**The transcriptional repressor Opi1 modulates the DNA Damage Response by downregulation of inositol pyrophosphates in *Saccharomyces cerevisiae***

Giovanna Marques Panessa[*]; Marina Rodrigues Pires[*]; Rodrigo Rodrigues Felix[*]; Rafaella Jekabson[*]; Eduardo Tassoni Tsuchida[†]; Nadja Cristhina de Souza-Pinto[‡]; Fernanda Marques da Cunha[*] and José Renato Rosa Cussiol[*]

[*] Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brasil

[†] Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brasil

[‡] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brasil

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**Corresponding author:**

José Renato R. Cussiol

Rua Três de Maio, 100

Instituto de Farmacologia e Biologia Molecular

Escola Paulista de Medicina – Universidade Federal de São Paulo

São Paulo, SP 04044-020

t. +55 11 5576-4444 Voip 1085

e-mail: cussiol@unifesp.br

## Abstract

Inositol is a six-carbon sugar that functions as a precursor for signaling molecules such as phosphoinositides and inositol polyphosphates, which are involved in the regulation of important biological processes such as energy metabolism, environmental stress response, phosphate signaling, among others. Given its role in a myriad of signaling pathways, regulation of inositol synthesis is essential for cellular homeostasis. In budding yeast, transcription of genes involved in inositol metabolism is regulated by the transcriptional repressor Opi1, which repress transcription of genes containing cis-acting inositol-sensitive upstream activation sequences ($UAS_{INO}$). Upon genotoxic stress, cells activate the DNA Damage Response (DDR) to coordinate DNA repair and cell cycle progression. It has been proposed that inositol containing molecules might act as modulators of the DDR, but evidences are still scarce. Herein, we report that *opi1*Δ cells fail to downregulate the inositol phosphate pathway leading to sensitivity to genotoxins and replication defects. Moreover, cells lacking Opi1 show decreased gamma-H2A levels which might indicate that Opi1 contributes to the activation of the DDR kinases Mec1/Tel1 (ATR/ATM in mammals). Importantly, we show that deletion of the inositol pyrophosphate kinase Kcs1 (IP6K1/2/3 in mammals), which leads to inhibition of inositol pyrophosphate synthesis, rescues the MMS sensitivity and replication defects of *opi1Δ* cells. Further, overexpression of Kcs1 recapitulates the MMS sensitivity of cells lacking Opi1. Therefore, we propose that cells must downregulate inositol pyrophosphate synthesis during replication stress in order to trigger an effective DNA Damage Response.

## 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). 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, transcription reprogramming, among others (Hanawalt 2015; Lanz *et al.* 2019; Cussiol *et al.* 2020). Although 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; Yi *et al.* 2017; Ferrari *et al.* 2017). Interestingly, it was proposed many years ago that might exist a crosstalk between the DDR and inositol metabolism in eukaryotes (Zewail *et al.* 2003). In line with that, many proteins from the inositol pathway are phosphorylated in response to DNA damage (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.* 2013), but the molecular basis for these effects is unknown. Moreover, it was recently proposed that phosphatidylinositol phosphate lipids (PIPs) are enriched in the nucleus after DNA damage and are important mediators of ATR signaling in mammalian cells (Wang *et al.* 2017). However, little is known about how DNA damage can regulate inositol metabolism and vice-versa.

Inositol is synthesized from glucose and is an essential precursor of a broad range of molecules that play important roles in cell signaling (Wilson *et al.* 2013; Henry *et al.* 2014; Shah *et al.* 2017). It has been proposed that the concentration of intracellular inositol is critical for higher eukaryotes, as perturbation of inositol levels is correlated with human diseases such as bipolar disorder, Alzheimer's disease, diabetes, and cancer (Winegrad 1987; Fruen and Lester 1991; Shi *et al.* 2006). In budding yeast, inositol biosynthesis is fine-tuned by the transcriptional repressor Opi1. In absence of inositol, Opi1 is localized to the perinuclear ER (endoplasmic reticulum) 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 a 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. Once intracellular inositol concentration increases, PA is redirected to the synthesis of phosphatidylinositol (PI) leading to its exhaustion. Subsequently, Opi1 migrates to the nucleus where it binds to the heterodimeric transcriptional activator 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.* 2017b) (Figure 1A). Deletion of Opi1 leads to constitutive activation of a large number of genes, most of which are regulated by Ino2-Ino4 (Santiago and Mamoun 2003; Jesch *et al.* 2005; Hoppen *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 might suggest that Ino2-Ino4 controls the expression of distinct biological processes (Wimalarathna *et al.* 2011). 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). 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; BastosdeOliveira *et al.* 2015; Lanz *et al.* 2021), which might suggest that Opi1 is involved in the DDR.

Herein, we propose that the transcriptional repressor Opi1 mediates, at least in part, the inositol pathway-DDR crosstalk. We find that cells lacking Opi1 under genotoxic stress show enhanced sensitivity, increased expression of *INO1*, cell cycle defects with delay in S-phase progression and decreased histone H2A phosphorylation. Interestingly, deletion of the inositol pyrophosphate kinase Kcs1 in an *opi1Δ* strain rescues MMS sensitivity and cell cycle defects. Moreover, overexpression of Kcs1 in wild-type cells phenocopies the genotoxin sensitivity of an *opi1Δ* strain. Together, these results indicate that modulation of PP-IPs synthesis during replication stress is part of the DDR and a key factor for cell survival in budding yeast.

## Material and Methods

**Yeast strains and plasmids.** Strains generated in this study are isogenic from BY4741 and S288C (where indicated). All yeast strains and plasmids used in this study are described in Table S1 and Table S2, respectively. All yeast strains were generated using yeast genetic tools for gene disruption and epitope tagging (Longtine *et al.* 1998). All yeast transformations were performed using the lithium acetate method (Gietz *et al.* 1992; Gietz and Woods 2006). Knockout strains were confirmed by genotyping using primers and epitope tagged strains were confirmed by western blotting. Restriction digestion cloning was used to generate the plasmids for this study. Briefly, a PCR product containing 250bp of the *OPI1* promoter + *OPI1* ORF with 3xHA sequence fused to the C-terminus + *ADH1* terminator sequence was cloned into the pRS416 vector using XhoI and SacII (New England Biolabs). For Ino1 and Kcs1 overexpression, the ORF coding for *INO1* and *KCS1* were PCR amplified from yeast genomic DNA using primers and cloned into pYES2-NTC using EcoRI and XhoI (New England Biolabs) under the control of the GAL1 promoter.

**Growth conditions.** Cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) and plasmid-bearing cells were grown in synthetic complete media lacking uracil (SC-URA) (0.17% YNB w/o aminoacids - Difco), 0.5% ammonium sulfate (MP Biomedicals) 0.07% CSM-URA dropout mix (Sunrise Science), 2% dextrose (Sigma). For experiments performed in the absence of inositol, SC-INO medium was prepared using an YNB without inositol (MP Biomedical). For expression of *INO1* 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).

**Serial dilution assays.** Overnight cultures were inoculated in the appropriate medium to an optical density ($OD_{600nm}$) 0.1 and cells were grown until log phase and normalized to an $OD_{600nm}$ of 1.0. Fourfold serial dilutions were spotted on yeast plates and grown for 1–3 days at 30ºC in the presence or absence of drugs. Plates were manually inspected and digitalized in an Uvitec Alliance 4.7.

**Real Time Quantitative PCR (RT-qPCR) Analysis.** Cells were grown overnight in YPD and diluted 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 two cell cycle divisions and immediately harvested at 4ºC, washed with sterile ultra-pure $H_2O$, and kept at -80ºC. Alternatively, log phase cells were treated with MMS (Sigma-Aldrich) 0.1% for 1 hour. Total RNA from $5x10^7$ cells was extracted using RNeasy mini kit (Qiagen). One μg total RNA was used for cDNA synthesis using Quantinova Reverse Transcription kit (Qiagen). Real time PCR was performed in an ABI Prism 7500 (Applied Biosystems) using 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 either after inositol depletion (Ye *et al.* 2013; Gaspar *et al.* 2017) or MMS treatment (Gasch *et al.* 2001). All reactions were

made with three biological replicates. Relative values of mRNA are described as fold change relative to a control condition indicated in the experiment.

**Western blot.** Whole-cell lysates were denatured with 3x SDS sample buffer (188mM Tris-HCL pH 6.8; 3% SDS; 30% glycerol; 0.01% bromophenol blue, 1x Bond-Breaker TCEP solution – Thermo Fischer Scientific) and resolved on SDS-PAGE gels. Proteins were transferred onto PVDF membranes (Amersham Hybond P – GE Healthcare). Phosphorylated histone H2A (gamma-H2A) and epitope tagged proteins were probed using specific antibodies: anti-gamma H2A (Ab17353-Abcam, 1:2500 dilution in TBST and 5% non-fat dry milk), anti-HA (12CA5; Roche, 1:10000 in TBST and 2% non-fat dry milk). Anti-PGK1 was used as a housekeeping control (22C5D8, Abcam, 1:15000 dilution in TBST and 2% non-fat dry milk). After primary antibody, PVDF membranes were incubated in the presence of ECL HRP-linked secondary antibody (NA931-GE, 1:10000 dilution in TBST and 2% non-fat dry milk). Blots were developed using the Amershan ECL Prime detection reagent and imaged in a Uvitec Alliance 4.7

**Cell cycle synchronization.** For arrest of cells in G1, yeast cells were grown in YPD medium at 30ºC until log phase followed by $\alpha$-factor (Zymo Research) treatment (30 ng/ml for *bar1Δ* background strains) for 2 h. To release from G1 arrest, cells were centrifuged and resuspended in fresh medium in the presence of MMS 0.033% (where indicated). For recovery of cells from MMS treatment, cells were treated with MMS 0.033% for 2 h, harvested and resuspended in fresh medium (YPD).

**Flow cytometry.** Logarithmic growing yeast cells were collected, harvested, fixed in 1 ml of 70% ethanol (Sigma-Aldrich), and incubated overnight at 4ºC. Cells were then centrifuged, and residual ethanol was dried in a speed-vac. After that, samples were solubilized in sodium citrate buffer (LabSynth) (50 mM, pH 7.2) and sonicated (three cycles of 3 sec, amplitude 30%) to release cell clumps. Samples were then incubated with 100 μg of RNAseA (Invitrogen) for 1 h at 37ºC followed by incubation with 500 μg of Proteinase K (Invitrogen) for 1 h at 42 ºC. Then, 1 μL of SYTOX Green (Thermo Fisher Scientific) was added to the samples and incubated for 2 h at 4ºC protected from the light. Data were acquired using a BD Accuri C6 Flow Cytometer.

**Respiratory capacity assay.** Oxygen consumption rates were measured using high-resolution O2K Fluorespirometer (Oroboros, Innsbruck, Austria). For each assay, $10^6$ cells were added to the Oroboros chamber containing a final volume of 2.0 ml of YPEG medium at 30ºC 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 hours at 30ºC. Finally, cells were transferred to O2K chambers for measurement of $O_2$ consumption rates. After basal respiration assessment, 1μM of the mitochondrial uncoupler FCCP (Sigma-Aldrich) was added to measure maximal respiration. At the end, 2μM of antimycin A (Sigma-Aldrich) was added to inhibit mitochondrial respiration. Non-mitochondrial oxygen consumption rates were subtracted from all measurements. Optimal concentrations of FCCP and antimycin A were previously determined by titration.

**Microscopy analysis.** Images were acquired in a Zeiss LSM 780 microscope. Laser lines at 561nm were used for the detection of Opi1-GFP tagged protein in yeast cells. For yeast Opi1-GFP analysis, cells were grown in synthetic complete media (SC +INO) until the log phase (OD = 0.3) and shifted to SC -INO for 1 hour. Finally, MMS (0.1%) was added to the cells and incubated for 30 min at 30°C. At each condition, cells were washed in sterile water and resuspended in fresh synthetic complete media lacking tryptophan (SC-TRP). Live yeast cultures were mounted on an agarose slide pad (1.2% agarose in SC-TRP media) and >100 cells were scored for each replicate. Cells were manually counted for Opi1-GFP localization (nuclear, transition from the nucleus to the ER and ER localization). Statistics were calculated based on the mean and SEM derived from two independent replicates.

## Data availability

Strains and plasmids are available upon request. Tables S1 and S2 contain the list of yeast strains and plasmids used in this study, respectively. Figure S1 contains supplemental data in support of Figure 3A and B. Figure S2 contains supplemental data in support of Figure 4. Figure S3 and S4 contains supplemental data in support of Figure 5.

## 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 genotoxins such as MMS and bleomycin (Figure 1B-C), thus indicating that Opi1 is important for the response to DNA damage. To confirm that this sensitivity to MMS was specific to *OPI1* deletion, we constructed a pRS416 plasmid expressing Opi1-HA from its endogenous promoter (Figure 1D) and show that Opi1-HA expression rescues the MMS sensitivity in *opi1Δ* (Figure 1E). Furthermore, deletion of genes that counteract Opi1 repressing functions, such as *INO1* and *SCS2*, does not lead to MMS sensitivity (Figure 1B). These results suggest that downregulation of inositol levels through Opi1 are important to trigger an effective response to genotoxic stress.

### Cells lacking Opi1 show replication defects and decreased gamma-H2A levels

Since *opi1*Δ mutants are sensitive to genotoxic stress induced by MMS (Figure 1B), we sought to investigate if these cells have defects in the DDR. First, we treated exponentially growing cells with MMS 0.033% for 2 hours to induce an intra S-phase arrest followed by release in fresh medium. Remarkably, *opi1Δ* cells arrest in early S-phase rather than mid S-phase and showed impaired S-phase progression during recovery from MMS treatment when compared to a wild-type strain (Figure 2A). Because cells lacking Opi1 show replication defects in the presence of MMS, we monitored gamma-H2A levels by immunoblot using a phospho-specific antibody. Upon replication

stress, the sensor kinases Mec1 and Tel1 catalyze the phosphorylation of long tracks of histone H2A at serine residue 129 (gamma-H2A) surrounding the DNA lesion creating a platform for the recruitment of multiple DNA damage response proteins (Rogakou *et al.* 1998; Downs *et al.* 2000), thus serving as a readout for DDR activation (Fernandez-Capetillo *et al.* 2004). Interestingly, gamma-H2A levels were significantly lower in *opi1Δ* relatively to wild-type after 2 hours of MMS treatment, but no significant difference was observed on kinetics of gamma-H2A disappearance during recovery (Figure 2B). Therefore, this result suggests that DNA damage signaling is downregulated in *opi1Δ*. To further address if DDR is downregulated in *opi1Δ* cells, we monitored the progressive phosphorylation of histone H2A upon MMS treatment. We arrested cells in G1 with α-factor and released in fresh YPD medium in the presence of MMS. Remarkably, cells lacking Opi1 have a delayed histone H2A phosphorylation and decreased intensity of gamma-H2A signal in latter time points (Figure 2C). Additionally, cells lacking Opi1 show slower S-phase progression in the presence of MMS (Figure 2D). Together, these results indicate that Opi1 is important for cells to cope with DNA damage during replication.

**Opi1 migrates to the nucleus upon genotoxic stress induced by MMS**

Knowing that transcriptional repression function of Opi1 is dependent on its cellular localization and because its absence causes MMS sensitivity (Figure 1B), we investigated Opi1 localization after genotoxic stress. Using a strain expressing Opi1-GFP, we recapitulated Opi1 cellular localization during inositol metabolism (Figure 3A panels i and ii). MMS treatment of cells supplemented with 100μM of inositol (+INO) did not cause any 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, suggesting that the nuclear localization of Opi1 is important to cope with genotoxic stress (Figure 3A-B and Supplementary Figure 1). Importantly, yeast cells treated with MMS in SC-INO are viable (Figure 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 RT-qPCR analysis. 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 both in +INO and -INO conditions (Figure 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.

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

Since Opi1 repress the transcriptional activator Ino2-Ino4 in the presence of inositol, we investigated if *INO2* and *INO4* deletions could rescue the MMS sensitivity of cells lacking Opi1. Remarkably, *opi1Δino2Δ* and *opi1Δino4Δ* strains are rescued for the MMS sensitivity, which is an indication that constitutive expression of Ino2-Ino4 responsive genes is causing the MMS sensitivity (Figure 4A and 4B). Of note, it has been

shown that the lack of Opi1 compromises mitochondrial function due to respiratory defects related to a decrease in mitochondrial membrane cardiolipin content (Luévano-Martínez *et al.* 2013). Interestingly, deletion of *INO*2 completely restores the respiratory capacity of cells lacking Opi1 (Figure 4C). To elucidate if the rescue of MMS sensitivity is related to a recovery of mitochondrial function we sought to investigate if *INO*2 deletion would also rescue the MMS sensitivity of *opi1Δ* in respiratory conditions (rich medium containing glycerol 3% as a carbon source). However, while *opi1Δino2Δ* fully rescues MMS sensitivity in fermentative conditions (Figure 4A), there is only a partial rescue in YPG (Figure 4D). It is known that ROS production during MMS treatment is exacerbated in the presence of high glucose (2%) with inhibition of glycolytic enzymes leading to growth arrest (Kitanovic *et al.* 2009; Yazgan and Krebs 2012). ROS leakage from mitochondria can be ameliorated by reducing glucose concentration in the medium. Therefore, we repeated the MMS sensitivity assay in the presence of 0.5% glucose with the expectation that if the lack of Opi1 affects the oxidative stress response we would see a rescue of MMS sensitivity. However, there was no change in MMS sensitivity (Supplementary Figure 2A). Furthermore, treatment with $H_2O_2$ does not sensitize *opi1Δ* cells (Supplementary Figure 2B). Therefore, we conclude that constitutive activation of genes regulated by Ino2-Ino4 affects both the response to genotoxic stress and mitochondrial function, but probably by independent mechanisms.

**Opi1 regulates inositol pyrophosphates synthesis during the DDR**

It is well established that the expression of *INO1* is positively regulated by Ino2-Ino4 and massively increases in the absence of Opi1 (Figure 3D) (Graves and Henry 2000;

Santiago and Mamoun 2003; Jesch *et al.* 2005). Consequently, we deleted *INO1* in an *opi1Δ* background with the expectation that it would phenocopy the MMS resistance of an *opi1Δino2Δ*. Surprisingly, an *opi1Δino1Δ* double mutant showed growth defects and increased MMS sensitivity (Figure 5A). This negative genetic interaction between Opi1 and Ino1 suggests that upregulation of one or more genes repressed by Opi1 in the absence of Ino1 negatively impacts the cell. To test if constitutive expression of *INO1* is causing MMS sensitivity, we overexpressed *INO1* by cloning it under the control of the strong inducible *GAL1* promoter with the expectation that it would phenocopy the MMS sensitivity of an *opi1Δ* strain. Expression of *INO1* from the *GAL1* promoter rescues the inositol auxotrophy of cells lacking *INO1* (Supplementary Figure 3A), but cells overexpressing *INO1* do not show sensitivity to MMS, which is an indication that increased levels of inositol do not affect the DDR (Supplementary Figure 3B).

Besides Ino1, Ino2-Ino4 upregulate the expression of several other genes counteracting Opi1 suggesting that constitutive expression of another Ino2-Ino4 target in *opi1Δ* cells is causing MMS sensitivity. Therefore, we conducted a screening of a small set of genes which expression is dependent on Ino2-Ino4 and that are upregulated in *opi1Δ* cells (Santiago and Mamoun 2003). Among them, we selected genes involved in the phosphatidylinositol and phosphatidylcholine synthesis (Itr1 - inositol transporter, Opi3 - phospholipid methyltransferase and Cki1 - choline kinase), polyphosphate metabolism (Pho84 - high-affinity inorganic phosphate transporter) and inositol pyrophosphate synthesis (Kcs1 - inositol pyrophosphate kinase). Similar to what was observed for Ino1, deletion of the inositol transporter Itr1, phospholipid methyltransferase Opi3, choline kinase Cki1 and inorganic phosphate transporter Pho84 also showed a

negative genetic interaction with Opi1 (Supplementary Figure 4). On the other hand, deletion of Kcs1 in an *opi1Δ* strain significantly rescued the MMS sensitivity and the S-phase progression after replication stress induced by MMS (Figure 5B and C). In budding yeast, Kcs1 catalyzes the synthesis of inositol pyrophosphates. Inositol polyphosphates $IP_5$ and $IP_6$ generated by Ipk2 and Ipk1 respectively are substrates of Kcs1 that catalyzes the pyrophosphorylation of the C5 $\alpha$-phosphate to 5-PP-$IP_4$ and 5-PP-$IP_5$ respectively (Saiardi *et al.* 1999). These results, suggest that overproduction of PP-IPs by Kcs1 is most probably the cause of genotoxin sensitivity (Figure 5B and C). Interestingly, cells lacking the Siw14 phosphatase that counteracts Kcs1-dependent PP-IPs synthesis have a mild sensitivity to MMS (Figure 5D). It has been shown that *siw14Δ* mutants have increased levels of 5-PP-$IP_5$ (Steidle *et al.* 2016). Consistent with that, wild-type cells overexpressing Kcs1 phenocopy an *opi1Δ* strain with cell growth delay and sensitivity to MMS (Figure 5E). Taken together, our results suggest that increased PP-IPs synthesis due to the lack of the transcriptional repressor Opi1 leads to impaired capacity to cope with replication stress induced by MMS.

## Discussion

In this manuscript, we demonstrated that the transcriptional repressor Opi1 modulates the expression of Ino2-Ino4 during genotoxic stress. Importantly, we propose that Opi1 controls PP-IPs levels through downregulation of genes from inositol phosphate pathway. Failure to downregulate PP-IPs in an *opi1Δ* strain causes downregulation of the DDR leading to sensitivity to genotoxic agents due to increased replication stress, reinforcing the notion that inositol metabolism affects the DDR.

### PP-IPs overproduction affects the response to replication stress

The fact that Opi1 regulates the expression of several genes involved in different metabolic pathways makes the task of identifying the cause of genotoxin sensitivity very challenging. However, we found that deletion of the transcription factor Ino2-Ino4 and the PP-IPs kinase Kcs1 restores viability of cells lacking Opi1. In budding yeast, PP-IPs are synthetized from the inositol polyphosphates $IP_5$ and $IP_6$ in a reaction catalyzed by the PP-IPs kinases Kcs1 and Vip1 (Saiardi *et al.* 1999; Mulugu *et al.* 2007; Lee *et al.* 2008). Kcs1 catalyzes the conversion of $IP_5$ and $IP_6$ to different isomers of PP-IPs such as 5-PP-$IP_4$ and 5-PP-$IP_5$ (Saiardi *et al.* 1999). Kcs1 and its mammalian orthologs IP6Ks have been implicated in several biological processes such as cell cycle progression, DNA repair, telomeric length, nutrient signaling, environmental stress response, polyphosphate metabolism among others (Saiardi *et al.* 2005; Auesukaree *et al.* 2005; Szijgyarto *et al.* 2011; Banfic *et al.* 2013, 2016; Worley *et al.* 2013; Wilson *et al.* 2013). It has been previously shown that *KCS1* possesses $UAS_{INO}$ sequences in its promoter region and that its expression is

upregulated when inositol concentration increases (Wimalarathna *et al.* 2011). Corroborating the notion that PP-IPs synthesis is upregulated in cells lacking Opi1, Kcs1 protein levels were shown to increase in *opi1Δ* cells (Ye *et al.* 2013). Additionally, the inositol polyphosphate multikinase Ipk2 that catalyzes the synthesis of $IP_4$ and $IP_5$ also contains $UAS_{INO}$ sequences in its promoter region, and its expression is dependent on Ino2-Ino4 (Wimalarathna *et al.* 2011). Therefore, an increase in PP-IPs levels might result whether from increased Kcs1 or Ipk2 expression. It is interesting to note that while Ino1 overexpression does not sensitize cells to MMS, that of Kcs1 does (Supplementary Figure 3B and Figure 5E respectively), suggesting that Ino1 overexpression does not lead to an increase in PP-IPs synthesis, which might indicate that there is a rate-limiting step reaction downstream of inositol-3-phophate synthesis restraining PP-IPs concentration in the cell.

Finally, is interesting to note that Opi1 shows negative genetic interactions with genes involved in inositol synthesis and transport (Ino1 and Itr1); phosphatidylcholine synthesis (Opi3 and Cki1) and phosphate metabolism (Pho84) (Figure 5A and Supp. Figure 4). For instance, deletion of the inorganic phosphate transporter Pho84 leads to a synthetic lethal/sick phenotype (Supp. Figure 4). Because inositol and phosphate metabolism are intimately related (Auesukaree *et al.* 2005; Mulugu *et al.* 2007; Lee *et al.* 2008; Kliewe *et al.* 2017a), this observation might give new insights for further exploration of these pathways.

**Genotoxin sensitivity in *opi1Δ* is directly associated with DNA damage**

Deletion of the transcriptional repressor Opi1 can induce pleiotropic effects in the cell. Therefore, we conducted a careful analysis to rule out the possibility that the genotoxin sensitivity of *opi1Δ* cells was due to a deregulation of other metabolic pathways not directly associated with the DDR. 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 nor yeast sensitivity to DNA damage (Harari *et al.* 2017). Consistent with this, PP-IPs were also shown to modulate cell cycle independently of telomere length (Banfic *et al.* 2016). MMS can also damage mitochondrial DNA 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). Moreover, deletion of Opi1 decreases mitochondrial cardiolipin content and it was proposed that overproduction of inositol might affect cardiolipin production probably through inhibition by phosphorylation of the Phosphatidylglycerol phosphate synthase, Pgs1 (Luévano-Martínez *et al.* 2013). We found, however, that genotoxin sensitivity of cells lacking Opi1 is probably not due to a defect in mitochondria homeostasis because: i) diauxic shift induced by incubation with lower glucose levels does not ameliorate MMS sensitivity (Supp. Figure 2A); ii) *opi1Δ* does not show sensitivity to $H_2O_2$ (Supp. Figure 2B); iii) deletion of *INO2* rescues the respiratory defects of cells lacking Opi1, but does not rescue the MMS sensitivity in the presence of a respiratory carbon source (Figure 4D). Despite that, we cannot entirely rule out the possibility that the genotoxin sensitivity of *opi1Δ* is dependent on mitochondria because it is known that

PP-IPs can also modulate mitochondrial respiration and ROS signaling (Onnebo and Saiardi 2009; Szijgyarto *et al.* 2011; Worley *et al.* 2013). Furthermore, MMS treatment can also induce ER stress leading to activation of the UPR (*Unfolded Protein* Response) and it was shown that Ino2-Ino4 also regulate expression of genes involved in the UPR (Jelinsky and Samson 1999; Jesch *et al.* 2005; Thibault *et al.* 2012). Nevertheless, it was shown that the absence of Opi1 causes an expansion of the ER membrane, alleviating the ER stress induced by DTT or tunicamycin in an UPR independent manner (Schuck *et al.* 2009).

**A working model for modulation of PP-IPs synthesis during replication stress induced by MMS**

How does inositol pyrophosphates deregulation affect DNA replication leading to genotoxin sensitivity? It is established that PP-IPs modulate protein function either through non-enzymatic 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. Therefore, we can speculate that proteins related to the DDR might being modified by PP-IPs. Of note, it has been shown that Kcs1 is important for S-phase progression, as PP-IPs levels increase after pheromone induced arrest by an unknown mechanism (Banfic *et al.* 2013). In fact, it was previously demonstrated that Kcs1 is required for 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 orthologous of Kcs1 (IP6K1/2/3) are important for homologous recombination repair (Jadav *et al.* 2013). Curiously, deletion of Kcs1 also confers sensitivity to MMS, suggesting that depletion of PP-IPs, which synthesis is dependent on Kcs1, is also

important for the DDR (Figure 5B and (Zhou *et al.* 2016)). Since MMS-induced lesions can be bypassed during replication by homologous recombination (HR) mechanisms (Cussiol *et al.* 2017), it is tempting to speculate that PP-IPs targets proteins related to HR in order to modulate the repair or bypass of MMS-induced replication blocks. Moreover, it has been shown that inositol pyrophosphates can compete with $PIP_3$ (PtdIns(3,4,5)P3) for its binding to pleckstrin-homology (PH) domains in proteins (Luo *et al.* 2003). In line with that, a recent report has revealed that $PIP_2$ rapidly accumulates in the nucleus after DNA damage in mammals and is important to mediate ATR recruitment to DNA lesions (Wang *et al.* 2017). Therefore, accumulation of inositol pyrophosphate has the potential to outcompete PIPs leading to downregulation of DNA damage signaling. This hypothesis can explain the lower levels of gamma-H2A observed in *opi1Δ* mutants (Figure 2B and C). Consequently, a decrease in Mec1/Tel1 signaling in cells lacking Opi1 might affect the ability of the cells to cope with replication stress.

We propose a model in which concerted downregulation of inositol pyrophosphates is important to cope with increased replication stress through a robust DNA Damage Response (Figure 6). In the presence of genotoxic stress, Opi1 translocates to the nucleus to repress the inositol phosphate pathway, probably through inhibition of *KCS1* and/or *IPK2* gene expression. In cells lacking Opi1, Ino2-Ino4 constitutively promotes transcription of genes from the inositol phosphate pathway, leading to the accumulation of Kcs1-dependent inositol pyrophosphates. PP-IPs accumulation might then modulate DDR targets either by allosteric interaction or pyrophosphorylation. Because replication stress is a hallmark of cancer cells (Hills and Diffley 2014; Gaillard *et al.* 2015) and the PP-IP pathway is highly conserved from yeast to

humans, the finding that PP-IP accumulation sensitizes cells to genotoxic stress might be relevant for future exploration in higher eukaryotes, especially in cancer cell lines.

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Author contributions: J.R.C. wrote the paper, designed the study, performed experiments and data analysis. G.M.P., M.R.P., R.J, E.T.T. performed experiments, data analysis and helped revised the manuscript. F.M.C. designed and performed experiments for measuring oxygen consumption and helped revised the manuscript. N.C.S-P provided reagents, infrastructure and helped revised the manuscript.

## Conflicts of interest

None declared

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## Figure Legends

**Figure 1. Cells lacking Opi1 show sensitivity to genotoxic stress.** (A) Working model for how Opi1 repress transcription of target genes; (B-C) Effect of deletion of genes involved in inositol metabolism in sensitivity to genotoxins; (D) Immunoblot showing expression of Opi1-HA from a pRS416 plasmid; (E) A pRS416 plasmid expressing Opi1 from its endogenous promoter complement an *opi1Δ* strain; For (B, C and E), fourfold serial dilutions were spotted on the media indicated in the figure and plates were grown for 2–3 days at 30°C.

**Figure 2. Cells lacking Opi1 show defects upon replication stress induced by MMS.** (A) S-phase progression analysis of wild-type and *opi1Δ* strains. Cells were treated with 0.033% MMS for 2 hours and then release in fresh YPD medium; (B and C) Immunoblot analysis of gamma-H2A in the indicated strains. For (B), cells were treated with 0.033% MMS for 2 hours and then release in fresh YPD medium until 6 hours. For (C), cells were arrested in G1 with α-factor and then released in medium containing MMS. Immunoblots were probed with anti-gamma-H2A and anti-Pgk1 (loading control) antibodies as described in *Material and methods*. (D) S-phase progression analysis of wild-type and *opi1Δ* strains. Cells were arrested in G1 with α-factor and then released in medium containing MMS.

**Figure 3. Opi1 is required to repress inositol synthesis during genotoxic stress.** (A-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 two 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, fourfold 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 three biological replicates and statistical significance of the differences observed was calculated using one one-way ANOVA with Tukey's multiple comparisons test. Of note, statistical significances between *wt + INO (MMS) vs. opi1Δ + INO (MMS) (p = 0.003); wt - INO (MMS) vs. opi1Δ - INO (MMS) (p = 0.0006); opi1Δ - INO vs. opi1Δ - INO (MMS) (p = 0.0129).*

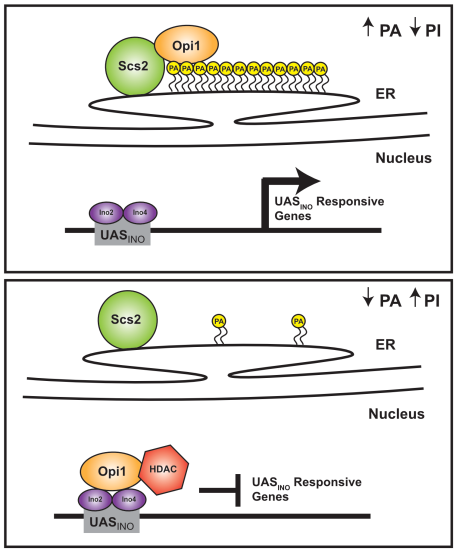
**Figure 4. Deletion of the transcriptional activators Ino2-Ino4 rescues genotoxin sensitivity and mitochondrial defects of cells lacking Opi1.** (A-B) Deletion of *INO*2 and *INO*4 completely rescue the MMS sensitivity of cells lacking Opi1; (C) Deletion of *INO*2 completely restores mitochondrial respiratory capacity of cells lacking Opi1; (D) – Deletion of *INO*2 does not completely rescue the MMS sensitivity of cells lacking Opi1 in respiratory medium (YPG). For A, B and D, fourfold serial dilutions were spotted on the media indicated in the figure and plates were grown for 2–3 days at 30°C.

**Figure 5. Deregulation of inositol pyrophosphate synthesis in cells lacking Opi1 is causing genotoxin sensitivity.** (A) Deletion of Ino1 does not rescues the MMS sensitivity of cells lacking Opi1; (B-C) Deletion of the inositol pyrophosphate kinase Kcs1 rescues the MMS sensitivity and replication defects in cells lacking Opi1. (D) Cells lacking Siw14 are sensitive to MMS; (E) Overexpression of Kcs1 causes MMS sensitivity. For (A-B, D and E), fourfold serial dilutions were spotted on the medium indicated in the figure and plates were grown for 2–3 days at 30°C.

**Figure 6. Proposed model illustrating how Opi1 modulate PP-IPs synthesis during genotoxic stress.**

# Figure 1

A

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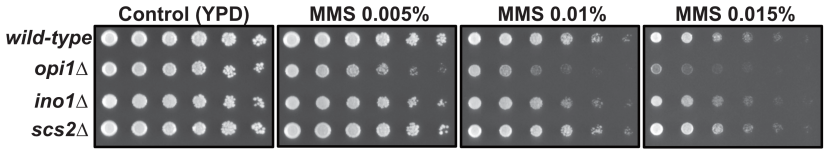


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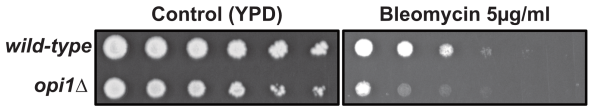


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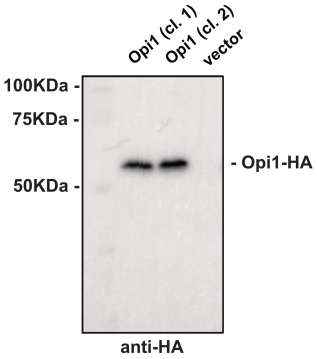


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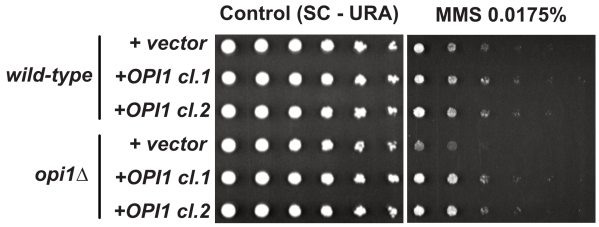
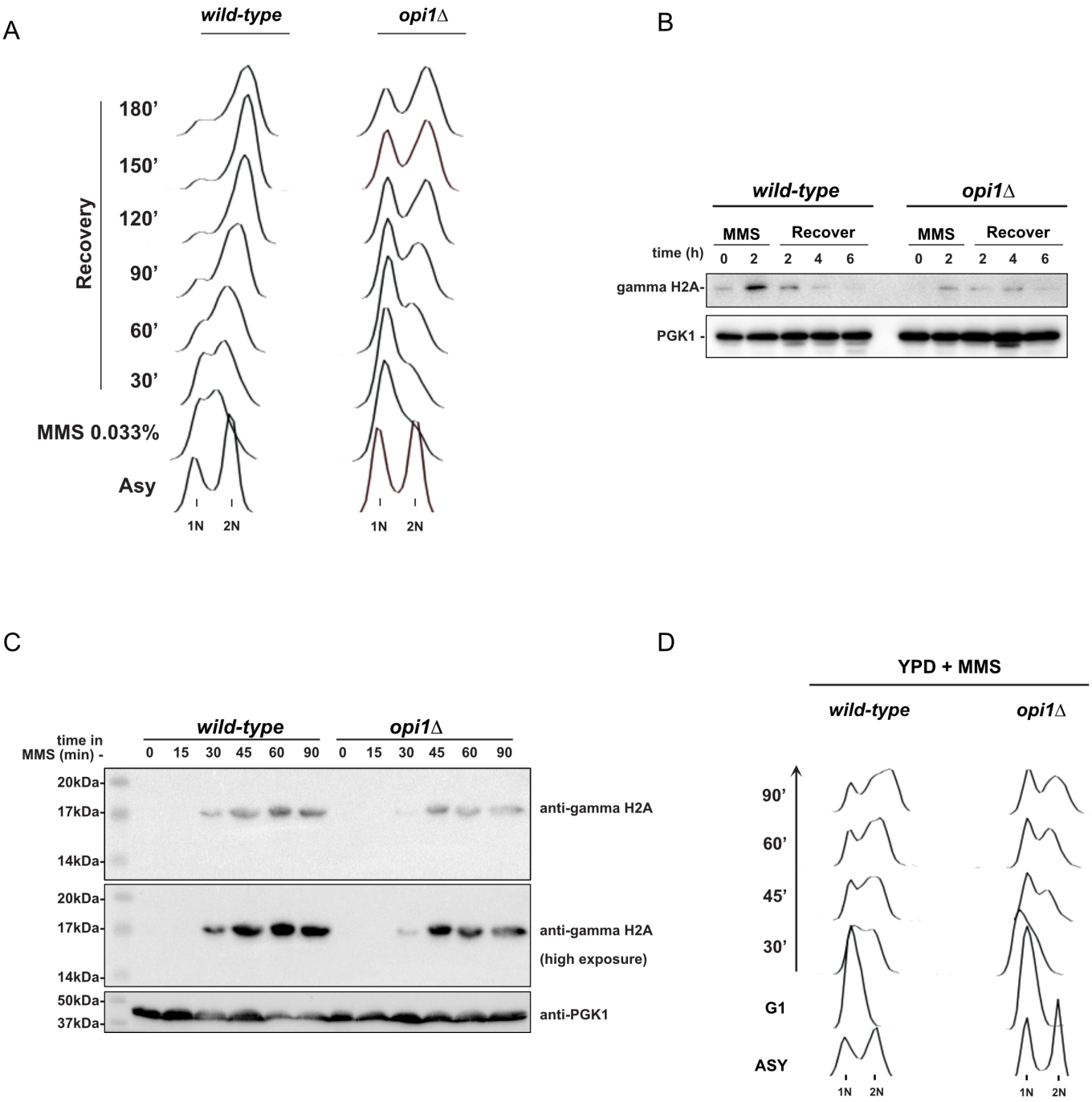
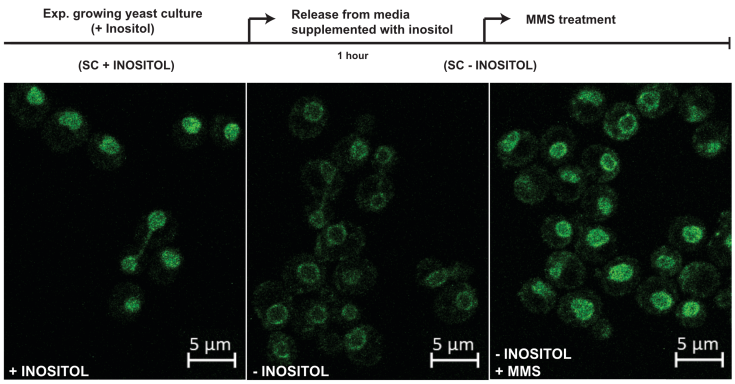


# Figure 2

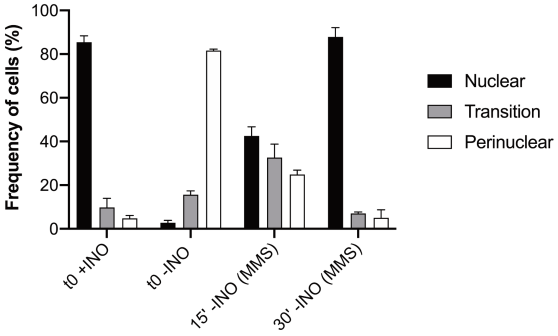

A
wild-type
opi1Δ
Recovery
180'
150'
120'
90'
60'
30'
MMS 0.033%
Asy
1N
2N
B
wild-type
opi1Δ
MMS
Recover
time (h)
0 2 2 4 6
gamma H2A-
PGK1 -
C
time in MMS (min) -
0 15 30 45 60 90
20kDa-
17kDa-
14kDa-
anti-gamma H2A
anti-gamma H2A (high exposure)
50kDa-
37kDa-
anti-PGK1
D
YPD + MMS
90'
60'
45'
30'
G1
ASY

# Figure 3

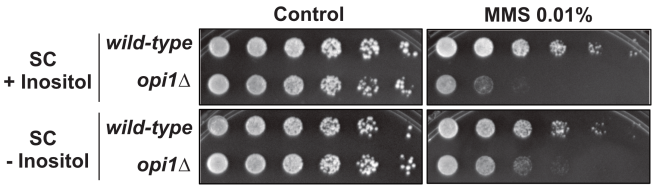
A




B




C




D

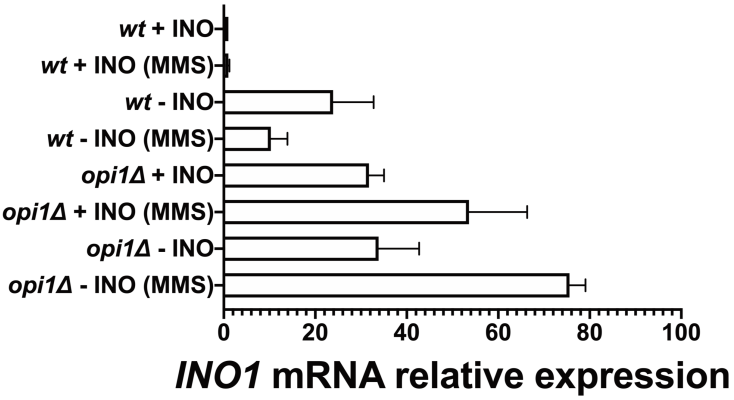
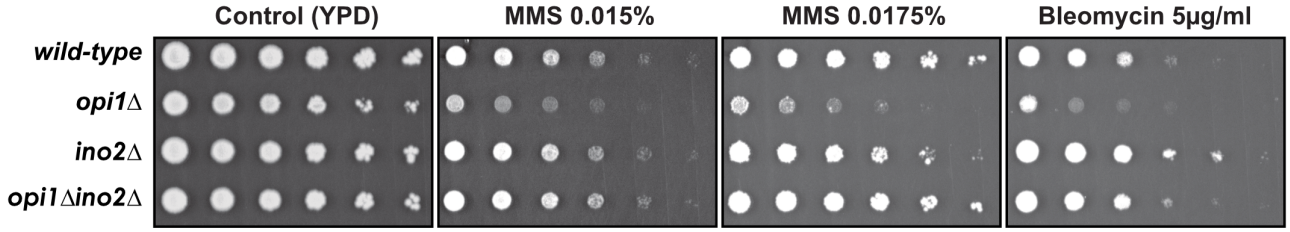
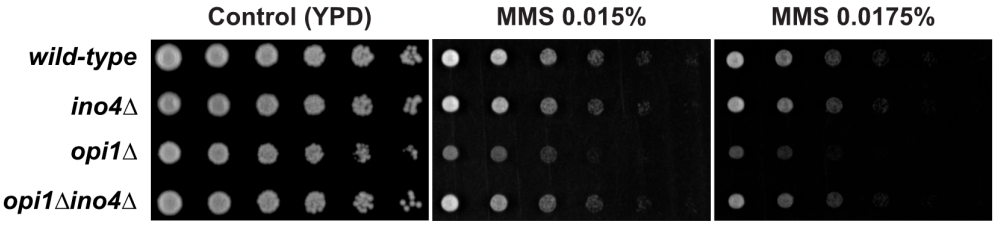


**Figure 4**

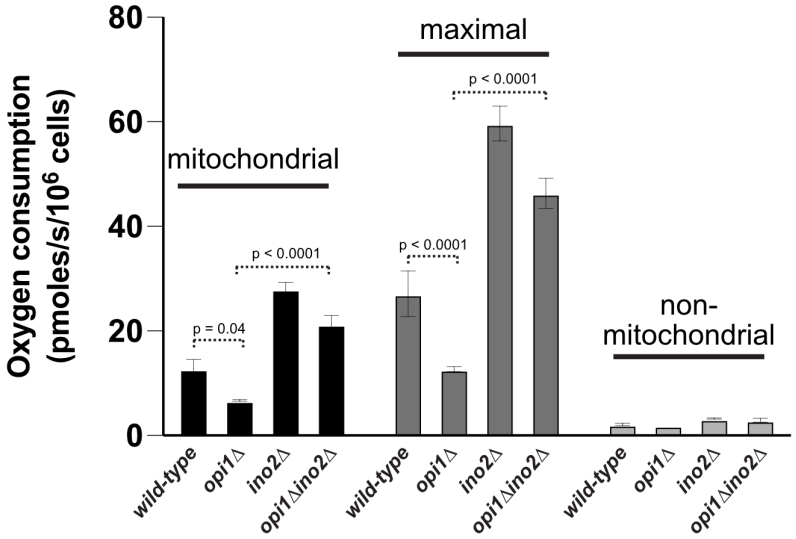
A




B




C




D

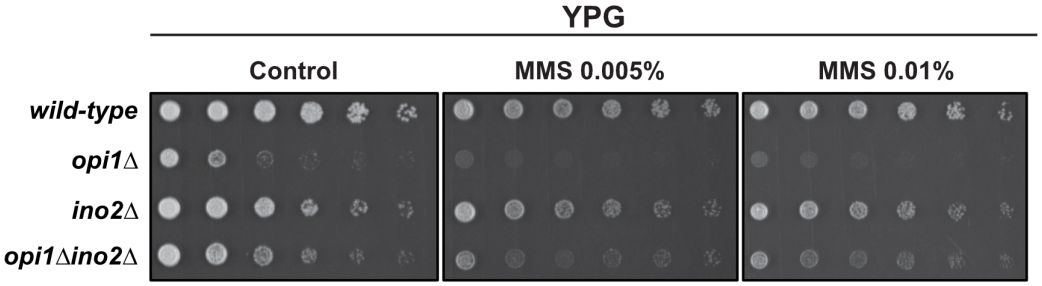
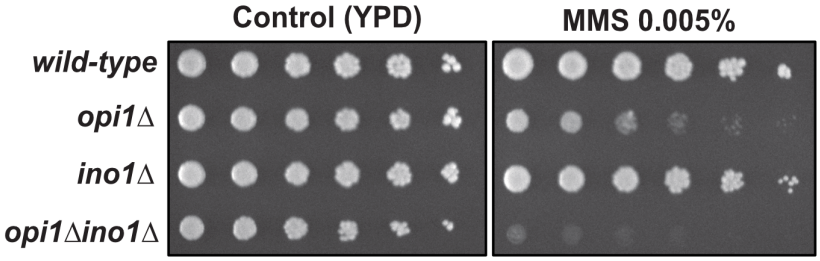
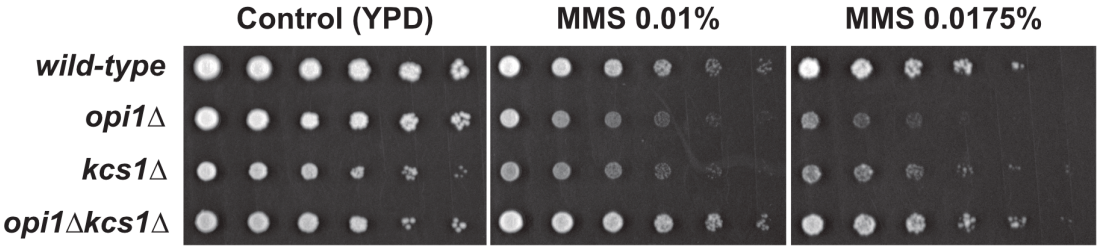


**Figure 5**

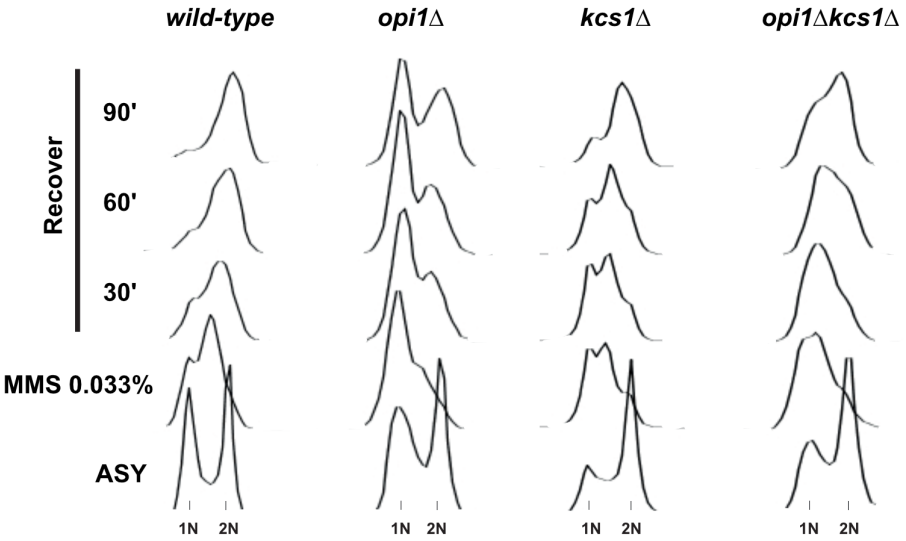
A



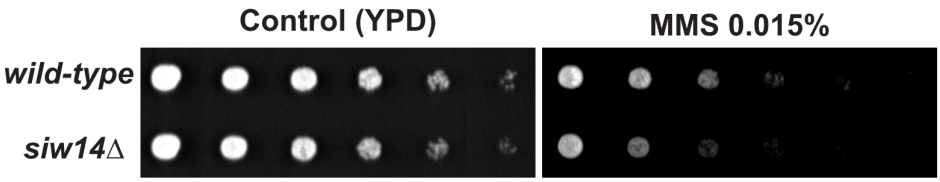

B




C




D




E

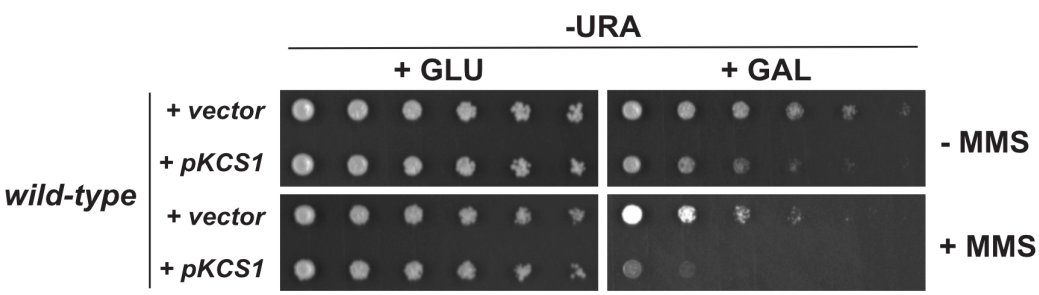
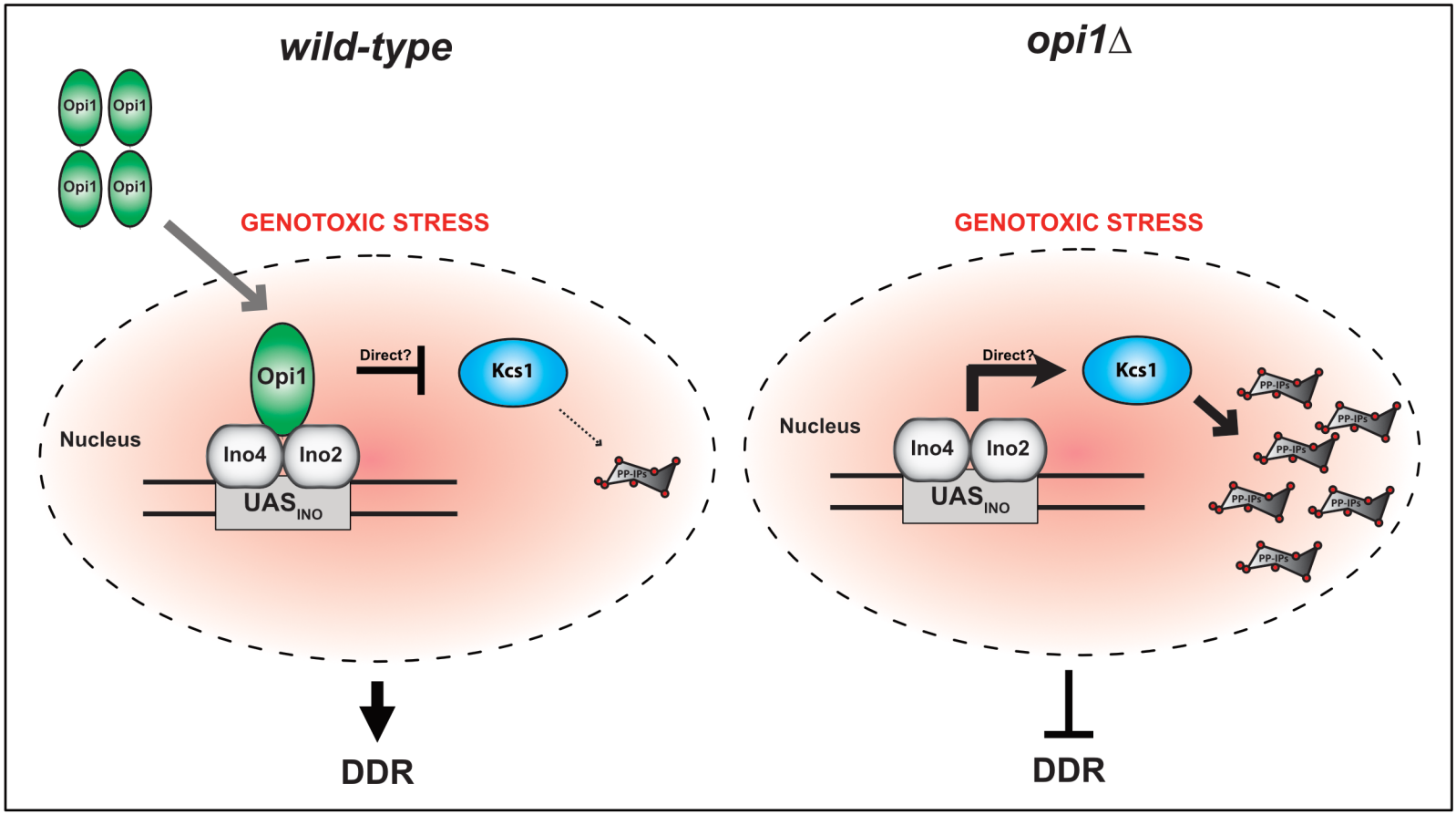


Figure 6


wild-type
opi1Δ
Opi1
Opi1
Opi1
Opi1
GENOTOXIC STRESS
GENOTOXIC STRESS
Opi1
Direct?
Kcs1
Nucleus
Ino4
Ino2
UAS INO
PP-IPs
Direct?
Kcs1
Nucleus
Ino4
Ino2
UAS INO
PP-IPs
DDR
DDR