

Opi1-mediated transcriptional modulation orchestrates genotoxic stress response in budding yeast

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Abstract

In budding yeast, the transcriptional repressor *Opi1* regulates phospholipid biosynthesis by repressing expression of genes containing inositol-sensitive upstream activation sequences. Upon genotoxic stress, cells activate the DNA damage response to coordinate a complex network of signaling pathways aimed at preserving genomic integrity. Here, we reveal that *Opi1* is important to modulate transcription in response to genotoxic stress. We find that cells lacking *Opi1* exhibit hypersensitivity to genotoxins, along with a delayed G1-to-S-phase transition and decreased gamma-H2A levels. Transcriptome analysis using RNA sequencing reveals that *Opi1* plays a central role in modulating essential biological processes during methyl methanesulfonate (MMS)-associated stress, including repression of phospholipid biosynthesis and transduction of mating signaling. Moreover, *Opi1* induces sulfate assimilation and amino acid metabolic processes, such as arginine and histidine biosynthesis and glycine catabolism. Furthermore, we observe increased mitochondrial DNA instability in *opi1Δ* cells upon MMS treatment. Notably, we show that constitutive activation of the transcription factor *Ino2-Ino4* is responsible for genotoxin sensitivity in *Opi1*-deficient cells, and the production of inositol pyrophosphates by *Kcs1* counteracts *Opi1* function specifically during MMS-induced stress. Overall, our findings highlight *Opi1* as a critical sensor of genotoxic stress in budding yeast, orchestrating gene expression to facilitate appropriate stress responses.

Keywords: DNA damage response, genotoxic stress, methyl methanesulfonate, *Opi1*, inositol pyrophosphates, mitochondrial function, transcriptional regulation

Introduction

The DNA molecule is constantly exposed to endogenous and exogenous genotoxins, leaving it subjected to a multitude of lesions that, if left unchecked, can lead to genomic instability, a hallmark of cancer and other diseases (Jackson and Bartek 2009). Methyl methanesulfonate (MMS) is a widely used genotoxic agent that induces DNA damage by alkylating DNA bases, resulting in the formation of DNA adducts. The most prevalent adducts formed by MMS are N7-methylguanine (N7-MeG) and N3-methyladenine (N3-MeA) (Beranek 1990), while N1-methyladenine (N1-MeA) and N3-methylcytosine (N3-MeC) can also be generated during DNA replication (Wyatt and Pittman 2006). These DNA adducts can interfere with DNA synthesis and replication by inducing replication fork stalling and subsequent DNA strand breaks (Branzei and Foiani 2010). Additionally, MMS has been shown to cause damage to mitochondrial DNA (mtDNA), which can disrupt oxidative phosphorylation and contribute to increased generation of reactive oxygen species (ROS) (Salmon et al. 2004; Kitanovic and Wöfl 2006; Kitanovic et al. 2009). Under such circumstances, a

comprehensive DNA damage response (DDR) is activated to tackle the consequences of DNA lesion accumulation, such as through DNA repair, telomere maintenance, chromatin remodeling, inhibition of DNA synthesis and cell cycle progression, and transcription reprogramming, among others (Hanawalt 2015; Lanz et al. 2019; Cussiol et al. 2020).

Although the DDR has been extensively studied at the molecular level, recent studies have proposed that the DDR is connected with metabolism of biomolecules such as carbohydrates and lipids (Simpson-Lavy et al. 2015; Ferrari et al. 2017; Yi et al. 2017). Remarkably, the existence of crosstalk between the DDR and phospholipid metabolism in eukaryotes was proposed several years ago (Zewail et al. 2003). Supporting this notion, numerous proteins involved in the phosphatidylinositol (PI) pathway were found to undergo phosphorylation in response to DNA damage induced by MMS (Zhou et al. 2016; Lanz et al. 2021). Inositol metabolites such as inositol polyphosphates (IPs) and inositol pyrophosphates (PP-IPs) have been implicated in cell cycle regulation and DNA damage repair (Banfic et al. 2013, 2016; Jadav et al.

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2013), but the molecular basis for these effects is unknown. Furthermore, it was proposed that PI phosphate lipids (PIPs) are enriched in the nucleus after DNA damage, serving as important mediators of ATR signaling in mammalian cells (Wang et al. 2017). Nonetheless, little is known about how DNA damage can regulate inositol metabolism and vice versa.

In budding yeast, inositol biosynthesis is fine-tuned by the transcriptional repressor *Opi1*. In the absence of inositol, *Opi1* is localized to the perinuclear endoplasmic reticulum (ER) membrane, establishing interactions with the integral membrane protein *Scs2* and the phospholipid precursor phosphatidic acid (PA) (Craven and Petes 2001; Brickner and Walter 2004; Gaspar et al. 2017; Hofbauer et al. 2018). As a result, the heterodimeric transcriptional activator *Ino2-Ino4* binds to cis-acting inositol-sensitive upstream activation sequences (UAS_{INO}) and upregulates expression of several genes related to phospholipid metabolism including the inositol-3-phosphate synthase (*INO1*), which promotes inositol de novo synthesis. Full repression of genes containing UAS_{INO} is also dependent on choline, leading to the designation of this sequence as inositol/choline-responsive elements (ICRE) (Schüller et al. 1992). Once intracellular inositol concentration increases, PA is redirected to the synthesis of PI leading to its exhaustion. Subsequently, *Opi1* migrates to the nucleus where it binds to the heterodimeric transcriptional factor *Ino2-Ino4* and promotes transcriptional repression through the interaction with the proteins *Sin3* and *Cyc8*, creating a scaffold for the recruitment of histone deacetylase (HDAC) complexes (Wagner et al. 2001; Jäschke et al. 2011; Kliewe et al. 2017) (Fig. 1a). Importantly, deletion of *Opi1* leads to constitutive expression of *INO1* with overproduction of inositol even when cells are supplemented with inositol (Graves and Henry 2000). Moreover, cells lacking *Opi1* show constitutive activation of a large number of genes, most of which are regulated by *Ino2-Ino4* (Santiago and Ben Mamoun 2003; Hoppen et al. 2005; Jesch et al. 2005). Many of these genes are involved in phospholipid biosynthesis, although UAS_{INO} motifs are found in several genes related to other metabolic processes, which implicates *Ino2-Ino4* in the control of the expression of distinct biological processes (Wimalaratna et al. 2011). Interestingly, phosphoproteomic analysis in budding yeast showed that the transcriptional repressor *Opi1* is phosphorylated in a *Mec1/Tel1*-dependent manner after MMS exposure (Balint et al. 2015; Bastos de Oliveira et al. 2015; Lanz et al. 2021), which might suggest that *Opi1* is involved in the DDR.

In this study, we employ an integrative approach combining RNA sequencing (RNA-seq) transcriptome analysis, yeast genetics, molecular biology, cell biology, and biochemistry techniques to investigate the role of the transcriptional repressor *Opi1* in the response to genotoxic stress. Our findings demonstrate that under MMS-induced stress, cells lacking *Opi1* exhibit enhanced sensitivity and cell cycle defects characterized by a delayed G1-to-S-phase transition, resulting in reduced histone H2A phosphorylation. Notably, RNA-seq analysis revealed that during MMS-induced genotoxic stress, *Opi1* is important to modulate expression of genes involved in important biological processes. Additionally, our study reveals that treatment with MMS leads to an increase in mtDNA instability in *opi1Δ* cells. This observation suggests a potential link between mtDNA instability and the genotoxin sensitivity exhibited by cells lacking *Opi1*. Importantly, deletion of the transcriptional activator *Ino2-Ino4* rescues the MMS sensitivity of *opi1Δ* cells showing that constitutive activation of *Ino2-Ino4* is the cause of MMS sensitivity. Finally, while deletion of the PP-IP kinase *Kcs1* in an *opi1Δ* strain rescues MMS sensitivity and cell cycle defects, overexpression of *Kcs1* in wild-type cells

phenocopies the MMS sensitivity of an *opi1Δ* strain. These findings suggest that PP-IPs may counteract the function of *Opi1* during MMS-induced stress in yeast.

Collectively, these results highlight the crucial role of *Opi1* as a key sensor of genotoxic stress and emphasize the significance of modulating PP-IP synthesis as an integral component of the DDR and a critical factor for proper cell function in budding yeast.

Materials and methods

Yeast strains and plasmids

Strains generated in this study are isogenic from BY4741 and S288C (where indicated). Detailed information on all yeast strains and plasmids used in this study can be found in [Supplementary Tables 1 and 2](#), respectively. To generate knockout and epitope-tagged strains, the 1-step gene disruption method, as described by Rothstein (Rothstein 1983, 1991; Longtine et al. 1998), was employed. All yeast transformations were performed using the lithium acetate method (Gietz et al. 1992; Gietz and Woods 2006). PCR genotyping using specific primers ([Supplementary Table 3](#)) was conducted to confirm the successful generation of knockout strains, while Western blotting was performed to verify the presence of epitope tags in the respective strains.

Growth conditions

Cells were grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose), and plasmid-bearing cells were grown in synthetic complete media lacking uracil (SC-URA) (0.17% YNB without amino acids, Difco), 0.5% ammonium sulfate (MP Biomedicals), 0.07% CSM-URA dropout mix (Sunrise Science), and 2% dextrose (Sigma). For experiments performed in the absence of inositol, SC – INO medium was prepared using YNB without inositol (MP Biomedicals). For expression of *INO1* and *KCS1* under the control of the *GAL1* promoter, cells were grown overnight in SC-URA medium with 2% dextrose and were plated in SC-URA plates in the presence of 2% dextrose (Sigma) or 2% galactose (Difco). For galactose induction in liquid cultures, the strains were initially grown in SC-URA medium supplemented with lactate (pH 5.5) as described by the Haber lab (Haber and Leung 1996). Once the cell density reached an optical density (OD_{600nm}) of 1.0, galactose was introduced into the culture to achieve a final concentration of 2%. To evaluate growth under respiratory conditions, cells were cultured in either YPGal (1% yeast extract, 2% peptone, and 2% galactose) or YPG medium (1% yeast extract, 2% peptone, and 3% glycerol). For the detailed concentrations and durations of specific chemical agents, including genotoxins and other drugs, please refer to the respective figure legends associated with the experimental data.

Serial dilution assays

Overnight cultures were inoculated in the appropriate medium to an OD_{600nm} of 0.1, and cells were grown until they reached the logarithmic growth phase and were subsequently normalized to an OD_{600nm} of 1.0. Four-fold serial dilutions were spotted on yeast plates and grown for 1–3 days at 30°C in the presence or absence of specific chemical agents, including genotoxins and other drugs. The specific concentrations of these agents used in the assay are provided in the corresponding figure and/or figure legends, serving as a clear reference for the experimental setup. Following the incubation period, the plates were manually inspected for growth changes and digitalized in an Uvitec Alliance 4.7 imaging system.

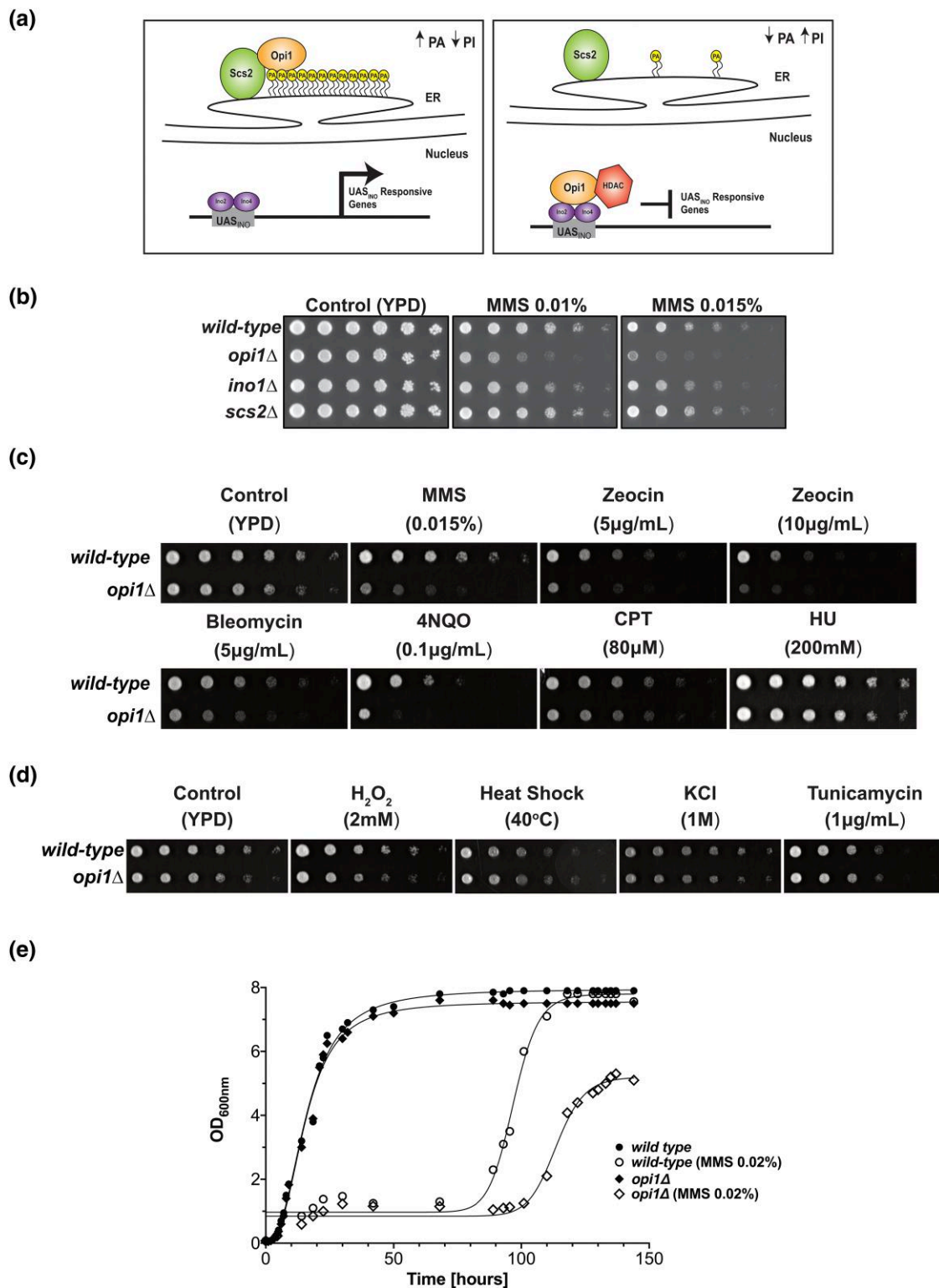


Fig. 1. Cellular response of *Opi1*-deficient cells to genotoxic and proteotoxic stress. a) Working model for how *Opi1* represses transcription of target genes. b) Effect of deletion of genes involved in inositol metabolism on sensitivity to genotoxins. c) Cells lacking *Opi1* show sensitivity to different genotoxins. d) Cells lacking *Opi1* do not show sensitivity to proteotoxic stress agents. e) Growth rate of wild-type and *opi1Δ* cells determined by monitoring OD_{600} over time. The data points (geometrical symbols) were fitted to a nonlinear regression curve using GraphPad Prism version 9.0.0 to analyze the growth kinetics. For b), c), and d), 4-fold serial dilutions were spotted on the media indicated in the figure, and plates were grown for 2–3 days at 30°C.

Western blotting

Proteins were transferred onto PVDF membranes (Amersham Hybond P, GE Healthcare). Phosphorylated histone H2A (gamma-H2A), total histone H2A, *Rad53*, and epitope-tagged

proteins were probed using specific antibodies: anti-gamma-H2A (ab17353, Abcam, 1:2,500 dilution in TBST and 5% nonfat dry milk), anti-histone H2A (ab188312, Abcam, 1:500 dilution in TBST and 5% nonfat dry milk), anti-HA (12CA5, Roche, 1:10,000

in TBST and 2% nonfat dry milk), and anti-Rad53 (ab104232, Abcam, 1:3,000 dilution in TBST and 5% nonfat dry milk). Anti-PGK1 was used as a housekeeping control (22C5D8, Abcam, 1:15,000 dilution in TBST and 2% nonfat dry milk). After primary antibodies, PVDF membranes were incubated in the presence of ECL HRP-linked secondary antibodies (mouse: NA931-GE or rabbit: NA934-GE, 1:10,000 dilution in TBST and 2% nonfat dry milk). Blots were developed using the Amersham ECL Prime detection reagent and imaged in an Uvitec Alliance 4.7 imaging system.

Cell cycle synchronization

Yeast cells were synchronized in the G1 phase as described before (Jablonski et al. 2015). In brief, cells were initially grown in YPD medium at 30°C until they reached the logarithmic growth phase. Subsequently, α -factor (Zymo Research) treatment was applied at a concentration of 30 ng/mL (for *bar1Δ* background strains) for 2 h. To release the cells from G1 arrest, they were centrifuged and re-suspended in fresh medium. In order to assess the impact of MMS-induced genotoxic stress on S-phase progression, MMS was added at a concentration of 0.033% during the release step. To induce an intra-S-phase arrest of the cell cycle, cells were exposed to MMS at a concentration of 0.033% for 2 h, which is sufficient to activate the intra-S-phase checkpoint (Shirahige et al. 1998). For recovery of cells following MMS treatment, cells were exposed to MMS at a concentration of 0.033% for 2 h, harvested, and subsequently resuspended in fresh YPD medium.

Flow cytometry

Cell cycle analysis was performed as described before (Jablonski et al. 2015). In brief, logarithmic growing yeast cells were collected, harvested, fixed in 1 mL of 70% ethanol (Sigma-Aldrich), and incubated overnight at 4°C. Cells were then centrifuged, and residual ethanol was dried in SpeedVac. After that, samples were solubilized in sodium citrate buffer (Labsynth) (50 mM, pH 7.2) and sonicated (3 cycles of 3 s, amplitude 30%) to release cell clumps. Samples were then incubated with 100 μ g of RNase A (Invitrogen) for 1 h at 37°C, followed by incubation with 500 μ g of Proteinase K (Invitrogen) for 1 h at 42°C. Then, 1 μ L of SYTOX Green (Thermo Fisher Scientific) was added to the samples and incubated for 2 h at 4°C protected from the light. Data were acquired using a BD Accuri C6 Flow Cytometer. For viability assessment, wild-type and *opi1Δ* cells were cultured in YPD liquid medium until the logarithmic phase of growth and treated with 0.1% MMS for 4 h. After treatment, the samples were normalized to a concentration of 1×10^6 cells/mL, harvested, and resuspended in 1 mL of 50 mM sodium citrate. Next, 0.4 μ M propidium iodide (Thermo Fisher Scientific) was added, and the cells were immediately analyzed using a BD Accuri C6 Flow Cytometer. To distinguish between dead and live cells, we used boiled cells (100°C for 10 min) as a control for dead cells and untreated cells to obtain a population of live cells. The data were obtained from 3 biological replicates, and multiple t-tests were used to determine statistical significance, comparing the mean viability values of the strains with the significance set at $\alpha = 0.05$.

Real-time quantitative PCR analysis

Cells were grown overnight in YPD and diluted the next day to an OD_{600nm} of 0.1 in synthetic complete medium either supplemented or lacking inositol (SC + INO or SC – INO). Cells were grown for at least 2 cell cycle divisions and immediately harvested at 4°C, washed with sterile ultrapure H₂O, and kept at –80°C. Alternatively, log-phase cells were treated with MMS (Sigma-Aldrich) 0.1% for 1 h. Total RNA from 5×10^7 cells was

extracted using an RNeasy Mini Kit (Qiagen). One microgram of total RNA was used for cDNA synthesis using a QuantiNova Reverse Transcription Kit (Qiagen). Real-time PCR was performed in an ABI Prism 7500 (Applied Biosystems) using a QuantiNova Probe PCR Kit (Qiagen) in the presence of TaqMan probes for *INO1* (Sc04136910_s1) and *ACT1* (Sc04120488_s1) (Thermo Fisher Scientific). The *ACT1* gene served as an internal standard for normalization as it was previously shown that there is no change in mRNA levels after either inositol depletion (Ye et al. 2013; Gaspar et al. 2017) or MMS treatment (Gasch et al. 2001). Relative values of mRNA are described as fold change relative to a control condition indicated in the experiment. The data were obtained from 3 biological replicates, and statistical analysis was performed using 1-way analysis of variance (ANOVA) with Tukey's post hoc test to determine the significant differences between the groups.

RNA-seq sample preparation and analysis

Cells were grown overnight in SC + INO media and diluted the next day to an OD_{600nm} of 0.1 in 250 mL of SC + INO media in biological duplicates. Cells were grown for at least 2 cell cycle divisions (\sim OD_{600nm} = 0.4), then vacuum filtered in 0.22- μ m nitrocellulose membranes to remove media and immediately “flash frozen” in liquid nitrogen. One milliliter of frozen 1 \times RIPA buffer (Millipore) was combined into each frozen pellet and lysed by mechanical grinding with a Spex 6750 Freezer Mill at a rate of 10 Hz for 2 min. The lysate was then thawed on ice and centrifuged at 2,000 g for 2 min at 4°C to remove debris; 250 μ L of cleared lysate was used for RNA extraction by combining it with 250 μ L of 2 \times AE Buffer (100 mM sodium acetate pH 5.2, 20 mM EDTA), 50 μ L of 10% SDS, and 500 μ L of acid phenol:chloroform pH 5.2. The mixture was incubated at 65°C for 10 min under 1,400 rpm agitation, then transferred to ice for 5 min. The solution was then spun at 15,000 g for 15 min at 4°C. The supernatant was then transferred to new microcentrifuge tubes, and 550 μ L of chloroform was added, followed by centrifugation at 15,000 g for 5 min at 4°C. The aqueous phase was then transferred to new microcentrifuge tubes, and RNA was precipitated in 0.3 M sodium acetate and 400 μ L of isopropanol after centrifugation at 15,000 g for 20 min at 4°C. RNA pellets were washed with 75% ethanol and centrifuged at 15,000 g for 5 min at 4°C. Finally, RNA pellets were resuspended in nuclease-free water and submitted to Bioanalyzer High Sensitivity DNA Assay (Agilent) for quality check and quantified using Qubit RNA High Sensitivity (Invitrogen). One microgram of total RNA was used as input for preparing the libraries for RNA-seq with Ultra RNA Library Prep II (New England Biolabs), following the recommended workflow. Final cDNA libraries after PCR were quantified by Qubit dsDNA High Sensitivity (Invitrogen). Samples were then equimolarly pooled and distributed on Illumina NextSeq High 500/550, resulting in \sim 7–8 million reads per sample. Reads were demultiplexed using bcl2fastq (version 2.20), and quality of reads was assessed using FastQC (version 0.11.8). Reads were then aligned to the S288C reference genome (release R64-2-1) using STAR (version 2.5.4b) (Dobin et al. 2013). Differential gene expression was calculated with DESeq2 (version 1.40.0) (Love et al. 2014) using the design “~genotype + condition + genotype: condition.” A threshold of 0.8 log₂ fold change with an adjusted P-value of 0.05 was used to analyze differentially expressed genes (DEGs) between samples. A principal component analysis (PCA) plot for the 2 biological replicates across genotypes and treatments is shown in Supplementary Fig. 1. Supplementary Table 4 (“genotype_OPI1_vs_WT.csv”) shows the DEGs between the *opi1Δ* and wild-type strains in an untreated condition.

Supplementary Table 5 (“treatment_MMS_vs_unt.csv”) shows the DEGs in the wild-type strain treated with MMS relative to an untreated condition. Supplementary Table 6 (“interaction.csv”) shows the differential effect of MMS treatment in the *opi1Δ* strain relative to the wild type (interaction term). Supplementary Table 7 (“treatment_MMS_vs_unt_OPI1.csv”) shows the DEGs in the *opi1Δ* strain treated with MMS relative to the *opi1Δ* without treatment. Supplementary Table 8 (“OPI1_vs_WT_treatment.csv”) shows the DEGs between the *opi1Δ* and wild-type strains both treated with MMS.

To identify enriched biological processes and cellular components among the list of DEGs, we utilized the PANTHER database (<http://pantherdb.org>) and the Saccharomyces Genome Database (SGD) with the following parameters: PANTHER version 17.0 over-representation test, FISHER test with false discovery rate (FDR) correction (Thomas et al. 2022), and Gene Ontology (GO) Slim term mapper (<https://www.yeastgenome.org/goSlimMapper>), respectively. Additionally, interaction networks of DEGs were generated using the STRING v11.5 database (<http://string-db.org>) (Szkarczyk et al. 2019).

Respiratory capacity assay

Oxygen consumption rates were measured using a modified protocol based on the method described by Zulkifli et al. (2020) using a high-resolution O₂k-FluoRespirometer (Oroboros, Innsbruck, Austria). For each assay, 10⁶ cells were added to the Oroboros chamber containing a final volume of 2.0 mL of YPEG medium at 30°C under agitation. Briefly, exponentially growing cells in YPD were harvested, resuspended in respiratory medium (YPEG; 2% glycerol and 1% ethanol), and incubated for at least 3 h at 30°C. Finally, cells were transferred to O₂k chambers for measurement of O₂ consumption rates. After basal respiration assessment, 5 μM of the mitochondrial uncoupler FCCP (Sigma-Aldrich) was added to measure maximal respiration. At the end, 2 μM of antimycin A (Sigma-Aldrich) was added to inhibit mitochondrial respiration. Nonmitochondrial oxygen consumption rates were subtracted from all measurements. Optimal concentrations of FCCP and antimycin A were previously determined by titration. The data were obtained from 2 biological replicates, and statistical analysis was performed using 1-way ANOVA with Tukey’s post hoc test to determine the significant differences between the groups.

Results

Opi1 is important for cell survival during genotoxic stress

To determine whether cells lacking *Opi1* are more sensitive to DNA damage, we performed sensitivity assays in the presence of genotoxins. We found that *opi1Δ* cells showed sensitivity to genotoxic stress induced by the DNA-alkylating agent MMS (Fig. 1b), indicating that *Opi1* is important for the response to genotoxic stress. To confirm that this sensitivity to MMS was specific to *OPI1* deletion, we constructed a pRS416 plasmid expressing *Opi1*-HA from its endogenous promoter and show that *Opi1*-HA expression rescues the MMS sensitivity in *opi1Δ* (Supplementary Fig. 2a and b, respectively). Furthermore, deletion of genes that counteract *Opi1*-repressing functions, such as *INO1* and *SCS2*, did not lead to MMS sensitivity (Fig. 1b).

Besides MMS, we performed a screening of several known DNA-damaging agents to assess for genotoxicity in *opi1Δ* cells (Fig. 1c). Interestingly, we found that *opi1Δ* cells are sensitive to different genotoxins such as the radiomimetic drugs bleomycin

and zeocin and 4-nitroquinoline 1-oxide (4NQO), while they did not show sensitivity to the replication stress inducers hydroxyurea (HU) and camptothecin (CPT). Importantly, cells lacking *Opi1* did not show sensitivity to agents that activate the environmental stress response (ESR) and the unfolded protein response (UPR), indicating that *Opi1* shows specificity to cope with increased DNA damage inflicted by genotoxins (Fig. 1d).

To determine if the increased sensitivity to genotoxins observed in cells lacking *Opi1* is due to loss of viability rather than a decreased growth rate, we used colony-forming unit assay and flow cytometry to monitor cell survival (Supplementary Fig. 2c and d, respectively). Our results show that *opi1Δ* cells do not exhibit a significant decrease in cell viability compared to wild-type cells, indicating that the hypersensitivity to genotoxins is likely due to delayed cell proliferation rather than cell death. Consistent with that, *opi1Δ* cells show growth defects in YPD liquid medium in the presence of 0.02% MMS, suggesting an increased arrest of the cell cycle in *opi1Δ* cells (Fig. 1e).

Cells lacking Opi1 show delayed G1-to-S-phase progression and decreased levels of gamma-H2A

Since *opi1Δ* mutants are sensitive to genotoxic stress (Fig. 1c) and show a prolonged arrest of growth (Fig. 1e), we sought to investigate if these cells have defects in the DDR. To investigate the potential involvement of *Opi1* in the regulation of the DDR, we subjected asynchronous yeast cells to genotoxic agents, such as MMS, zeocin, and 4NQO, and monitored the activation of *Rad53*, a pivotal kinase in the signal transduction pathway responsible for DDR regulation (Pelliccioli and Foiani 2005; Branzei and Foiani 2006; Cussiol et al. 2020). Our findings revealed no significant differences in *Rad53* activation between wild-type and *opi1Δ* cells in asynchronous conditions, suggesting that *Opi1* may not play a critical role in the regulation of the *Rad53* axis within the DDR (Supplementary Fig. 3).

Given the indications from our results that genotoxic stress leads to delayed cell proliferation in *opi1Δ* cells (Fig. 1e), we proceeded to investigate the role of *Opi1* in the regulation of the cell cycle. To accomplish this, we utilized flow cytometry to monitor cell cycle progression. Initially, we arrested cells in the G1 phase using α -factor and subsequently released them into fresh YPD medium in the presence or absence of 0.033% MMS, a concentration known to reduce S-phase progression and induce a robust activation of the intra-S-phase checkpoint (Tercero and Diffley 2001). Our findings reveal that cells lacking *Opi1* exhibit a noticeable delay in S-phase progression under both unchallenged conditions and in the presence of MMS, as depicted in Fig. 2a. Furthermore, it is noteworthy that cells lacking *Opi1* consistently display a discernible G1 peak, indicating a G1/S arrest in the cell cycle after release from pheromone arrest. Interestingly, cells lacking *Opi1* show a delayed phosphorylation of histone H2A (gamma-H2A) and a reduced intensity of the gamma-H2A signal at later time points (Fig. 2b). Importantly, a previous study has demonstrated that gamma-H2A is enriched in regions containing newly replicated DNA, particularly behind the replication forks at early-firing replication origins following MMS treatment (Balint et al. 2015). The observed decrease in gamma-H2A levels in cells lacking *Opi1* may be attributed to a decrease in the firing of replication origins. Moreover, our results reveal a delayed activation of *Rad53* in *Opi1*-deficient cells (Fig. 2c), which aligns with the experimental observations regarding gamma-H2A (Fig. 2b). Specifically, we observed the appearance of a phosphorylated isoform of *Rad53* at 30 min of MMS treatment in wild-type cells, while this band was not detected at this time point in *opi1Δ* cells (Fig. 2c).

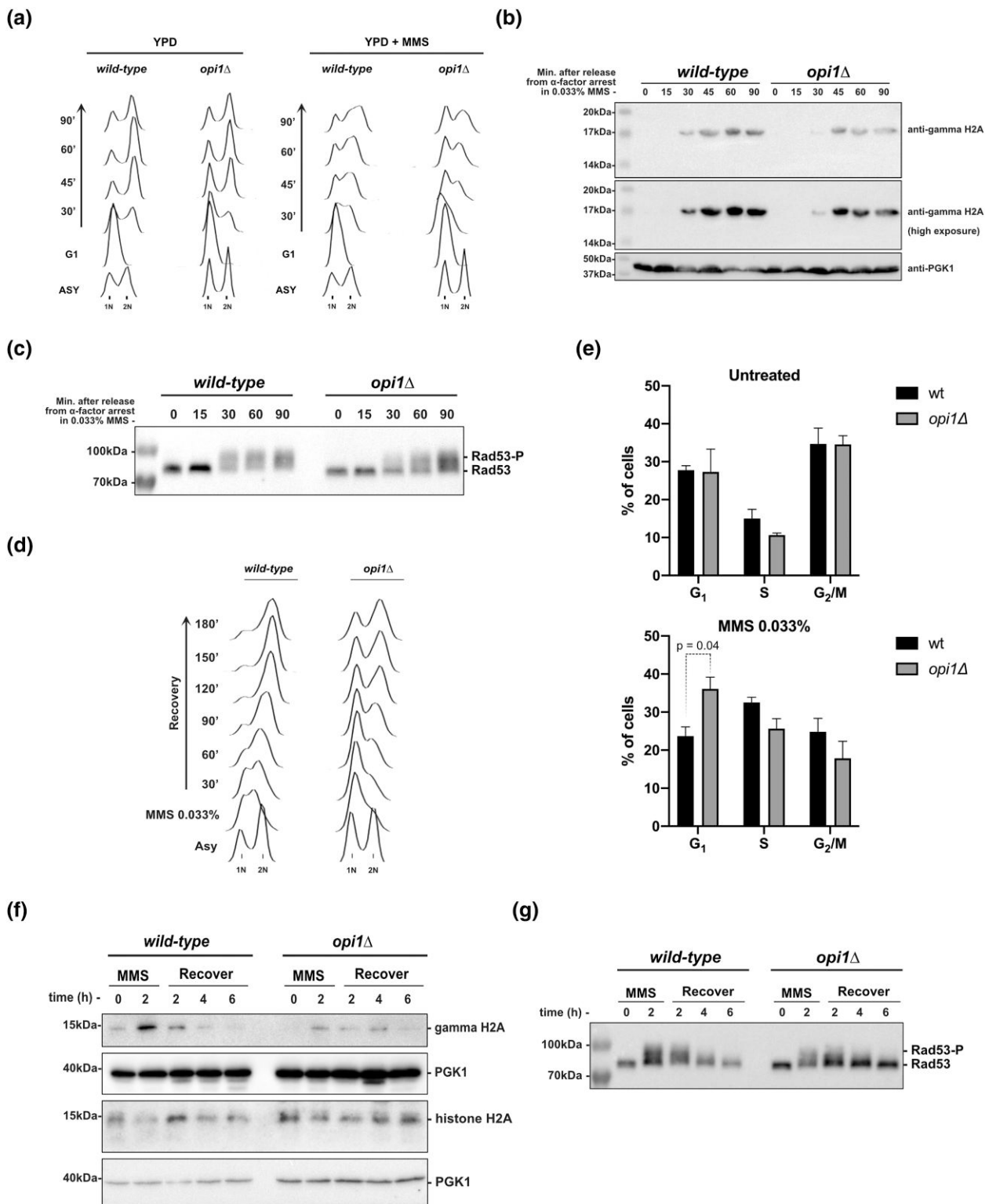


Fig. 2. Cells lacking *Opi1* show a delayed G1-to-S-phase transition and activation of the DDR upon genotoxic stress induced by MMS. a) Flow cytometry analysis depicting S-phase progression in wild-type and *opi1Δ* strains. b) Western blot analysis of gamma-H2A (histone H2A phosphorylation) in the indicated strains. c) Western blot analysis showing the MMS-induced *Rad53* phospho-shift in the indicated strains. d) Flow cytometry analysis illustrating the resumption of S-phase progression in wild-type and *opi1Δ* strains after MMS treatment (0.033% for 2 h). e) Quantification of the percentage of cells in the G₁, S, and G₂/M phases in untreated cells and after MMS treatment (0.033% for 2 h). The results shown are representative of 3 independent experiments, and error bars indicate the standard deviation. Two-way ANOVA with Tukey's post hoc test was used for significance analysis between samples. f) Western blot analysis of gamma-H2A and total histone H2A in the indicated strains. Note: For a) to c), cells were arrested in G₁ after 2-h treatment with α -factor and then released in fresh YPD medium \pm 0.033% MMS. All strains are *bar1Δ*. For d) to g), cells were treated with 0.033% MMS for 2 h and then released in fresh YPD medium. Western blots were probed with anti-gamma-H2A, anti-histone H2A, anti-*Rad53*, and anti-PGK1 (loading control) antibodies as described in Materials and methods.

Next, we assessed the ability of cells to recover from genotoxic stress induced by MMS. Exponentially growing cells were treated with 0.033% MMS for 2 h to induce an intra-S-phase arrest (Gasch et al. 2001). Afterward, cells were released into fresh medium, and cell cycle progression and DDR deactivation were monitored. We observed that MMS-treated *opi1Δ* cells had a significantly increased population of G1 cells, indicating a persistent G1/S arrest of the cell cycle (Fig. 2d and e). In contrast, wild-type cells displayed an intra-S phase arrest, which is consistent with the replication stress induced by MMS (Fig. 2d). It is noteworthy that, consistent with the result from Fig. 2b, gamma-H2A levels were significantly lower in *opi1Δ* cells relative to wild-type cells after 2 h of MMS treatment. However, no significant difference was observed in the disappearance of gamma-H2A during the recovery period (Fig. 2f). Notably, the decrease in gamma-H2A levels observed in *opi1Δ* cells was not due to histone depletion since total histone H2A levels were not decreased in *opi1Δ* cells after genotoxic stress (Fig. 2f). Also, we found that both wild-type and *opi1Δ* cells exhibited comparable levels of Rad53 activation after 2 h of MMS treatment, and there was no difference in Rad53 deactivation during recovery (Fig. 2g). Our results suggest that DNA damage signaling downregulation is not altered in *opi1Δ* cells but indicate that MMS treatment induces cell cycle defects related to delayed G1-to-S-phase progression.

Opi1 migrates to the nucleus upon genotoxic stress induced by MMS

Knowing that transcriptional repression function of *Opi1* is dependent on its cellular localization (Fig. 1a) and because its absence causes genotoxin sensitivity and cell cycle defects (Figs. 1c and 2, respectively), we investigated *Opi1* localization after treatment with 0.1% MMS since this concentration was previously used to monitor global changes in gene expression (Jelinsky and Samson 1999). Using a strain expressing *Opi1*-GFP, we recapitulated *Opi1* cellular localization during inositol metabolism (Fig. 3a panels I and II). MMS treatment of cells supplemented with 100 μM of inositol (+INO), a concentration known to sufficiently repress *INO1* (Hirsch and Henry 1986), did not cause any significant alteration in *Opi1*-GFP intensity or localization (data not shown). Surprisingly, MMS treatment of cells kept without inositol supplementation (–INO) induced a rapid translocation of *Opi1*-GFP to the nucleus (Fig. 3a and b and Supplementary Fig. 4). Since *Opi1* mediates gene repression when it translocates to the nucleus, this observation might suggest that the nuclear localization of *Opi1* is important to mediate MMS-induced stress resistance. Importantly, we found that yeast cells treated with MMS in SC – INO remained viable (Fig. 3c), which implies that *Opi1* does not fully repress *INO1* in these conditions; otherwise, cells would die.

Subsequently, to understand if *Opi1* migration to the nucleus in the presence of MMS causes gene repression, we looked at *INO1* expression during MMS treatment by real-time quantitative PCR (RT-qPCR) analysis (Fig. 3d). While MMS treatment does not change *INO1* expression in wild-type cells supplemented with inositol (+INO), there is a substantial decrease in *INO1* expression in the absence of inositol (–INO). Strikingly, *INO1* expression significantly increases in an *opi1Δ* strain upon MMS treatment in both +INO and –INO conditions (Fig. 3d). These results show that under genotoxic stress, *Opi1* migrates to the nucleus to repress *INO1* expression in order to downregulate inositol synthesis. Because *Opi1* represses genes containing *UAS_{INO}*, it is possible that in its absence, several other genes are also upregulated upon MMS treatment.

Opi1 modulates gene expression during genotoxic stress induced by MMS

In order to gain a deeper understanding of the impact of *Opi1*-mediated gene expression regulation in response to MMS treatment on a transcriptome-wide scale, we conducted RNA-seq experiments using exponentially growing cells (wild-type vs *opi1Δ*) supplemented with 100 μM inositol (SC + INO) in the presence of ±0.1% MMS for 60 min (Fig. 4a). We selected this specific experimental condition based on 2 primary reasons. First, our experiments have demonstrated the heightened sensitivity of *Opi1*-deficient cells to MMS when grown in medium supplemented with inositol (Fig. 3c). Second, our own observation revealed that MMS-induced upregulation of *INO1* expression in *opi1Δ* cells is independent of inositol supplementation (Fig. 3d).

Previous studies utilizing transcriptome analysis have characterized genes whose expression is modulated by *Opi1* (Santiago and Ben Mamoun 2003; Jesch et al. 2005). As anticipated, the results of our differential expression analysis revealed the upregulation of several canonical *Opi1* targets in *opi1Δ* cells (Fig. 4b top panel and Supplementary Table 4). Interestingly, our results demonstrated that the expression of several *Opi1* canonical targets was further augmented following MMS treatment in *opi1Δ* cells (Supplementary Table 8 and Fig. 4b, compare top and bottom panels), indicating the crucial role of *Opi1* in modulating gene expression during such conditions. Moreover, to reinforce this hypothesis, we observed that genes highlighted in Fig. 4b were repressed in wild-type cells exposed to MMS (Fig. 4c and Supplementary Table 5). This finding further supports the notion that *Opi1* plays a critical role in regulating gene expression in response to MMS treatment and that its absence leads to misregulation of several genes, ultimately resulting in MMS sensitivity. Curiously, contrary to the observed trend in *INO1* expression determined by RT-qPCR (Fig. 3d), *INO1* expression did not follow the same pattern as other *Opi1* targets, as MMS treatment did not increase *INO1* expression in *opi1Δ* cells (Fig. 4b, compare top and bottom panels). The observed discrepancy in *INO1* expression between the RT-qPCR and RNA-seq analyses could stem from various factors, including technical variability inherent to the methods, biological variability between samples, and differences in the sensitivity and dynamic range.

To clarify the role of *Opi1* during MMS treatment, we sought to identify genes exhibiting differential expression in an *Opi1*-dependent manner (Supplementary Table 6). We then selectively screened genes showing a ±0.8 log₂ fold change with an adjusted P-value of ≤0.05 and conducted a statistical overrepresentation analysis using the PANTHER database (<http://pantherdb.org>) to investigate biological processes that were enriched within our gene list. With that, we were able to see which biological processes are misregulated upon MMS treatment in an *Opi1*-dependent manner. Notably, we observe a significant enrichment of phospholipid biosynthesis genes within our dataset (Fig. 4d). Moreover, we observe that *Opi1* is pivotal in repressing the expression of proteins associated with MAPK signaling such as cellular conjugation, transduction of mating signaling (pheromone response), and filamentous growth (Fig. 4d), thereby indicating its significance in modulating these biological processes during genotoxic stress. In addition, we generated an interactome of the DEGs, which aligns in clusters using the STRING database (<http://string-db.org>) (Fig. 4e). Importantly, the upregulation of genes involved in the mating signaling pathway is associated with the inhibition of the G1-to-S-phase transition. This finding is particularly relevant since we observed that cells lacking *Opi1* exhibit a delayed transition from the G1 phase to the S phase

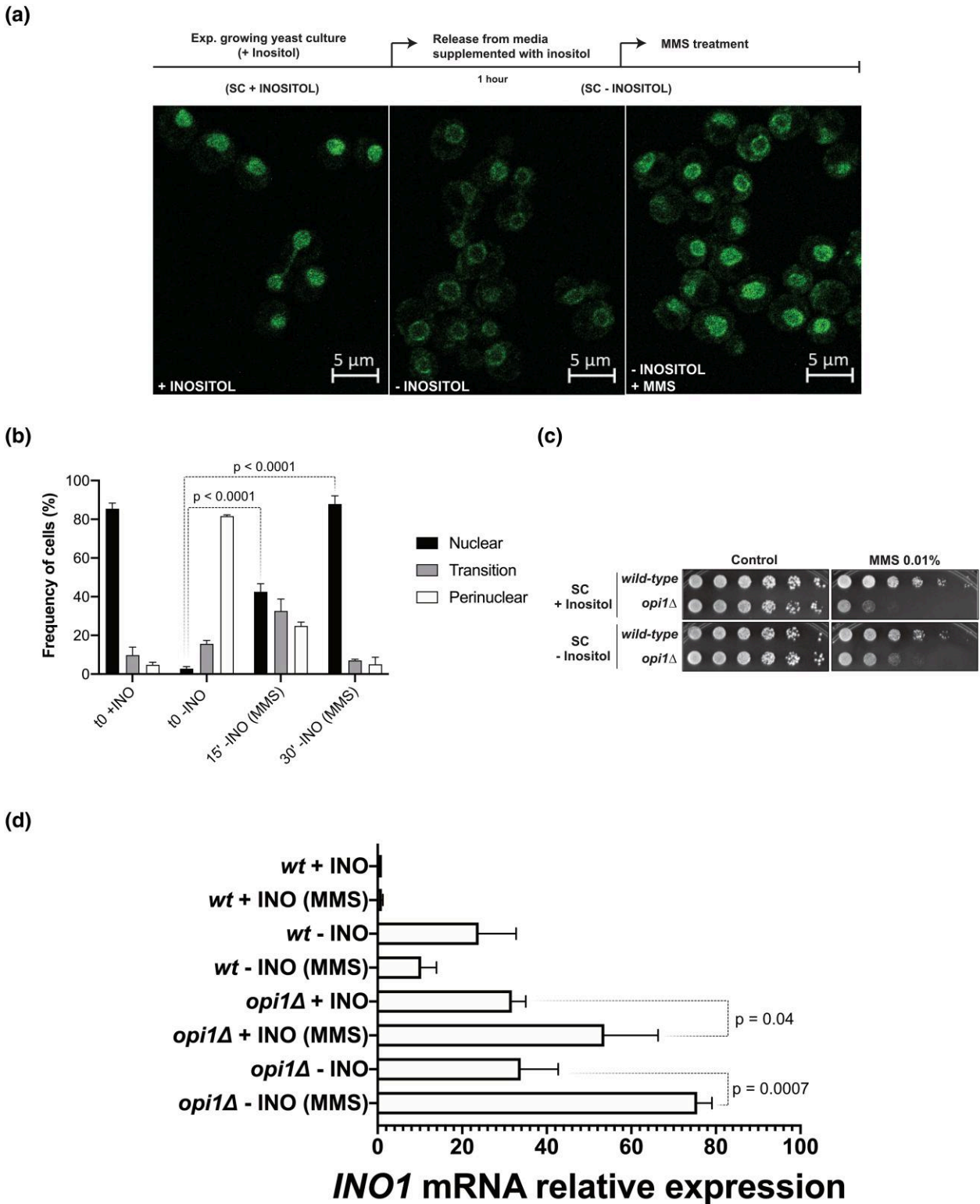


Fig. 3. *Opi1* translocates to the nucleus after MMS treatment and is required to repress *INO1* expression during genotoxic stress. a) and b) Effect of inositol availability and MMS treatment on *Opi1*-GFP localization. Cellular localization of *Opi1*-GFP was scored manually, and the means and standard deviations from 2 independent experiments are shown. c) Cells treated with MMS in medium lacking inositol are still viable. d) Effect of inositol availability and MMS treatment on *INO1* expression by RT-qPCR. Relative values of mRNA are described as fold change relative to a control condition (wt + INO). For c), 4-fold serial dilutions were spotted on the media indicated in the figure, and plates were grown for 2–3 days at 30°C. For d), experiments were made with 3 biological replicates, and statistical significance of the differences observed was calculated using 1-way ANOVA with Tukey’s multiple comparison test.

under genotoxic stress induced by MMS (Fig. 2a, d, and e), which can be correlated with the observed delay in cell growth in both solid and liquid cultures (Fig. 1c and e, respectively).

Finally, our findings challenge the canonical role of *Opi1* as a gene repressor by also demonstrating its involvement in the upregulation of several genes related to amino acid biosynthesis,

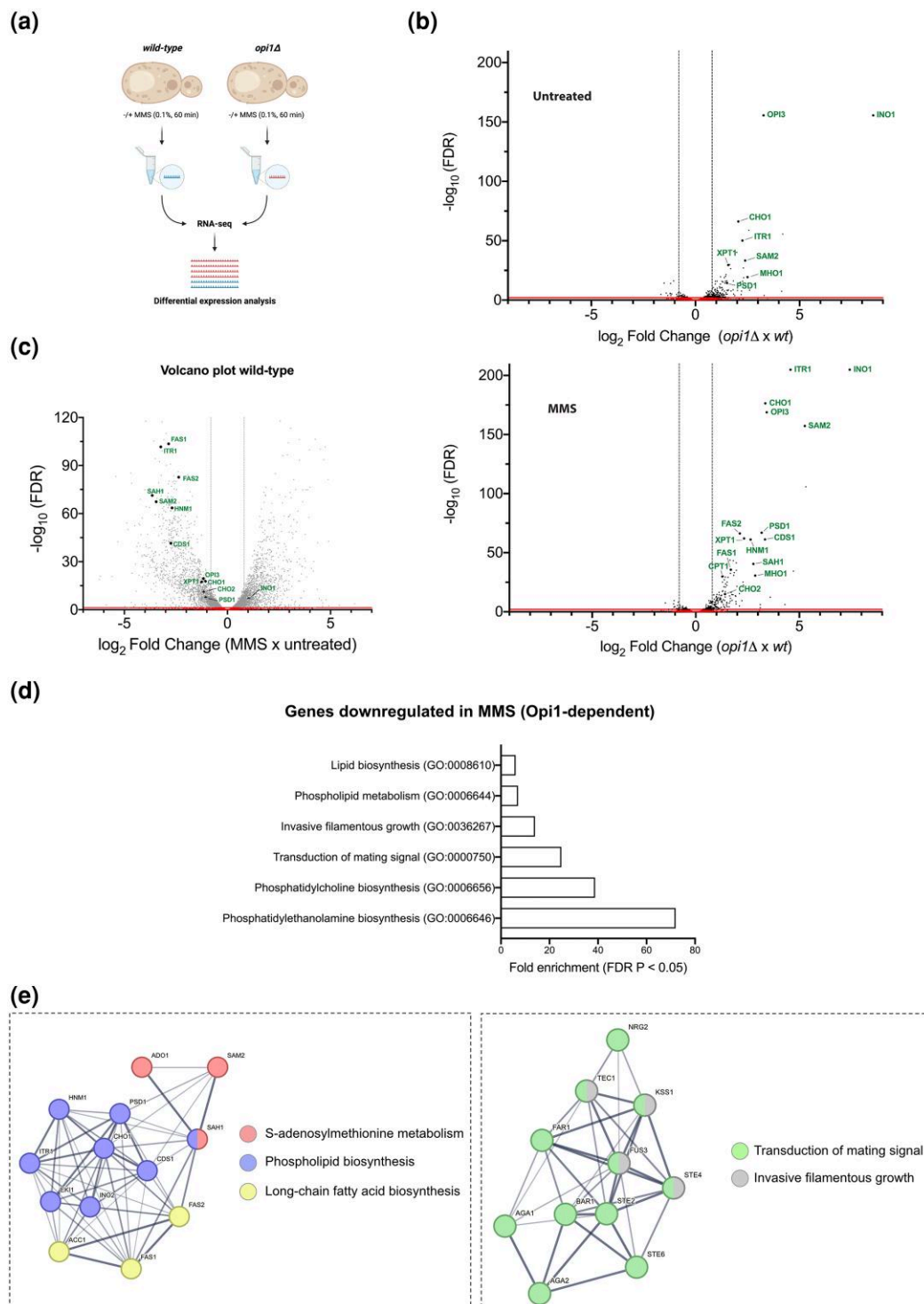


Fig. 4. *Opi1* is important to modulate gene expression during MMS-induced genotoxic stress. a) Schematic representation of RNA-seq performed in wild-type and *opi1Δ* cells cultured in minimal medium (SC + INO) with or without 0.1% MMS treatment for 60 min. Created with BioRender.com. b) Volcano plot illustrating the differential gene expression between *opi1Δ* and wild-type cells (top panel) and between *opi1Δ* + MMS and wild-type + MMS cells (bottom panel). c) Volcano plot displaying the differential gene expression between wild-type + MMS and wild-type cells. For b) and c), genes with significant differential expression are plotted based on a threshold of ± 0.8 \log_2 fold change and an adjusted P-value of 0.05. The red line (x-axis) and dotted line (y-axis) represent the significant cutoff. Known genes regulated by *Opi1* are labeled in both plots. d) Bar plot illustrating the top enriched biological processes identified in the gene set that are repressed during MMS treatment in an *Opi1*-dependent manner (Supplementary Table 6). Each bar represents a specific biological process, and the height of the bar corresponds to the fold enrichment with a P-adjusted value of 0.05. GO analysis was conducted in PANTHER using the overrepresentation test with *S. cerevisiae* serving as the reference list, which includes all genes in the database. The test type used was FISHER, and the FDR correction was applied. e) GO term enrichment network depicting the relationships between genes grouped in clusters that are repressed during MMS treatment in an *Opi1*-dependent manner (Supplementary Table 6). The enrichment analysis was performed using the STRING database, which integrates various sources of functional information with a confidence score of >0.4 (medium confidence). Nodes in the network represent genes, while edges indicate the connections between them. The color of the nodes represents a biological process, and the thickness of the edges represents the increased confidence of the interaction between nodes.

including arginine and histidine biosynthesis, the mitochondrial glycine cleavage system, and sulfate assimilation (Supplementary Fig. 5a and b). However, it is important to emphasize that we cannot exclude the possibility that *Opi1* indirectly regulates many of these targets by influencing the expression of genes involved in transcriptional regulation.

The constitutive activation of *Ino2-Ino4*-responsive genes is the cause of MMS sensitivity in cells lacking *Opi1*

A previous study by Heyken et al. (2005) provided evidence for a physical interaction between *Opi1* and a functional domain within the transcriptional factor *Ino2*. This interaction was shown to result in the repression of *Ino2*'s ability to promote gene expression. Moreover, our RNA-seq analysis revealed that *INO2* and *INO4* expression is upregulated in *opi1Δ* cells following MMS treatment (Supplementary Table 8), indicating a role for *Opi1* in *INO2* repression during MMS-induced stress. In line with that, we investigated if *INO2* and *INO4* deletions could rescue the genotoxin sensitivity of cells lacking *Opi1*. Remarkably, *opi1Δino2Δ* and *opi1Δino4Δ* strains are rescued for the sensitivity to MMS and 4NQO, which indicates that constitutive expression of *Ino2-Ino4*-responsive genes is causing the hypersensitivity to these genotoxins (Fig. 5a). Interestingly, our findings demonstrate that the deletion of *INO4* alleviates the sensitivity to zeocin and bleomycin, while the deletion of *INO2* does not have the same effect. Moreover, we notice that *ino4Δ* cells display sensitivity to heat shock but not to other types of proteotoxic stress inducers (Supplementary Fig. 6). Intriguingly, *ino2Δ* cells do not exhibit sensitivity to heat shock, and the deletion of *Opi1* rescues the heat shock sensitivity of *ino4Δ* cells while conferring sensitivity to *ino2Δ* cells (Supplementary Fig. 6). These findings provide further support for previous evidence demonstrating the distinct roles of *Ino2* and *Ino4* in transcriptional regulation under various stress conditions (Chumnanpuen et al. 2013). These consistent findings underscore the unique and context-dependent roles of *Ino2* and *Ino4* in transcriptional regulation.

The transcriptional activator *Ino2-Ino4* upregulates the expression of several other genes counteracting *Opi1*, suggesting that constitutive expression of another *Ino2-Ino4* target in *opi1Δ* cells is causing MMS sensitivity. Thus, we conducted a screening of a small set of genes whose expression is dependent on *Ino2-Ino4* and that are upregulated in *opi1Δ* cells treated with MMS (Supplementary Table 8). We selected genes involved in PI (*INO1*, inositol-3-phosphate synthase; *ITR1*, inositol transporter) and phosphatidylcholine (PC) biosynthesis (*OPI3*, phospholipid methyltransferase; *CKI1*, choline kinase) since these pathways are upregulated in *opi1Δ* cells under genotoxic stress (Fig. 4d). Consequently, we deleted these genes in an *opi1Δ* background with the expectation that it would phenocopy the MMS resistance of *opi1Δino2Δ* and *opi1Δino4Δ* strains. Surprisingly, all double mutants showed growth defects and increased MMS sensitivity (Supplementary Fig. 7a and b). This negative genetic interaction between *Opi1* and genes from PI and PC biosynthesis suggests that upregulation of 1 or more genes repressed by *Opi1* in the absence of *Ino1*, *Itr1*, *Opi3*, and *Cki1* negatively impacts the cell.

It is well established that the expression of *INO1* is positively regulated by *Ino2-Ino4* and massively increases in the absence of *Opi1* (Figs. 3d and 4b) (Graves and Henry 2000; Santiago and Ben Mamoun 2003; Jesch et al. 2005). To test if constitutive expression of *INO1* in cells lacking *Opi1* is causing MMS sensitivity, we overexpressed *INO1* by cloning it under the control of the strong inducible *GAL1* promoter. Overexpression of *INO1* from the

GAL1 promoter rescues the inositol auxotrophy of an *ino1Δ* strain (Supplementary Fig. 7c) but does not show sensitivity to MMS (Supplementary Fig. 7d), which is an indication that increased levels of inositol do not affect the response to MMS-induced stress.

PP-IPs counteract the effects of *Opi1* on gene expression during MMS-induced stress

In budding yeast, *Kcs1* catalyzes the pyrophosphorylation of the C5 α -phosphate of IPs to form 5-PP-IP₄ and 5-PP-IP₅, respectively (Saiardi et al. 1999). Miriam Greenberg's group has proposed that inositol pyrophosphate 5-PP-IP₄ may play a role in recruiting transcriptional activators, such as *Ino2-Ino4*, to the promoter region of *INO1*, thus regulating its expression (Ye et al.'s (2013). This suggests that deletion of *Kcs1*, involved in the synthesis of PP-IPs, could lead to a reduction in the expression of genes that are upregulated by *Ino2-Ino4* in cells lacking *Opi1*. We consequently conducted a genetic rescue experiment by deleting *Kcs1* in an *opi1Δ* strain to determine whether it would ameliorate the MMS sensitivity observed in the absence of *Opi1*. Remarkably, *Kcs1* deletion significantly rescues the MMS sensitivity observed in the *opi1Δ* strain (Fig. 5b). Additionally, *Kcs1* deletion also rescues the G1/S arrest observed in *opi1Δ* cells during recovery from MMS treatment (Fig. 5c). These findings might indicate that in the presence of stress induced by MMS, *Opi1* functions in opposition to PP-IPs to facilitate the modulation of gene expression in an *Ino2-Ino4*-dependent manner. In agreement with that, cells lacking the *Siw14* phosphatase that counteracts *Kcs1*-dependent PP-IP synthesis have a mild sensitivity to MMS (Supplementary Fig. 8a). It has been shown that *siw14Δ* mutants have increased levels of 5-PP-IP₅ (Steidle et al. 2016).

To assess the impact of elevated PP-IP levels on MMS sensitivity, we constructed a strain in which *KCS1* was placed under the control of the *GAL1* promoter. Our data demonstrate that induction with galactose results in elevated levels of *Kcs1* mRNA and protein (see Supplementary Fig. 8b and c). Remarkably, wild-type cells overexpressing *Kcs1* phenocopy an *opi1Δ* strain with cell growth delay and sensitivity to MMS (Fig. 5d). Taken together, our results suggest that increased PP-IP levels due to the lack of the transcriptional repressor *Opi1* lead to impaired capacity to cope with stress induced by MMS.

Intriguingly, while the deletion of *Ino2-Ino4* rescues the sensitivity to other genotoxic agents (Fig. 5a), the deletion of *Kcs1* in *opi1Δ* cells instead increases sensitivity to zeocin/bleomycin and 4NQO (Fig. 5e). Furthermore, *opi1Δkcs1Δ* cells show sensitivity to HU and CPT (Supplementary Fig. 8d). These findings suggest a complex interplay between *Opi1* and PP-IPs in the cellular response to different sources of stress. The specific mechanisms underlying the distinct responses to genotoxic agents warrant further investigation.

Opi1 regulates mitochondrial function upon MMS-induced stress

Remarkably, our RNA-seq data, combined with GO analysis, demonstrate a significant enrichment of genes with mitochondrial localization that show significant upregulation or downregulation (\log_2 fold change ≥ 0.8 or ≤ -0.8 , respectively) in response to MMS treatment in *opi1Δ* cells (Fig. 6a). Of note, it has been shown that the lack of *Opi1* causes a decrease in mitochondrial membrane cardiolipin content leading to respiratory defects and a petite-negative phenotype (*pet*⁻) due to the inability to survive in the presence of damaged mtDNA (Dunn et al. 2006; Luévano-Martínez et al. 2013). Interestingly, the expression of *Opi1* is upregulated in cells lacking mtDNA (ρ^0), emphasizing the critical role of *Opi1* in managing mitochondrial dysfunction (Singh et al. 2004). Our

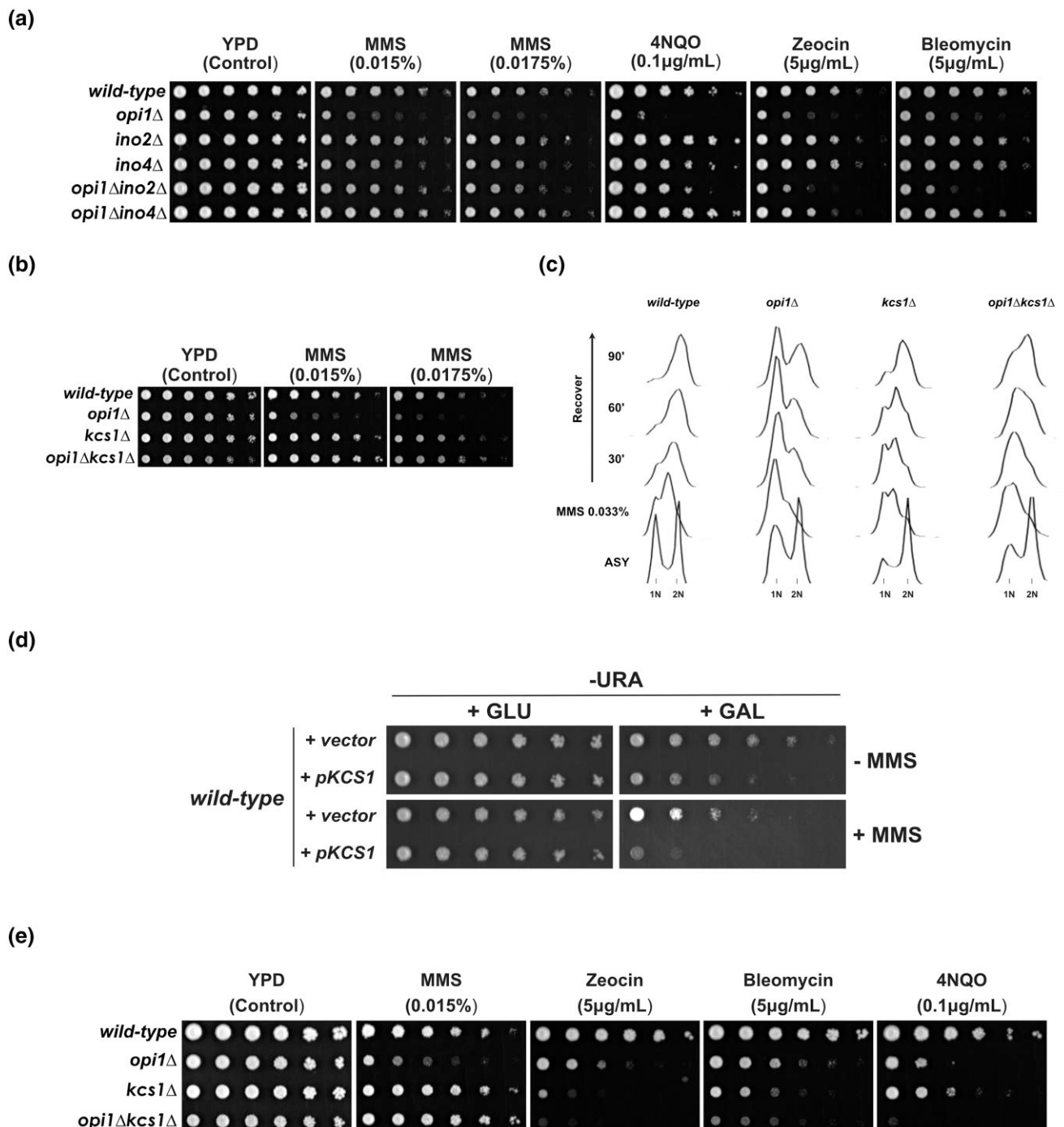


Fig. 5. *Opi1* counteracts the function of the transcriptional repressor *Ino2-Ino4* and the PP-IP kinase *Kcs1* during MMS-induced genotoxic stress. a) Deletion of *INO2* and *INO4* rescues the genotoxin sensitivity of cells lacking *Opi1*. b) and c) Deletion of the PP-IP kinase *KCS1* rescues the MMS sensitivity b) and the delayed G1-to-S-phase transition c) of cells lacking *Opi1*. d) Overexpression of *Kcs1* phenocopies the MMS sensitivity of an *opi1*Δ strain. e) Different from the effect on MMS, deletion of *Kcs1* causes a synergistic increase in sensitivity of *opi1*Δ cells in the presence of several DNA damage agents. For a), b), d), and e), 4-fold serial dilutions were spotted on the media indicated in the figure, and plates were grown for 2–3 days at 30°C.

results confirm the sensitivity of *Opi1*-deficient cells to ethidium bromide (EtBr), a known inducer of mtDNA damage, and we demonstrate that deletion of *Ino2-Ino4* and *Kcs1* rescues the *pet*⁻ phenotype observed in *opi1*Δ cells (Fig. 6b). Moreover, cells lacking *Opi1* are sensitive to antimycin A, which can also be reverted by deletion of *INO2* and *INO4* (Supplementary Fig. 9). Antimycin A, an inhibitor of complex III of the electron transport chain, can induce oxidative damage to mtDNA by increasing ROS leakage (Doudican et al. 2005).

Together, these data strongly suggest that constitutive activation of *Ino2-Ino4* is also involved in mitochondrial dysfunction. Therefore, we hypothesize that the sensitivity of *opi1*Δ cells to MMS is more likely due to increased mtDNA instability rather than direct damage to nuclear DNA, as MMS is also known to induce damage to mtDNA (Kitanovic et al. 2009). Notably, the combined treatment of MMS and EtBr did not result in an additive or synergistic increase in the sensitivity of *opi1*Δ cells, suggesting that the MMS sensitivity of these cells may be attributed to

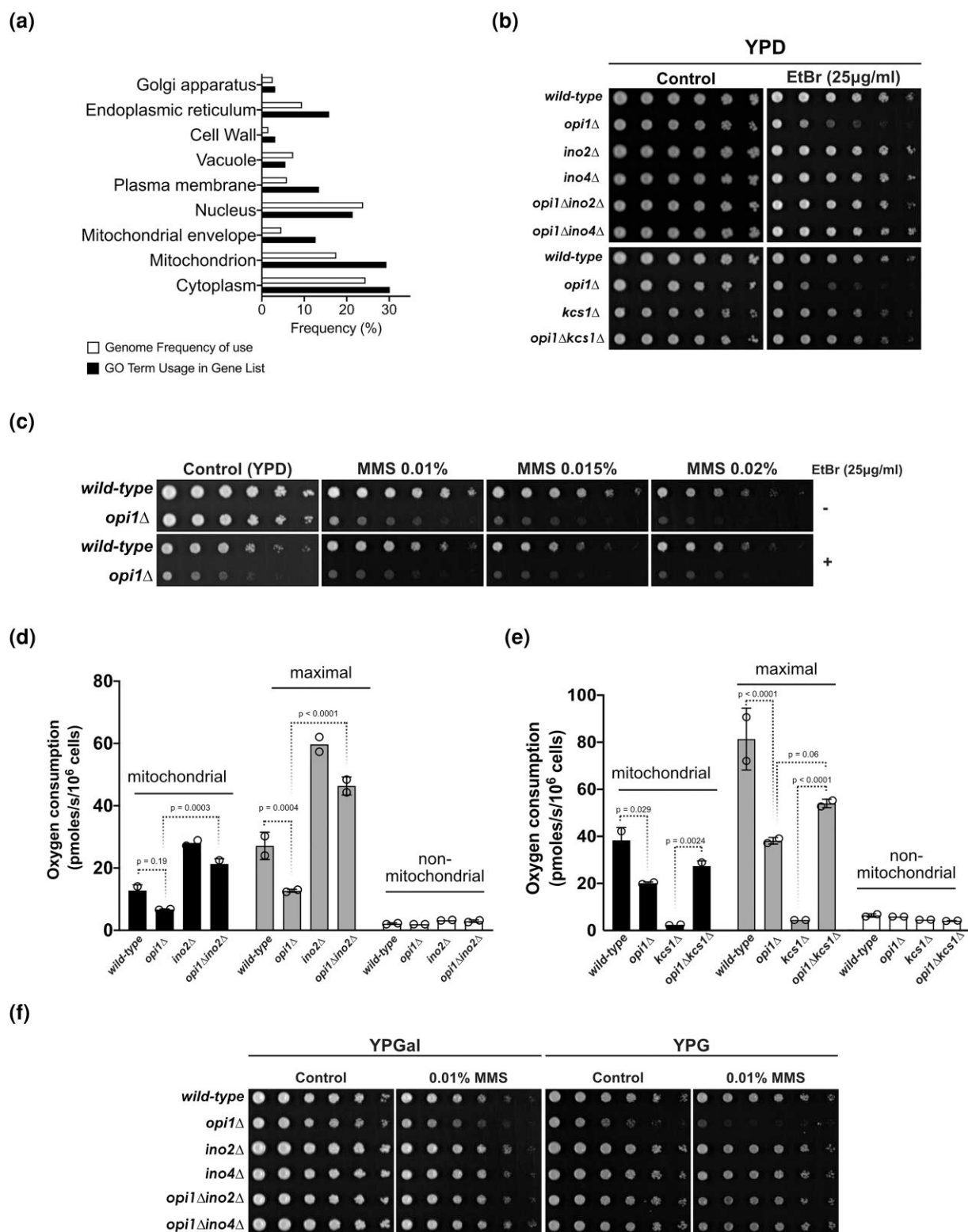


Fig. 6. *Opi1* is important to prevent mtDNA instability and to promote respiratory function during MMS-induced genotoxic stress. a) GO Slim term analysis using the Yeast Genome Database's GO Slim Mapper revealing enrichment of genes associated with specific cellular components that are misregulated in cells lacking *Opi1* upon MMS treatment (Supplementary Table 8). All genes from *S. cerevisiae* were included as a reference list. b) Deletion of *INO2*, *INO4*, and *KCS1* rescues the EtBr sensitivity of cells lacking *Opi1*. c) Combined treatment of MMS and EtBr does not synergistically enhance the sensitivity phenotype of an *opi1*Δ strain beyond the sensitivity conferred by each drug independently. d) Deletion of *INO2* completely restores mitochondrial respiratory capacity of cells lacking *Opi1*. e) The *opi1*Δ*kcs1*Δ double mutant exhibits restored mitochondrial respiratory capacity, which is impaired in the single *opi1*Δ and *kcs1*Δ mutants. f) Deletion of *INO2* and *INO4* rescues the MMS sensitivity of cells lacking *Opi1* in medium containing respiratory carbon sources (galactose and glycerol). For b), c), and f), 4-fold serial dilutions of the samples were spotted onto the designated growth medium as indicated in the figure. The plates were then incubated at 30°C for 2–3 days, except for plates containing YPG medium, which were photographed after 4 days of incubation.

increased mtDNA damage rather than direct nuclear DNA damage (Fig. 6c).

To elucidate if the rescue of MMS sensitivity is related to a recovery of mitochondrial function, we sought to investigate if *INO2* deletion would also rescue the respiratory defect and MMS sensitivity of *opi1Δ* cells in the presence of a respiratory carbon source. Utilizing the Oroboros system, we measured the rate of oxygen consumption in respiratory medium (YPEG; 2% glycerol and 1% ethanol) and found that deletion of *Opi1* led to a reduction in mitochondrial respiration, which is consistent with a defect in oxidative phosphorylation. Interestingly, the respiratory defect in *Opi1*-deficient cells was fully restored upon the deletion of *INO2* (Fig. 6d). Surprisingly, the *opi1Δkcs1Δ* strain showed a significant rescue of mitochondrial respiration (Fig. 6e). It is worth noting that cells lacking *Kcs1* are typically unable to survive in a nonfermentable carbon source medium due to the critical role of PP-IPs in mediating the diauxic shift through the modulation of *Gcr1*, a key transcription factor involved in glycolysis regulation (Szijgyarto et al. 2011). Furthermore, we tested if the deletion of *Ino2-Ino4* would rescue the MMS sensitivity of *opi1Δ* cells in the presence of a respiratory carbon source (Fig. 6f). In the presence of galactose, which is known to induce simultaneous respiration and fermentation (Fendt and Sauer 2010), deletion of *Ino2* and *Ino4* completely restored the MMS sensitivity of *opi1Δ* cells. Importantly, in the presence of glycerol, there was a significant rescue of MMS sensitivity, which indicates that constitutive expression of genes regulated by *Ino2-Ino4* increases mtDNA instability in the presence of MMS.

Overall, our findings indicate that under MMS-induced stress, *Opi1* is important to the fine-tuning of gene expression to ensure appropriate cellular responses. Future investigations into these mechanisms will deepen our understanding of how cells maintain homeostasis and cope with genotoxic stress.

Discussion

Through transcriptomic and functional analyses, our study provides evidence supporting the role of the transcriptional repressor *Opi1* as a crucial regulator of gene expression during MMS-induced stress. Our findings indicate that the constitutive activation of the transcriptional activator *Ino2-Ino4* is associated with increased MMS sensitivity, cell cycle defects, and mitochondrial dysfunction. Importantly, reduction of PP-IP synthesis resulting from the deletion of *Kcs1* can ameliorate the MMS-induced defects observed in *opi1Δ* cells. In contrast, the overexpression of *Kcs1* in wild-type cells mimics the phenotype of cells lacking *Opi1*, suggesting that PP-IPs compete with *Opi1* for the expression of genes in response to MMS treatment.

Opi1 modulates different biological processes during MMS-induced stress

The regulation of multiple metabolic pathways by *Opi1* poses a significant challenge in identifying the underlying cause of MMS sensitivity. In this study, we employed RNA-seq analyses to uncover *Opi1*'s modulation of diverse biological processes during MMS treatment. Importantly, our findings underscore the pivotal role of *Opi1* in the repression of phospholipid biosynthesis after MMS-induced stress. It is worth noting that despite the involvement of *Opi1* in regulating genes involved in phospholipid biosynthesis, the deletion of several genes related to this process did not rescue the MMS sensitivity of *opi1Δ* cells. Surprisingly, *Opi1* exhibited negative genetic interactions with genes involved in PI (*Ino1* and *Itr1*) and PC synthesis (*Opi3* and *Cki1*), as revealed by

Supplementary Fig. 7a and b. One explanation is that impairment of PC synthesis leading to an unbalance of the phosphatidylethanolamine (PE)/PC ratio causes ER stress (Thibault et al. 2012). Furthermore, accumulation of the PC metabolic intermediate phosphatidylmonomethylethanolamine (PMME) has been shown to exacerbate ER stress in yeast cells (Ishiwata-Kimata et al. 2022). Given the increased expression of genes upstream of *Opi3*, such as *CDS1*, *CHO1*, *PSD1*, and *CHO2* (Supplementary Table 8), it is conceivable that PMME levels are even higher in *opi1Δ* cells. Significantly, previous research has demonstrated that exposure to MMS leads to lipid stress specifically at the inner nuclear membrane of RPE-1 cells. This stress response is mitigated through a combination of nuclear membrane deformation and the emission of nuclear lipid droplets (Ovejero et al. 2021). Given that alterations in the nuclear membrane can impact critical nuclear processes including DNA replication, transcription, and repair (Mekhail and Moazed 2010), it is of particular interest to explore the potential involvement of *Opi1* in the preservation of nuclear membrane integrity. Further investigation could help to clarify the specific mechanisms by which *Opi1* and proteins involved in phospholipid biosynthesis are involved in the response to MMS-induced stress.

Furthermore, our study revealed that multiple proteins involved in the transduction of mating signaling with cellular conjugation and invasive filamentous growth are significantly downregulated during genotoxic stress in an *Opi1*-dependent manner (Fig. 4d). Filamentous growth in yeast is triggered by nutrient limitation and involves various pathways including MAPK, Ras/PKA, *Snf1*, and TOR. A comprehensive review of filamentous growth response is available in Cullen and Sprague (2012). Interestingly, it has been shown that genotoxic stress induces filamentous growth in budding yeast (Jiang and Kang 2003) and in *Candida* species (Shi et al. 2007; Bravo Ruiz et al. 2020). Noteworthy, it has been proposed that *Saccharomyces cerevisiae* *Opi1* regulates filamentous growth through the upregulation of *Flo11*, a glycosylphosphatidylinositol (GPI) anchored cell surface glycoprotein that determines colony morphology in yeast (Reynolds 2006). While we did not observe significant changes in *Flo11* expression in *opi1Δ* cells, our RNA-seq analysis revealed significant upregulation of several genes that inhibit filamentous growth, especially after MMS treatment, including the *Fus3* MAP kinase, the *Nrg2* transcriptional repressor, and *Mho1*, a protein of unknown function. Interestingly, while *Fus3* inhibits filamentous growth by causing degradation of the transcription factor *Tec1* via phosphorylation (Bao et al. 2004; Chou et al. 2004), it promotes mating signaling by phosphorylating the CDK inhibitor *Far1*, leading to its association with *Cln-Cdc28* (Peter et al. 1993; Tyers and Futcher 1993; Peter and Herskowitz 1994). Consequently, *Far1* impedes the function of the *Cln-Cdc28* complex in the G1 phase, thus preventing cells from advancing from G1 to START (reviewed in Bardwell 2005; Sieber et al. 2023). Our findings are intriguing as they provide evidence for the involvement of *Opi1* in promoting the suppression of mating signaling under MMS-induced stress conditions. To begin with, *FAR1* and *FUS3* expression are downregulated in an *Opi1*-dependent manner (Fig. 4e). Moreover, we observed an increase in the percentage of cells in the G1 phase following a 2-h MMS treatment (Fig. 2d and e). Furthermore, upon release from MMS treatment, *opi1Δ* cells exhibited a delayed G1-to-S-phase transition, indicating that mating signaling is hyperactivated (Fig. 2e).

It is important to note that all of our results were obtained using BY4741, which is known to be a filamentation-deficient yeast strain. Thus, any phenotypes related to filamentous growth,

such as biofilm formation and invasive growth on agar, were not observable in our study.

PP-IPs generated by *Kcs1* dampen *Opi1*-mediated gene expression during genotoxic stress

Our findings reveal that the MMS sensitivity and cell cycle defects observed in *opi1Δ* cells can be rescued by deletion of the PP-IP kinase *Kcs1* (Fig. 5b and c), which provides compelling evidence that PP-IP synthesis in the absence of *Opi1* is detrimental for cells under MMS-induced stress. It has been previously shown that *KCS1* possesses UAS_{INO} in its promoter region and that its expression is upregulated when inositol concentration increases (Wimalarathna et al. 2011). Additionally, the IP multikinase *Ipk2* (*ARG82*) that catalyzes the synthesis of IP₄ and IP₅ also contains UAS_{INO} in its promoter region, and its expression is dependent on *Ino2-Ino4* (Wimalarathna et al. 2011). However, while we did not observe changes in *KCS1* and *ARG82* expression levels in our dataset for *opi1Δ* cells (Supplementary Tables 4, 6, and 8), it is plausible that PP-IP synthesis is elevated in these cells, as previous studies have demonstrated an increase in *Kcs1* protein levels in the absence of *Opi1* (Ye et al. 2013). It is interesting to note that while *INO1* overexpression does not sensitize cells to MMS, that of *KCS1* does (Supplementary Fig. 7d and Fig. 5d, respectively), suggesting that *INO1* overexpression probably does not lead to an increase in PP-IP synthesis, which might indicate that there is a rate-limiting step reaction downstream of inositol-3-phosphate synthesis restraining PP-IP concentration in the cell.

How do PP-IPs affect the DDR leading to MMS sensitivity in the absence of *Opi1*? In budding yeast, PP-IPs are synthesized from IP₅ and IP₆ in a reaction catalyzed by the PP-IP kinases *Kcs1* and *Vip1* (Saiardi et al. 1999; Mulugu et al. 2007; Lee et al. 2008). *Kcs1* catalyzes the conversion of IP₅ and IP₆ to different isomers of PP-IPs such as 5-PP-IP₄ and 5-PP-IP₅ (Saiardi et al. 1999). It is established that PP-IPs modulate protein function through either nonenzymatic pyrophosphorylation of proteins or allosteric regulation through its interaction with protein domains (Wilson et al. 2013). However, up until now, only a few targets have been characterized. *Kcs1* and its mammalian orthologs IP6Ks have been implicated in several biological processes such as cell cycle progression, DNA repair, telomere length, nutrient signaling, ESR, and polyphosphate metabolism, among others (Auesukaree et al. 2005; Saiardi et al. 2005; Sziogyarto et al. 2011; Banfic et al. 2013, 2016; Wilson et al. 2013; Worley et al. 2013). In light of these considerations, our findings suggest a potential role for PP-IPs in modulating the function of *Opi1*-dependent genes during MMS-induced stress. One plausible explanation is the previous proposal that PP-IPs generated by *Kcs1* are important to induce *INO1* expression, indicating their potential involvement in recruiting *Ino2-Ino4* to UAS_{INO} regions (Ye et al. 2013). However, this hypothesis requires further investigation, as there is currently no evidence suggesting that PP-IPs directly target *Ino2-Ino4* for either allosteric regulation or pyrophosphorylation. It is also important to note that while *Kcs1* deletion rescues MMS sensitivity of *opi1Δ* cells, it shows a synergistic increase in sensitivity to other genotoxins (Fig. 5e). It has been shown that *Kcs1* is required for the DNA hyperrecombination phenotype in yeast cells with defects in protein kinase C1 (*Pkc1*) (Luo et al. 2002). Moreover, it was shown in mammalian cells that the orthologs of *Kcs1* (IP6K1/2/3) are important for homologous recombination repair (Jadav et al. 2013). Hence, it is important to conduct future investigations to examine the potential cooperative role between *Opi1* and *Kcs1* in dealing with specific types of DNA lesions and their impact on DNA repair pathways. By looking into these aspects, we can gain a deeper

understanding of the role of *Opi1* and *Kcs1* for cellular responses to genotoxic stress.

Genotoxin sensitivity in *opi1Δ* is associated with mtDNA damage

Deletion of the transcriptional repressor *Opi1* can induce pleiotropic effects in the cell. First, we ruled out the possibility that the heightened sensitivity of *opi1Δ* cells to MMS is linked to deregulation of the ESR (Fig. 1d). Besides that, tunicamycin treatment did not sensitize *opi1Δ* cells (Fig. 1d), which is in line with previous evidence that the absence of *Opi1* causes an expansion of the ER membrane, alleviating the ER stress induced by dithiothreitol (DTT) or tunicamycin in an UPR-independent manner (Schuck et al. 2009). Furthermore, it was previously shown that cells lacking *Opi1* possess short telomeres (Askree et al. 2004). In contrast, cells lacking *Kcs1* have longer telomeres (York et al. 2005). This finding could indicate that deletion of *Kcs1* rescues MMS sensitivity due to restoration of telomere length. However, this is probably not the case as it was shown that telomere length does not affect cellular fitness or yeast sensitivity to DNA damage (Harari et al. 2017).

MMS can also damage mtDNA leading to direct inhibition of the respiratory chain with increased ROS leakage, oxidative inactivation of glycolytic enzymes, and cell cycle arrest (Kitanovic et al. 2009). Importantly, cells lacking *Opi1* exhibit a decrease in mitochondrial cardiolipin content and are unable to survive in the absence of mtDNA (*pet⁻* phenotype) (Luévano-Martínez et al. 2013). Here, we have discovered that the sensitivity of cells lacking *Opi1* to MMS may be linked to mtDNA instability. This conclusion is supported by 2 observations: (1) deletion of *Ino2-Ino4* genes rescues the *pet⁻* phenotype, antimycin A hypersensitivity, and respiratory capacity of cells lacking *Opi1*; and (2) the combined treatment of MMS and EtBr does not increase the sensitivity of *opi1Δ* cells. It is worth noting that a previous study has demonstrated that cells lacking mtDNA exhibit defects in the G1-to-S-phase progression (Crider et al. 2012). Therefore, it would be important to investigate whether *Opi1* is involved in a mitochondria-to-nucleus retrograde signaling pathway that promotes the transition from the G1 phase to the S phase.

Furthermore, we observed that there is a significant enrichment of mitochondrial proteins among the genes that are differentially expressed in *opi1Δ* cells following MMS treatment (Fig. 6a). For instance, we observed upregulation of *Psd1* in *opi1Δ* cells upon genotoxic stress, as depicted in Fig. 4d and Supplementary Tables 6 and 7. *Psd1* is an enzyme located in the mitochondrial inner membrane that converts phosphatidylserine to PE and plays a crucial role in regulating mitochondrial fusion and morphology (Chan and McQuibban 2012). In contrast, *Aim17*, a protein with a mitochondrial localization and unknown function, was found to be downregulated in *opi1Δ* cells upon genotoxic stress, as shown in Supplementary Fig. 5b and Supplementary Tables 6 and 7. Although limited information is available regarding the precise role of *Aim17* in mitochondrial homeostasis, it seems to be important for mtDNA integrity (Hess et al. 2009). Considering that *Psd1* and *Aim17* are among the top hits of DEGs in our screening (Supplementary Table 6), it would be valuable to explore in future investigations the specific contributions of these genes to the observed genotoxin sensitivity and mitochondrial dysfunction in *opi1Δ* cells.

A working model for *Opi1* function during genotoxic stress

We propose a model in which *Opi1* functions as a crucial sensor of genotoxic stress in budding yeast (Fig. 7). Upon genotoxic stress,

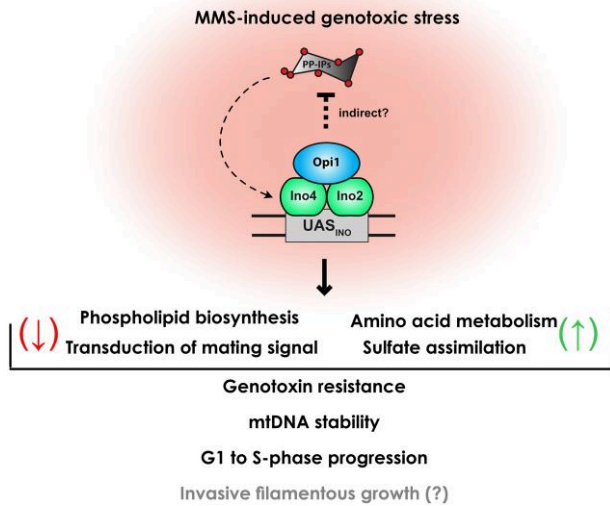


Fig. 7. Model summarizing the *Opi1* role as a key sensor of genotoxic stress in budding yeast. Briefly, upon genotoxic stress induced by MMS, *Opi1* physically interacts with the *Ino2-Ino4* transcriptional activator and counteracts PP-IPs, thereby modulating the expression of several genes. *Opi1* plays an important role in repressing phospholipid biosynthesis and transduction of mating signaling while promoting the expression of genes involved in amino acid metabolism and sulfate assimilation. The coordinated modulation of these genes contributes to genotoxin resistance by preventing mtDNA instability, delaying G1-to-S-phase progression, and potentially promoting invasive filamentous growth. See Discussion for more details.

Opi1 translocates to the nucleus and regulates the expression of multiple genes either directly or indirectly. Specifically, during MMS-induced genotoxic stress, *Opi1* represses phospholipid biosynthesis and MAPK signaling, potentially suppressing cellular conjugation with mating while promoting invasive filamentous growth. Additionally, *Opi1* fosters the expression of genes involved in amino acid metabolism and sulfate assimilation. In cells lacking *Opi1*, the constitutive activation of *Ino2-Ino4* promotes gene transcription, which is further potentiated by PP-IPs produced by *Kcs1*. Consequently, the deletion of *Opi1* results in pleiotropic defects affecting cell cycle progression and mitochondrial function, ultimately reducing cellular fitness. Understanding the interconnections between these processes and determining their relative contributions to MMS resistance will be crucial for future investigations.

In conclusion, this study provides insights into the intricate molecular mechanisms underlying *Opi1*'s role in cellular response to genotoxic stress. The significance of these findings is magnified by the presence of *Opi1* in various pathogenic fungal species, which have become a global concern due to their widespread distribution and increasing antifungal resistance. Importantly, as *Opi1* is absent in mammals, our results highlight its potential as a target for the development of more effective treatments against pathogenic fungal infections.

Data availability

The raw and processed RNA-seq transcriptome data were deposited to the ArrayExpress database at EMBL-EBI (<https://www.ebi.ac.uk/biostudies/>) under the accession number E-MTAB-13057. Strains and plasmids are available upon request. [Supplementary Tables 1 to 3](#) contain the list of yeast strains, plasmids, and primers used in this study, respectively. [Supplementary](#)

[Tables 4 to 8](#) contain the RNA-seq dataset as described in *Materials and methods*. [Supplementary Fig. 1](#) contains the PCA showing the variation among biological replicates in RNA-seq. [Supplementary Fig. 2](#) contains supplemental data in support of [Fig. 1](#). [Supplementary Fig. 3](#) contains supplemental data in support of [Fig. 2](#). [Supplementary Fig. 4](#) contains supplemental data in support of [Fig. 3a and b](#). [Supplementary Fig. 5](#) contains supplemental data in support of [Fig. 4](#). [Supplementary Figs. 6 and 7](#) contain supplemental data in support of [Fig. 5](#). [Supplementary Fig. 8](#) contains supplemental data in support of [Fig. 5d and e](#). [Supplementary Fig. 9](#) contains supplemental data in support of [Fig. 6b](#).

[Supplemental material](#) available at GENETICS online.

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Conflicts of interest

The author(s) declare no conflict of interest.

Author contributions

JRRC conceived the project and wrote the paper. JRRC, GMP, ET-T, MRP, RRF, and RJ designed the experiments, analyzed the data, and helped revise the manuscript. FMdC designed and performed experiments for measuring oxygen consumption and helped revise the manuscript. NCdS-P and OB provided reagents and infrastructure and helped revise the manuscript.

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