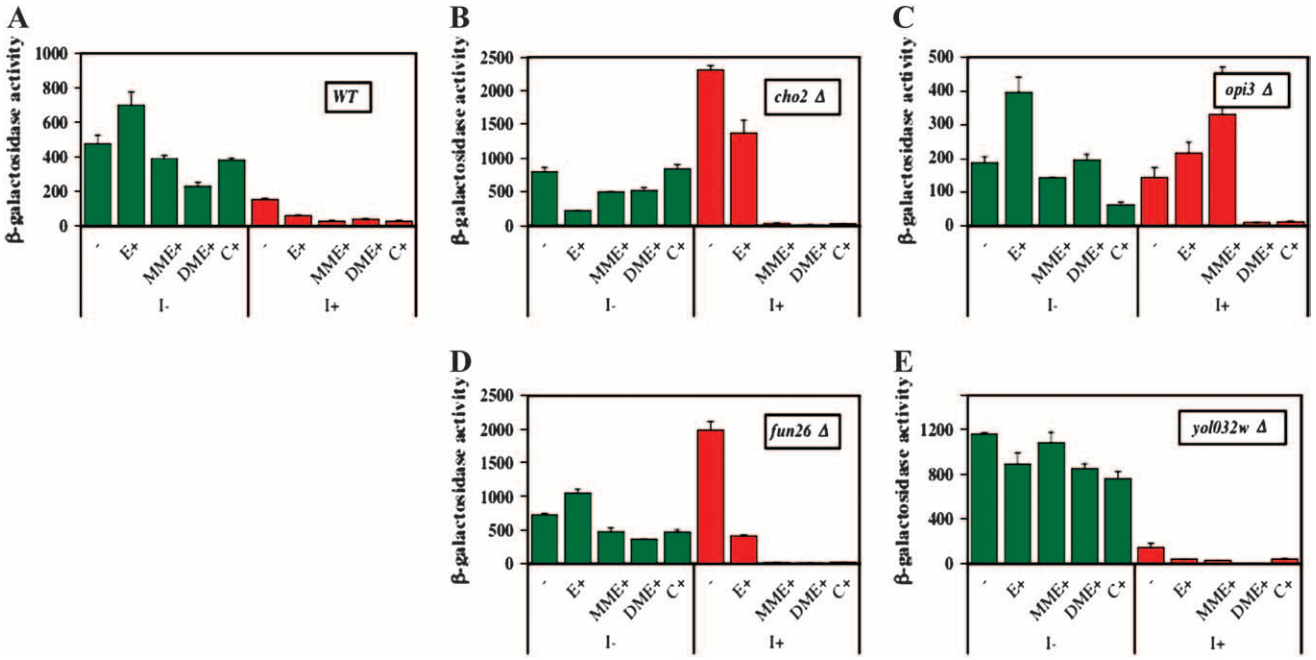

Figure 7.—Effect of Kennedy pathway on regulation of the *INO1-lacZ* reporter. Strains containing the *INO1-lacZ* reporter in pJH330 were grown in media either lacking or containing inositol ± E, MME, DME, or C. Red bars represent cells grown in I− media and green bars in I+ media. Each bar is the mean of at least three independent transformants. A wild-type strain (BY4742) and an *opi1*Δ strain were used as controls.

I+DME+, and I+C+ media (Figure 7E). Thus, in general the pattern of regulation of *INO1-lacZ* expression correlates with the Opi⁻ phenotype suppression pattern.

Opi⁻ mutants affected the UPR: Inositol depletion and accumulation of unfolded proteins in the ER affect expression of both phospholipid biosynthetic genes and UPR target genes (Travers *et al.* 2000; Jesch *et al.* 2005). This is due to the fact that both responses are coordinated through the *IRE1* and *HAC1* gene products. Previous reports restrict the activity of Opi1p to the inositol response (Cox *et al.* 1997). Microarray studies show that Opi1p is not required for inositol-mediated regulation of UPR target genes (Jesch *et al.* 2005) but does affect a small set of genes regulated in response to accumulation of unfolded proteins (Travers *et al.* 2000). Thus, it was reasonable to assume that some of the Opi⁻ mutant genes might affect the UPR. To test this, the expression of an UPRE-driven *lacZ* reporter was examined in the Opi⁻ mutant strains.

The 89 Opi⁻ mutant strains were transformed with pJC104 (Cox and Walter 1996) to determine their response to UPR activation. Plasmid pJC104 contained four repeats of the UPRE fused to *lacZ*. Wild-type and *hac1* mutant strains were used as controls and an *opi1* mutant strain was also tested. Transformants were grown on X-gal I+C+ medium in the absence (Tm−) and presence of Tm (Tm+; induces UPR). The wild-type strain grew dark blue on Tm+ medium after an overnight incubation. As expected the *hac1* mutant strain grew white on Tm+ medium. Several other mutant strains grew as white colonies (including *vps74*) and some as light-blue colonies (including *opi1*, *yhi9*, and *ede1*). The complete data set will be reported in a separate publication.

To quantify the effect of the Opi⁻ mutants on the UPR, the wild-type strain (BY4742) and the *opi1*, *yhi9*, *hac1*, *vps74*, and *ede1* mutant strains were assayed for β-galactosidase activity. Transformants were grown at 30° to midlogarithmic phase in I+C+ medium and shifted to I+C+, I−C−, or I+C+Tm+. Following the shift, cells were allowed to grow for 1 and 3 hr at which times samples were collected.

In general, the most dramatic effects were observed 3 hr after the shift (compare Figure 8, A and B). In the wild-type strain, β-galactosidase activity increased 72-fold after the 3-hr shift in I+C+Tm+ and 12-fold after the 3-hr shift in I−C− (Figure 8B). This is consistent with previously published results (Cox *et al.* 1997; Chang *et al.* 2002). Also as expected, the *hac1* strain was defective in responding to inositol depletion and Tm addition (Cox and Walter 1996; Brickner and Walter 2004) (Figure 8). Consistent with its plate phenotype, the effect of *vps74* resembled that of *hac1* (Figure 8B). In contrast to microarray studies (Travers *et al.* 2000; Jesch *et al.* 2005), *opi1* did appear to play a role in activation of UPRE in response to Tm and inositol depletion (Figure 8). The *opi1* mutant was able to initiate the response to both conditions (Figure 8A) but was not able to reach the wild-type level of induction (Figure 8B). The *yhi9* mutant was completely defective

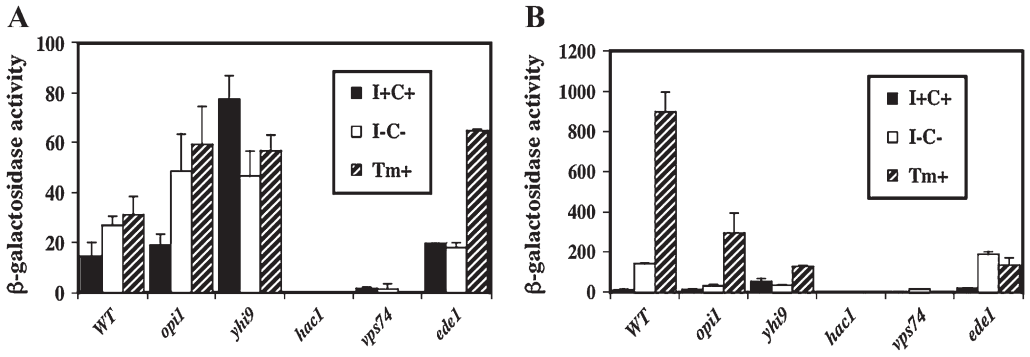

Figure 8.—Opi⁻ mutants affected the UPR. Wild type and a sample of Opi⁻ mutants were transformed with pJC104. Transformants were grown in I+C+ conditions at 30° and shifted to I+C+ or, to induce stress, I−C− and I+C+Tm+. (A) 1-hr and (B) 3-hr samples were collected and β-galactosidase activity was quantified. Each bar is the mean of three independent transformants.

in the response to inositol and displayed a delayed and abated response to Tm (2.4-fold induction in Tm+) (Figure 8B). The *ede1* mutant exhibited a normal early response to Tm but an abated 3-hr response to Tm (6.5-fold induction); however, the response to inositol depletion was normal (Figure 8). These data show that the Opi⁻ mutants are likely to provide significant insight into understanding the response to accumulation of unfolded proteins in the ER.

DISCUSSION

Recent findings have significantly advanced our understanding of the mechanism of Opi1p repression (Loewen *et al.* 2003, 2004; Gardenour *et al.* 2004; Heyken *et al.* 2005; Jesch *et al.* 2005; Loewen and Levine 2005). However, many questions remain unanswered. A powerful and effective way to deal with this is to screen the VYDS (Huang and O'Shea 2005). Thus, we identified 89 genes from the VYDS that display the Opi⁻ phenotype. These 89 genes affect multiple biological processes (Figure 2), including six that are involved in phospholipid biosynthesis, the five nonessential members of the NuA4 complex, and seven that are the members of the Rpd3p HDAC complex. The screen also identified 43 mutants that alter protein trafficking and, notably, the four members of the AP-3 complex (Table 1). The screen successfully identified all predictable Opi⁻ mutants. These include *cho2*, *opi1*, *opi3*, *ume6*, *rpd3*, *sin3*, and *reg1* (Table 1). As previously reported, mutations in the Ume6p–Sin3p–Rpd3p HDAC displayed the Opi⁻ phenotype (Elkhaimi *et al.* 2000). The Opi⁻ phenotype was most severe in the *ume6* strain and least severe in the *rpd3* strain (Figure 3) (Elkhaimi *et al.* 2000). More recently, it has been shown that Pho23p, Dep1p, Rxt2p, and Eaf3p are also associated with the Rpd3p HDAC (Loewith *et al.* 2001; Carrozza *et al.* 2005; Gavin *et al.* 2006) and the VYDS screen found that mutants in each of these genes display the Opi⁻ phenotype (Figure 3) and misregulate *INO1-lacZ* expression (Figure 5B). However, the screen did not identify two other members of this complex (*ume1* and *sap30*) that turned out to have a weak Opi⁻ phenotype (Figure 3B) and four other members of the complex do not appear to yield an Opi⁻ phenotype.

Some of the Opi⁻ mutants identified in the screen overlap other ORFs. In those situations, the disruption of one of the two genes may be the cause of the Opi⁻ phenotype. In some cases, the screen identified both genes that overlap. For example, the screen identified *vid21* and the overlapping *ydr360w* (*opi7*), as well as *vps61* and *rgp1*. Examination of the *INO1-lacZ* plate phenotypes and the β-galactosidase activity of the *vid21/ydr360w* mutant pair revealed different phenotypes. The *vid21* mutant has a medium-blue-plate phenotype and β-galactosidase activity of 823 units in I−C− and 97 units in I+C+ compared to the *ydr360w* mutant, which has a light-blue-plate phenotype and β-galactosidase activity of 351 units in I−C− and 4 units in I+C+ (Table 2 and Figure 5). *YDR360W* (*OPI7*) is listed as a dubious gene in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Thus, while mutation of either gene may yield an Opi⁻ phenotype, a more likely explanation for the Opi⁻ phenotype of the *ydr360w* mutant is that it partially affects expression of *VID21* because it only partially overlaps *VID21*. The *vps61* and *rgp1* mutants both have a medium-blue-plate phenotype and comparable β-galactosidase activities in I−C− (494 and 382 units, respectively) and I+C+ (26 and 20 units, respectively) media. This suggests that only one of the two mutants actually yields the Opi⁻ phenotype. *APL6* and *TNA1* transcribe toward each other; however, the *apl6* mutant shows a medium-blue-plate phenotype with β-galactosidase activities of 731 units and 28 units (I−C− and I+C+, respectively) while the *tna1* mutant shows a dark-blue-plate phenotype with β-galactosidase activities of 87 units and 149 units (I−C− and I+C+, respectively). Thus, the different phenotypes suggest that both mutants have the Opi⁻ phenotype. In a few instances, only one of the two overlapping genes was identified. For example, the screen identified *ydl096c* (*opi6*), *ykr035c* (*opi8*), *ylr338w* (*opi9*), and *ypr044c* (*opi11*) but not the overlapping *pmt1* (*ydl095w*), *did2* (*ykr035w-a*), *vrp1* (*ylr337c*), and *rpl43a* (*ypr043w*), respectively. In each of these cases, the screen identified a dubious gene. However, because each of these mutants had a relatively weak Opi⁻ phenotype, we screened mutants in the

corresponding overlapping genes and found them to also have a weak Opi⁻ phenotype. In another case, the screen identified *lbd16* and *ycl006c*. The *YCL006C* ORF has since been removed from the database (http://www.yeastgenome.org/); however, it was originally located immediately upstream of and divergent from the *LBD16* gene. Thus, the *ycl006c* mutant probably removes the promoter of the *LDB16* gene. In every one of these cases, additional experiments will be needed, and are ongoing, to determine which mutant is responsible for the phenotype.

Curiously, a subset of the Opi⁻ mutants did not overexpress the *INO1-lacZ* reporter under repressing conditions (Figure 5B). Transcriptional regulation of *INO1* is dependent on growth phase (Lamping *et al.* 1995). *INO1* transcript levels increase during exponential growth in wild-type strains grown in the absence of inositol and drop precipitously when cells enter stationary phase. In an *opi1*Δ strain, *INO1* transcript levels are elevated during all growth stages (Jiranek *et al.* 1998). *INO1* transcription is repressed in the presence of inositol during all phases of growth (Lamping *et al.* 1995). However, in the *opi1*Δ strain, *INO1* expression remains elevated at all growth phases in the presence of inositol (Jiranek *et al.* 1998). Thus, it is possible that some of the Opi⁻ mutants affect growth-phase regulation, yielding the Opi⁻ phenotype and dark-blue colonies in the X-gal plate assay (Table 2) without demonstrating a phenotype in the β-gal assay, which reflects a single time point in the growth of the culture (Figure 5).

Yet another explanation for the Opi⁻ mutants that do not affect *INO1-lacZ* expression is that they may affect inositol excretion directly. The mechanism for inositol transport is understood and requires two membrane-associated transporters encoded by the *ITR1* and *ITR2* genes (Nikawa *et al.* 1991). Mutations in the *INO2*, *INO4*, and *OPI1* genes alter the transcription of the *ITR1* gene (Lai and McGraw 1994). An *itr1* mutant has been shown to have a defect in *INO1* repression (Lai and McGraw 1994). Consistent with this, the VYDS screen identified the *itr1* mutant (Table 1). Unlike inositol uptake, the mechanism for inositol excretion is unknown. Thus, some of the Opi⁻ mutants may help to elucidate this mechanism.

Several membrane trafficking pathways exist in yeast, including secretory, endocytic, vacuolar protein sorting, and multivesicular body sorting (Deamilli *et al.* 1996; Odorizzi *et al.* 1998, 2000; Lee *et al.* 2004; Bowers and Stevens 2005). The VYDS identified several *vps* mutants that are known to affect these pathways. While some of these *vps* mutants affected the regulation of *INO1-lacZ*, many had no effect (Table 2 and Figure 5). The screen also identified all four components (Apl5p, Apl6p, Apm3p, and Aps3p) of the AP-3 complex that is involved in protein transport to the vacuole (Odorizzi *et al.* 1998). However, mutants of the AP-3 complex did not affect regulation of *INO1-lacZ* (Table 2 and Figure 5). It is tempting to speculate that the Opi⁻ phenotype of the *vps* and AP-3 complex mutants could be indirect and due to misregulation of intracellular stores of inositol. However, it is also possible that some of these mutants have direct effects since phospholipids are known to be integrally involved in the regulation of membrane trafficking (McGee *et al.* 1994; Deamilli *et al.* 1996; Kagiwada *et al.* 1996; Odorizzi *et al.* 2000; Yanagisawa *et al.* 2002).

The NuA4 HAT complex is associated with global and targeted acetylation of H4 tails *in vivo* (Doyon and Coté 2004). This complex consists of 13 proteins (Carrozza *et al.* 2003; Utley and Coté 2003; Doyon and Coté 2004). Of these, Tra1p, Epl1p, Esa11p, Eaf2p, Yng2p, Arp4p, Act1p, and Eaf6p are essential while Eaf3p, Eaf5p, Eaf7p, Vid21p, and Yaf9p are nonessential. All of the nonessential genes were identified as Opi⁻ mutants (Table 1, Figure 4). The NuA4 complex is primarily associated with acetylation of histone H4, which relaxes chromatin and allows transcription to take place. Therefore, mutants in NuA4 would be expected to reduce *INO1* expression. One model to explain why the NuA4 complex mutants affected repression is that NuA4 may function indirectly via regulation of the *OPI1* gene. However, we found that *OPI1-cat* expression was not reduced in four different strains containing NuA4 mutants (Figure 4C). Consequently, NuA4 may regulate *INO1* indirectly through another transcription factor or it may also regulate *INO1* directly via a different chromatin-modifying activity.

Of the 89 Opi⁻ mutants, 9 (7 new mutants) were suppressed by the addition of choline, suggesting that they are involved in PC biosynthesis. These included 3 mutants that were suppressed by the addition of E (*mrpl38*, *mrpl49*, and *yol032w*), 4 that were suppressed by MME (*cho2*, *fun26*, *nup84*, and *kex1*), and 2 that were suppressed by DME (*opi3* and *tps1*) (Table 3 and Figure 6). Because these mutants have phenotypes similar to those of *cho2* and *opi3* mutants, it is reasonable to predict that they play different roles in the biochemical steps of PC biosynthesis. It is well established that *CHO2* is responsible for the methylation of PE to phosphatidylmonomethylethanolamine (PMME) and *OPI3* is responsible for the final two methylation steps from PMME to PDME to PC (Kodaki *et al.* 1987; Summers *et al.* 1988; McGraw and Henry 1989). In most cases, the annotation of the 7 new conditional Opi⁻ mutants does not provide obvious explanations for the Opi⁻ phenotype. *MRPL38* and *MRPL49* encode mitochondrial ribosomal proteins of the large subunit that are involved in protein biosynthesis (Graack and Wittmann-Liebold 1998). *YOLO32W* (*OPI10*) is of unknown function and *KEX1* is required for protein processing (Latchinian-Sadek and Thomas 1993). *FUN26* is of unknown function; however, it has been shown to be an intracellular nucleoside transporter with broad specificity (Vickers *et al.* 2000). However, the *nup84* Opi⁻ phenotype may be explained by some recent

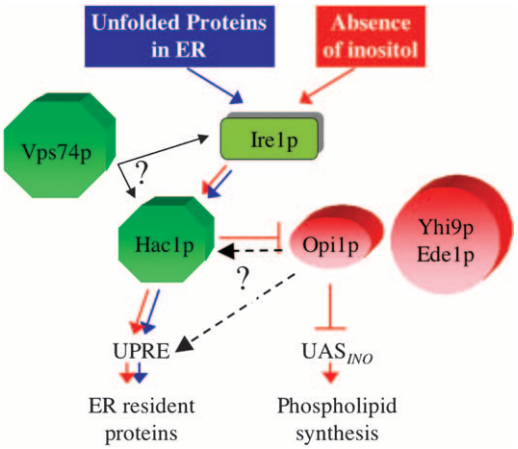

FIGURE 9.—Schematic depicting the UPR regulatory cascade. Refer to text for description.

observations. *NUP84* encodes a subunit of the nuclear pore complex (NPC) that has recently been shown to be required for activation of transcription via Rap1p, Gcr1p, and Gcr2p by recruiting these factors to the NPC (MENON *et al.* 2005). Expression from the *OPI3* (*PEM2*) promoter has been shown to be dependent on Rap1p (Grf1p) (KODAKI *et al.* 1991). Therefore, it is conceivable that expression of the *CHO2* gene is dependent on Rap1p or on some other transcription factor that is recruited to the Nup84 NPC. Thus, while it is not yet possible to explain the PC biosynthesis-dependent Opi$^-$ phenotype mutants, it is likely that these mutants may be affecting the export, processing, or function of the known PC biosynthesis enzymes.

The inositol depletion signal and the UPR-signaling pathway converge via the activation of Ire1p. Activation of Ire1p leads to the splicing of the *HAC1* transcript, producing a functional Hac1p that is able to bind to target genes containing the UPRE (Figure 9). Hac1p has been shown to antagonize Opi1p from binding to the Ino2p/Ino4p heterodimer (BRICKNER and WALTER 2004), thereby allowing induction of the phospholipid biosynthetic genes. However, Opi1p was not reported to play a role in the UPR. Contrary to published reports (TRAVERS *et al.* 2000), this study shows that Opi1p appears to act as an activator of the UPR (Figures 8 and 9). This effect could be due to a direct effect on UPRE-regulated genes or an indirect effect through Hac1p (Figure 9). Some of the other Opi$^-$ mutants were also found to affect the UPR (Figure 8). For example, the *vps74* mutant phenotype resembles the *hac1* (and *ire1*) mutant phenotype. This suggests that *VPS74* is required for the function/expression of either *HAC1* or *IRE1* (Figure 9). Likewise, *yhi9* and *ede1* resemble the *opi1* mutant (Figures 8 and 9), suggesting that they may affect Opi1p function. Further analysis of the effects of the Opi$^-$ mutants in the UPR may therefore reveal how these mutants affect regulation of both the UPR target genes and the phospholipid biosynthetic genes.

We thank Meng Chen, Linan Chen, Ying He, and Niketa Jani for critical reading of the manuscript. We thank Peter Walter (University of California at San Francisco) for providing the *UPRE-lacZ* reporter. The authors also thank Miriam Greenberg (Wayne State University) for developing the Opi$^-$ plate screen. This work was supported by a grant from the National Science Foundation to J.M.L. (MCB 0415511). L.C.H. was the recipient of the William A. Turner Jr. Memorial Foundation Scholarship and R.P.B. was the recipient of the Nicolette Therese Keller Endowed Fellowship in Biological Sciences.

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Communicating editor: B. J. Andrews