RESEARCH ARTICLE | Physiological Genomics of Cell States and Their Regulation and Single Cell Genomics

Genome reprogramming in *Saccharomyces cerevisiae* upon nonylphenol exposure

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**Nonylphenol** (NP) is a hydrophobic and nonionic surfactant commonly used in industrial applications. It is a polychlorinated biphenyl (PCB) derivative, which is an environmental estrogen that can affect human health. Since genomes of eukaryotes share many structural and functional similarities, we investigated subcellular effects of NP on *S. cerevisiae* BY4742 cells by analyzing genome-wide transcriptional profiles. We examined effects of low (1 mg/l; <15% cell number reduction) and high (5 mg/l; >65% cell number reduction) inhibitory concentration exposures for 120 or 180 min. After 120 and 180 min of NP exposure, 187 (63 downregulated, 124 upregulated) and 103 genes (56 downregulated, 47 upregulated) were differentially expressed. Similarly, 678 (168 repressed, 510 induced) and 688 genes (215 repressed, 473 induced) were differentially expressed in cells exposed to 5 mg/l NP for 120 and 180 min, respectively. Only 15 downregulated and 63 upregulated genes were common between low and high NP inhibitory concentration exposure for 120 min, whereas 16 downregulated and 31 upregulated genes were common after the 180-min exposure. Several processes/pathways were prominently affected by either low or high inhibitory concentration exposure, while certain processes were affected by both inhibitory concentrations, including ion transport, response to chemicals, transmembrane transport, cellular amino acids, and carbohydrate metabolism. While minimal expression changes were observed with low inhibitory concentration exposure, 5 mg/l NP treatment induced substantial expression changes in genes involved in oxidative phosphorylation, cell wall biogenesis, ribosomal biogenesis, and RNA processing, and encoding heat shock proteins and ubiquitin-conjugating enzymes. Collectively, these results provide considerable information on effects of NP at the molecular level.

Nonylphenol; genome-wide transcriptional profiles; *Saccharomyces cerevisiae*; exposure; downregulation; upregulation
identification and characterization of cellular responses to toxicants at the genomic, transcriptomic, proteomic, and/or metabolic levels (31, 91). Transcriptomics via DNA microarrays is a promising toxicogenomic tool since thousands of gene expression patterns can be analyzed in a single hybridization assay (44, 122). The budding yeast *Saccharomyces cerevisiae* is widely used as a eukaryotic model organism for such studies owing to its simple growth conditions, rapid reproductive rates, and the ease with which it can be manipulated. Moreover, the whole genome of this organism has been extensively annotated and characterized, and the data deposited in various publicly available databases (21, 44). Moreover, the *S. cerevisiae* genome shares many structural and functional similarities with those of higher eukaryotes, including humans. As such, this organism could potentially be used to clarify the molecular mechanisms and functional pathways involved in the cellular responses to NP toxicity in higher eukaryotes (82).

Therefore, in the present study, we analyzed genome-wide transcriptional changes in the *S. cerevisiae* strain BY4742 upon exposure to NP to identify differentially expressed genes, biological processes, metabolic pathways, and cellular compartments affected by this compound. For these analyses, we focused on two NP exposure scenarios 1) exposure to a low inhibitory concentration and 2) exposure to a high inhibitory concentration.

**MATERIALS AND METHODS**

*Strain, growth conditions, and chemical exposure. S. cerevisiae* BY4742 (Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0) was obtained from the Euroscarf collection (Frankfurt, Germany). For precultures, 25 ml of yeast extract-peptone-glucose (YPD) medium [2% (wt/vol) D-glucose, 2% (wt/vol) peptone, 1% (wt/vol) yeast extract] was inoculated with a single colony of cells obtained from agar plates and incubated overnight at 30°C with orbital shaking (180 rpm). A 1% volume fraction of the preculture was then used to inoculate 100 ml YPD in 500 ml flasks and grown to an optical density at 600 nm (OD₆₀₀) of 0.8 ± 0.05 (midexponential phase). Subsequently, a nonylphenol (NP; 4-nonylphenol) stock solution was prepared in ethanol, and then it was added to the cultures to reach various final concentrations of NP (0.1, 0.5, 1, 3, 5, 7.5, 10, and 20 mg/l NP). The final concentration of ethanol in the medium was maintained at 1% (vol/vol), which did not affect growth. Other cultures containing 1% (vol/vol) ethanol, but lacking NP were used as untreated.

The growth inhibition rates after NP exposure were determined using cell viability analysis by vital staining with methylene blue solution (0.01% methylene blue, 2% sodium citrate), as described previously (117). Briefly, aliquots of yeast cultures exposed to NP for 120 and 180 min beginning at midexponential phase (t = 0) were diluted and mixed with an equal volume of methylene blue solution. The number of dead (stained blue) and live (unstained) cells were determined microscopically within 10 min of staining. Under sublethal doses, the percent inhibition of yeast growth was defined as [(N₀ - Nₜ)/N₀] × 100, where N₀ and Nₜ are the numbers of yeast cells in cultures containing X mg/l of NP and untreated samples, respectively. Inhibition rates were categorized as low (<15% reduction in cell number) and high (>65% reduction in cell number). For further analyses, one concentration appropriate for each category was chosen. Growth curves for selected NP inhibitory concentrations were constructed by monitoring the OD₆₀₀. Briefly, optical density measurements were conducted every 120 min with a spectrophotometer (DR 2800 Portable Spectrophotometer-Hach, Loveland, CO). Three independent toxicity experiments were performed for each inhibitory concentration. Finally, after exposure to determined concentrations of NP for 120 and 180 min, samples were harvested for microarray analysis, immediately flash-frozen in liquid nitrogen, and stored at −80°C until RNA isolation.

*RNA isolation and DNA microarray analysis. RNA isolation was performed according to the enzymatic lysis protocol provided with the RNeasy Mini Kit (cat. no.: 74104; Qiagen, Venlo, Netherlands). The quantity and quality of the RNA samples were analyzed with a NanoDrop ND-100 UV-vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity (RIN) was determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) device using an RNA 6000 Nano Assay kit (Agilent Technologies), and samples with RIN values between 7 and 10 were used for further analysis. Approximately 100 ng of total RNA for each sample was then used to synthesize first-strand cDNA, which was then converted into double-stranded cDNA using a GeneChip 3’ IVT Express Kit (Affymetrix, Santa Clara, CA). In vitro transcription and synthesis of biotin-labeled antisense RNA (aRNA) were performed using the double-stranded cDNA as a template. The final product was purified and quantified using a NanoDrop ND-100 UV-vis spectrophotometer (Thermo Fisher Scientific). The purification and fragmentation steps were performed using GeneChip reagents, and fragmented aRNA was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Affymetrix Yeast 2.0 arrays were prepared for hybridization with the GeneChip Hybridization, Wash, and Stain Kit reagents (Affymetrix). A total of 5 μg of aRNA was hybridized to the arrays for 16 h at 45°C. For washing and staining, the arrays were loaded into a fluidics station using the Affymetrix Command Consoloe Software 3.0.1 Fluidics Control Module with the Mini-Euk 2v3 protocol. Finally, arrays were scanned on an Affymetrix GeneChip Scanner 3000 device. Independent biological triplicates were processed and analyzed for each exposure condition and untreated. The microarray data have been submitted to Gene Expression Omnibus under accession number GSE96651.

The raw microarray data (presented as .CEL files) were preprocessed using Robust multiarray (13) as implemented in the affy package (42) in R/Bioconductor (43). Differentially expressed genes were identified from the normalized log-expression values using the Linear Models for Microarray Data package (103). P < 0.01 was used to identify significantly differentially expressed genes, which were used as inputs for gene set enrichment analysis (GSEA) based on Gene Ontology (GO) annotations. All GO enrichment analyses were performed with the GO Slim Mapper web-tool of the *Saccharomyces* Genome Database (21), with a P value threshold of 0.05, CIMiner (98) was used for the clustering analysis of significantly enriched biological processes. Pathway enrichment analysis was conducted using the DAVID Bioinformatics Database (https://david.ncifcrf.gov/home.jsp) and P values were corrected using the Benjamini-Hochberg method. Common genes were found by comparing data from low and high NP inhibitory concentration exposures and presented using a Venn diagram.

*Quantitative real-time reverse transcription PCR. cDNA samples were synthesized from equivalent amounts of the total RNA samples used in the microarray experiments with a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was then performed on a LightCycler device using a LightCycler FastStart DNA Master PLUS SYBR Green I Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. Most of the specific primers used for these analyses were designed using Primer Premier 6.0 software (PREMIER Biosoft International, Palo Alto, CA); however, several primer sets were obtained from previous studies (Supplemental Table S1). (The online version of this article contains supplemental material.) All samples were analyzed in triplicate. Fold changes were calculated according to the ΔΔCt method using the averages of these results as described by Livak and Schmittgen (2001, 69). CCW12 normalization relative to control samples was performed to calculate differences in gene expression levels.

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RESULTS AND DISCUSSION

NP inhibits the growth of S. cerevisiae BY4742 cells. The transcriptional profiles of S. cerevisiae BY4742 cells exposed to low and high inhibitory concentrations of NP for 120 and 180 min beginning at midexponential phase (i.e., OD$_{600} = 0.8 \pm 0.05$) were analyzed. The exposure period was based on results of previous studies showing that gene expression alterations in yeast typically appear a few hours after exposure to chemicals, although the exact timing and magnitude of the observed changes depend on the gene and stress conditions (10, 41, 44).

The NP inhibitory concentrations used in the microarray analysis were determined by exposing yeast cells to various NP concentrations (0.1, 0.5, 1, 3, 5, 7.5, 10, and 20 mg/l NP) and then analyzing cell viability by methylene blue staining (data not shown). The results showed that growth inhibition became apparent after 1 mg/L NP (1.4 and 11.5% reduction in cell number after 120 and 180 min, respectively), while cell death occurred at concentrations $>5$ mg/l NP (54.5 and 68.4% reduction after 120 and 180 min, respectively. For simplicity, 1 and 5 mg/l treatments will henceforth be referred to as low and high inhibitory concentrations of NP, respectively. Finally, growth curves were constructed for the determined inhibitory concentrations by measuring OD$_{600}$ (Fig. 1).

Gene expression analyses of yeast cells exposed to low inhibitory concentration of NP. Microarray analysis revealed a total of 187 gene transcripts differentially expressed in yeast exposed to low inhibitory concentration of NP for 120 min compared with those in untreated (Supplemental Table S2). Of these genes, 63 were downregulated and 124 were upregulated. Alternatively, a total of 103 genes were differentially expressed in yeast exposed for 180 min compared with untreated (Supplemental Table S3) and, of these genes, 56 were repressed while 47 were induced (Fig. 2).

GSEA showed that several downregulated genes were associated with transport processes, particularly, transmembrane and ion transport processes and metabolic processes such as cellular amino acid and monocarboxylic acid metabolism at both exposure times. In addition, transcription from the RNA polymerase II promoter was downregulated at 120 min of exposure, while carbohydrate metabolic processes were repressed at 180 min of exposure (Table 1).

NP may negatively affect amino acid biosynthesis. ARO9 and ARO10 encode enzymes required for biosynthesis of the aromatic amino acids tryptophan, phenylalanine, or tyrosine that are used as the main source for cellular nitrogen by S. cerevisiae. Notably, both genes were significantly downregulated by NP exposure for both 120 and 180 min (Table 1). Interestingly, ARO9 was induced in the presence of these amino acids but repressed in their absence (53).

Several genes related to branched-chain amino acid biosynthesis were also repressed after 120 min of exposure, whereas these genes were not affected after 180 min of exposure (Table 1). Branched-chain amino acids are important nutrients metabolized by both eukaryotic and prokaryotic microorganisms as various sources such as carbon, nitrogen, and energy (101). Two genes, BAT1 and BAT2, encoding mitochondrial and cytosolic branched-chain amino acid aminotransferases required for branched-chain amino acid biosynthesis and Ehrlich pathway amino acid catabolism (60), and BAP2, encoding a branched-chain amino acid permease involved in leucine, isoleucine, and valine uptake, were significantly downregulated (45). Deletion of BAT1 and/or BAT2 causes branched-chain amino acid auxotrophy and severe growth retardation (60). Moreover, ILV3 and ILV5, also involved in branched-chain amino acid biosynthesis (101), were significantly downregulated. LEU4 and LEU1, two genes responsible for the first and second steps in the leucine biosynthesis pathway respectively (6), were also significantly repressed upon exposure to low inhibitory concentration of NP for 120 min (Table 1). This finding indicates that yeast cells may adapt to low levels of NP in time, because expression of the affected genes returned to basal levels after 180 min of exposure.

NP inhibits the expression of genes involved in phosphate metabolism. Inorganic phosphate is essential for the synthesis of many cellular components, including nucleic acids and
phospholipids that are used in biomolecule synthesis, energy metabolism, and protein modification (100, 120). Cells undergo certain cellular processes based on nutrient availability in the environment, and inorganic phosphate is a growth-limiting substrate for organisms (100). Phosphate depletion causes the induction of several genes encoding phosphate transporters, which then increase phosphate uptake. These genes are regulated by PHO operon (58, 100, 120); however, in S. cerevisiae, cell division and growth can get arrested in the case of prolonged phosphate deficiency (100). In the present study, three genes involved in phosphate metabolism, PHO5, PHO8, and PHO11, were significantly downregulated upon exposure to low inhibitory concentration of NP for 120 min (Supplemental Table S2). Two of these genes, PHO5 and PHO8, were also downregulated after 180 min of exposure to low inhibitory concentration of NP (Supplemental Table S3).

Exposure to a low inhibitory concentration of NP hinders major facilitator superfamilies gene expression. Major facilitator superfamilies (MFS) transporters are involved in multidrug resistance (MDR) and classified into groups such as the 12-spanner drug:H⁺ antiporter DHA1 family, according to the xenobiotics to protect from their possible toxic effects (29, 97). We found that four genes belonging to the 12-spanner drug:H⁺ antiporter DHA1 family, TPO2, TPO4, QDR2, and AQR1, were downregulated in response to low inhibitory concentration of NP after 180 min of exposure (Table 1). This result conflicts with a previous report that alachlor toxicity enhanced the expression of several MFS genes (44). Moreover, these genes convey resistance to several cytotoxic compounds in S. cerevisiae (29). As such, we speculated that low inhibitory concentration of NP may inhibit MFS transporter activity rather than induce these genes. Besides, yeast cells overexpress drug resistance genes as is discussed in subsequent sections, whereas MFS genes are repressed.

On the other hand, many of the genes upregulated in response to a low inhibitory concentration of NP were associated with carbohydrate metabolic process, the generation of precursor metabolites and energy, response to chemicals, and ion transport in both exposure times. In addition, monocarboxylic acid and lipid metabolic processes were significantly upregulated after 120 min of exposure time, while organelle fission

<table>
<thead>
<tr>
<th>GOID</th>
<th>GO Term</th>
<th>Frequency</th>
<th>Gene(s)</th>
</tr>
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<tbody>
<tr>
<td>6520</td>
<td>cellular amino acid metabolic process</td>
<td>11/63 genes 17.5%</td>
<td>ARO10, LEU1, ARO9, BAT1, ILV3, BAT2, MAE1, ALT1, ILV5, LEU4, GLN1</td>
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<td>6811</td>
<td>ion transport</td>
<td>10/63 genes 15.9%</td>
<td>BAP2, GIT1, PMP2, ZRT1, TPO2, OAC1, DIC1, SAM3, OPT2, ARR3</td>
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<td>55085</td>
<td>transmembrane transport</td>
<td>7/63 genes 11.1%</td>
<td>FU1, BAT2, GIT1, HXT13, HXT15, ZRT1, MDH2</td>
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<tr>
<td>6366</td>
<td>transcription from RNA polymerase II promoter</td>
<td>6/63 genes 9.5%</td>
<td>NRG1, MIG3, MIG1, RPH1, ZAP1, CUP9</td>
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<tr>
<td>32787</td>
<td>monocarboxylic acid metabolic process</td>
<td>4/63 genes 6.3%</td>
<td>ALD5, MAE1, MCT1, EEB1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>GOID</th>
<th>GO Term</th>
<th>Frequency</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6811</td>
<td>ion transport</td>
<td>11/56 genes 19.6%</td>
<td>GIT1, ZRT1, TPO2, AVT7, QDR2, ZRT2, DIC1, AQR1, TPO4, SAM3, OPT2</td>
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<td>55085</td>
<td>transmembrane transport</td>
<td>8/56 genes 14.3%</td>
<td>FU1, GIT1, HXT13, ZRT1, QDR2, ZRT2, AQR1, MDH2</td>
</tr>
<tr>
<td>6520</td>
<td>cellular amino acid metabolic process</td>
<td>5/56 genes 8.9%</td>
<td>ARO10, ARG5, BAT2, ALTI</td>
</tr>
<tr>
<td>32787</td>
<td>monocarboxylic acid metabolic process</td>
<td>4/56 genes 7.1%</td>
<td>ALD5, POT1, EEB1, PDI1</td>
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<td>5975</td>
<td>carbohydrate transport</td>
<td>3/56 genes 5.4%</td>
<td>HXT13, HXT1, HXT11</td>
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</table>

Table 1. Selected biological processes significantly enriched among the genes downregulated in Saccharomyces cerevisiae cells exposed to 1 mg/l nonylphenol for 120 and 180 min

Fig. 2. Number of differentially expressed genes in response to NP exposure.
and cellular ion homeostasis were induced after 180 min of exposure to a low inhibitory concentration of NP (Table 2).

Exposure to a low inhibitory concentration of NP upregulates genes associated with iron and copper acquisition. Iron and copper are important cofactors with roles in several biochemical reactions, such as electron transfer during respiration, iron acquisition, and protection against oxidative stress. However, a certain amount of these environmentally insoluble elements can be toxic in the presence of oxygen because of the generation of destructive hydroxyl free radicals. On the other hand, organisms have developed sophisticated systems to transport iron and copper into the cell while maintaining intracellular concentrations at nontoxic levels. While iron is reduced at the cell surface by membrane-spanning ferric reductases, copper uptake requires high-affinity copper transporters that are functionally redundant to ferric reductases. Consistently, depletion of either iron or copper results in the upregulation of ferric reductases and copper transporters (74). Notably, several genes associated with iron and copper acquisition were significantly induced in response to a low inhibitory concentration of NP at both exposure times (Table 2). Specifically, FRE2 (iron uptake), ENB1 (ferric enterobactin transport), and LSO1 (iron uptake) were affected after 120 min of exposure, while FRE1 and FRE7, involved in iron uptake; FIT1 and SIT1, involved in iron transport; and FET3, involved in the transcriptional regulation of ferric reductase, were significantly upregulated upon exposure to a low inhibitory concentration of NP for 180 min. Moreover, two genes associated with copper transport, CTR1 and CTR3, were significantly induced after 180 min of exposure to a low inhibitory concentration of NP, while only CTR3 was upregulated upon 120 min of exposure time. Based on this observation and that of our previous study (10), we speculate that low-level NP may directly inhibit cellular iron and copper uptake or interfere with these ions indirectly, thereby reducing nutrient allocation to the appropriate compartments.

Exposure to low inhibitory concentration of NP upregulates genes involved in fatty acid biosynthesis. Fatty acids are key components used in several processes, such as synthesis of biological membranes, energy storage, and protein modification. Fatty acid biosynthesis is conserved among organisms (110). Two genes (FASI and FAS2) encoding fatty acid synthetases that catalyze long-chain saturated fatty acid synthesis were significantly upregulated upon exposure to a low inhibitory concentration of NP for 120 min (Table 2). Considering the role of fatty acids in membrane biosynthesis and the upregulation of related genes, we speculated that NP may functionally disrupt the yeast cell membrane. It was also speculated that the induction of genes involved in fatty acid metabolism in response to amphotericin B might be a reflection to cell membrane disruption caused by the chemical (2).

NP induces autophagy-related gene expression. Autophagy is a highly conserved pathway among eukaryotes and induced under stressful conditions. In this pathway, cytoplasmic contents are transported in bulk to lysosomes for degradation into amino acids and fatty acids, which are then utilized in the synthesis of new proteins or oxidized by mitochondria to produce energy for cell survival during starvation (20). In S. cerevisiae, ~30 autophagy-related (Atg) proteins have been identified, and null mutations in many of these proteins result in cell death under nutrient starvation conditions by inhibiting autophagy (109). Here, we found that four Atg genes, ATG7, ATG34, ATG39, and ATG40, were significantly upregulated in response to low inhibitory concentration of NP after 120 min of exposure (Supplemental Table S2), in contrast to our previous findings with bisphenol A (BPA) treatment (10). These data

Table 2. Selected biological processes significantly enriched among the genes upregulated in Saccharomyces cerevisiae cells exposed to 1 mg/l nonylphenol for 120 and 180 min

<table>
<thead>
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<th>GOID</th>
<th>GO Term</th>
<th>Frequency</th>
<th>Gene(s)</th>
</tr>
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<tbody>
<tr>
<td>5975</td>
<td>carbohydrate metabolic process</td>
<td>18/124 genes 14.5%</td>
<td>MAL33, MAL32, GLC3, GIP2, GSY1, IGD1, HXK1, SUC2, TDH1, YJR096W, XIL2, PGM2, GID8, NDE1, CAT8, MLS1, GAC1, GPH1, HXK1, OLE1, TDH1, ELO1, FAS1, CAT2, NDE1, CAT8, MLS1, GOR1, ACC1, GLO4, CRC1, FAS2, GLC3, GIP2, GSY1, IGD1, HXK1, LSC2, TDH1, PGM2, NDE1, GAC1, NCA2, GPH1</td>
</tr>
<tr>
<td>32787</td>
<td>monocarboxylic acid metabolic process</td>
<td>14/124 genes 11.3%</td>
<td>PDR3, UPC2, HSP12, YGL039W, VMR1, YJR096W, GDX1, CSU1, GAD1, CAT8, PDR5, UPC2, OLE1, ELO1, FAS1, ICT1, PLB2, ACC1, GRE2, CRC1, FAS2, GEX1, MFC3, JEN1, FRE2, CTR3, PRM6, ENB1, RSB1, PUT4</td>
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<tr>
<td>6091</td>
<td>generation of precursor metabolites and energy</td>
<td>12/124 genes 9.7%</td>
<td>PDR3, UPC2, HSP12, YGL039W, VMR1, YJR096W, GDX1, CSU1, GAD1, CAT8, PDR5, UPC2, OLE1, ELO1, FAS1, ICT1, PLB2, ACC1, GRE2, CRC1, FAS2, GEX1, MFC3, JEN1, FRE2, CTR3, PRM6, ENB1, RSB1, PUT4</td>
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<tr>
<td>42221</td>
<td>response to chemical</td>
<td>11/124 genes 8.9%</td>
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<tr>
<td>6629</td>
<td>lipid metabolic process</td>
<td>10/124 genes 8.1%</td>
<td>PDR3, UPC2, HSP12, YGL039W, VMR1, YJR096W, GDX1, CSU1, GAD1, CAT8, PDR5, UPC2, OLE1, ELO1, FAS1, ICT1, PLB2, ACC1, GRE2, CRC1, FAS2, GEX1, MFC3, JEN1, FRE2, CTR3, PRM6, ENB1, RSB1, PUT4</td>
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<tr>
<td>6811</td>
<td>ion transport</td>
<td>9/124 genes 7.3%</td>
<td>PDR3, UPC2, HSP12, YGL039W, VMR1, YJR096W, GDX1, CSU1, GAD1, CAT8, PDR5, UPC2, OLE1, ELO1, FAS1, ICT1, PLB2, ACC1, GRE2, CRC1, FAS2, GEX1, MFC3, JEN1, FRE2, CTR3, PRM6, ENB1, RSB1, PUT4</td>
</tr>
<tr>
<td>6811</td>
<td>ion transport</td>
<td>7/47 genes 14.9%</td>
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<td>generation of precursor metabolites and energy</td>
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<td>MBR1, PET10, PGM2, GAC1</td>
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<td>48285</td>
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<td>cellular ion homeostasis</td>
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<td>5975</td>
<td>carbohydrate metabolic process</td>
<td>3/47 genes 6.4%</td>
<td>MAL32, PGM2, GAC1</td>
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showed that NP exposure may upregulate Atg genes to balance the lack of essential amino acids necessary for cell survival, since several genes associated with amino acid biosynthesis were significantly repressed with the same treatment.

**Exposure to low inhibitory concentration of NP upregulates genes involved in glycogen metabolism.** Glycogen metabolism was significantly affected by exposure to low inhibitory concentration of NP for 120 min. Six genes, GLC3, IGD1, GIP2, GAC1, GSY1, and GPH1, related to glycogen synthesis, accumulation, and phosphorylation were significantly upregulated (Table 2). In *S. cerevisiae* and most other organisms, glycogen is the main storage form of glucose. Previous studies demonstrate that glycogen metabolism is regulated according to growth phase of the cell. For instance, glycogen is rarely found in actively growing cultures but accumulates in cells under starvation, passing in diauxic growth phase, or reaching stationary phase. Specifically, accumulation begins when glucose levels in fall below 50% of that typically in the environment (95). Thus, we speculated that glucose may be quickly depleted and starvation starts in cells exposed to NP compared with untreated cells, resulting in the induction of these genes. We also suggest that NP may induce glycogen metabolism to increase ATP consumption, which may prevent cell death caused by a hypothesized mechanism called "turbo design pathway" (12). As part of this assumption, osmotic stress decreases cell growth rates and ATP demand, which causes inorganic phosphate depletion that may ultimately lead to cell death.

**NP promotes the expression of drug resistance genes.** Four pleiotropic drug resistance genes, *PDR3, PDR5, YOR1*, and *VMR1*, were significantly upregulated upon exposure to a low inhibitory concentration of NP for 120 min (Table 2). The pleiotropic drug resistance (PDR) network is constitutively regulated by the transcription factors *Pdr1p* and *Pdr3p* in *S. cerevisiae* (8). *PDR5*, one of four upregulated genes, encodes the plasma membrane ATP-binding cassette (ABC) transporter *Pdr5p*, which regulates resistance to many xenobiotic compounds including mutagens, fungicides, steroids, and antican- cer drugs (8). The ABC superfamily is conserved from bacteria to humans (8), and the human ABC transporter *ABCB1/MDR1* overexpression is involved in tumor resistance to drug therapy (28). This finding is consistent with previous toxicity studies on BPA (10), cycloheximide and isocitrate (81), alachlor (44), and p-anisaldehyde (122), which are also known to induce the expression of several pleiotropic drug resistance genes.

**Gene expression analyses of yeast cells exposed to high inhibitory concentration of NP.** DNA microarray analysis revealed 678 genes differentially expressed in *S. cerevisiae* cells exposed to high inhibitory concentration of NP compared with untreated cells after 120 min. Of these genes, 168 were downregulated and 510 were upregulated (Fig. 2). On the other hand, a total of 688 genes were differentially expressed upon exposure to a high inhibitory concentration of NP for 180 min. Of these, 215 were downregulated, while 473 were upregulated. Supplemental Tables S4 and S5 show the complete list of the differentially expressed genes and their expression changes in response to exposure to high inhibitory concentration of NP.

A significant proportion of the downregulated genes at both exposure times were involved in transmembrane transport, cellular amino acid metabolic processes, and ion transport. Moreover, several downregulated genes after 120 min of exposure were involved in rRNA processing, mitotic cell cycle, and ribosomal small subunit, whereas most of the repressed genes with 180 min of exposure were associated with mitochondrion organization, nucleobase-containing small molecule metabolic process, and protein complex biogenesis (Table 3).

**Exposure to high inhibitory concentration of NP downregulates 12-spanner drug:H\textsuperscript{+} antiporter DHA1 family expression.** Four 12-spanner drug:H\textsuperscript{+} antiporter DHA1 family genes, *TP02, TPO4, QDR2*, and *HOL1*, were significantly downregulated in response to a high inhibitory concentration of NP at both exposure times (Table 3). In addition to these genes, *TPO3* was affected from exposure at the 180 time point. This finding showed that both low and high inhibitory concentrations of NP negatively affected MFS transporters involved in MDR.

**Exposure to high inhibitory concentration of NP inhibits the expression of genes associated with amino acid biosynthesis.** As observed in response to a low inhibitory concentration of NP, genes associated with aromatic amino acid biosynthesis were significantly repressed upon exposure to high inhibitory concentration of NP for both treatment times. Specifically, high inhibitory concentration of NP induced the expression of *ARO7, ARO9*, and *ARO10* (Table 3).

Moreover, some genes associated with histidine biosynthesis were also significantly downregulated upon exposure to a high inhibitory concentration of NP for 180 min (Table 3). *HIS1* and HIS4 are two genes related to histidine biosynthesis. *HIS1* encodes an ATP phosphoribosyltransferase that catalyzes the first step in histidine biosynthesis (4), while *HIS4* encodes a multifunctional polypeptide that catalyzes the second, third, ninth, and 10th steps (57). Notably, mutations in *HIS1, HIS4*, and other histidine biosynthesis enzyme genes lead histidine auxotrophy and sensitivity to copper, cobalt, and nickel salts (88).

**Exposure to high inhibitory concentration of NP downregulates genes associated with O-linked glycosylation.** Two mannosyltransferase genes, *MNN1* and *MNN5*, were downregulated after 120 min of exposure to NP (Supplemental Table S4), whereas five mannosyltransferase genes, *MNT3, MNT4, MNN10, KTR7*, and *PMT4*, were repressed after 180 min of exposure (Supplemental Table S5). Glycoproteins are organized with N-linked and O-linked glycans in the *S. cerevisiae* cell wall. Of these, the O-linked structures contain four or five mannoses that are attached to serines and threonines. Notably, 40% of the dry weight of the cell wall results from mannan synthesis, which are believed to increase the structural integrity of the cell wall by forming an external layer (55). Mutations causing mannan deficiency results in synthetic lethality with protein kinase C pathway components that regulate the expression of cell wall components (55, 80). Moreover, yeast cells must organize the actin cytoskeleton and have a functional mitotic checkpoint to survive without mannan (55). Thus, the downregulation of these genes suggests that high inhibitory concentration of NP may cause defects in the yeast cell wall. Moreover, the upregulation of other cell wall components may support this speculation and will be discussed further in the subsequent sections.

**Exposure to high inhibitory concentration of NP downregulates genes involved in cell cycle progression.** In contrast to results obtained from BPA exposure, we observed that three cell cycle progression genes, *CLB1, CLB6*, and *CLN1*, were...
downregulated after 120 min of NP exposure (Table 3 and Supplemental Table S4). In addition to \( CLB1 \), \( CLB2 \) was also significantly downregulated after 180 min (Supplemental Table S5). Cyclins activate cyclin-dependent kinases, which are conserved regulatory units involved in cell cycle progression. \( CLN1 \) encodes a G\(_1\) cyclin involved in cell cycle regulation and activates the Cdc28p kinase to promote G\(_1\)/S phase transition. \( CLB1 \) and \( CLB2 \) encode B-type cyclins involved in cell cycle progression that also activate Cdc28p, but promote the G\(_2\)/M transition. Transcripts of these genes generally accumulate during the G\(_2\) and M phases and repressed at the end of mitosis (36). Moreover, \( CLB6 \) encodes a B-type cyclin involved in DNA replication during S phase and activates Cdc28p to initiate DNA synthesis (65). Therefore, these findings suggest that NP may have an inhibitory effect on cell cycle progression.

**Exposure to high inhibitory concentration of NP hinders the expression of genes involved in ribosomal activity.** Ribosomal biogenesis consists of a complex series of processes, such as synthesis, processing, and modification of RNA and ribosomal proteins, which are followed by assembly of the individual components. Moreover, these processes are highly regulated and coordinated with the help of other fundamental cellular activities, like cell division and growth. Two ribonucleoprotein subunits are found in the ribosomes of \( S.\ cerevisiae \), the small subunit (SSU, 40S) and the large subunit (LSU, 60S). In addition, there are one ribosomal RNA (18S rRNA) and 33 ribosomal proteins (r-proteins) in the SSU, while the LSU consists of three rRNAs (5S, 5.8S, and 25S) and 46 r-proteins. The SSU brings the mRNA and aminoacylated transfer RNAs (tRNAs) together by acting as a decoding center, whereas the LSU mediates peptidyltransferase reactions that catalyze peptide bond formation (119). Several ribosomes are necessary for cell growth and/or increases in cell bulk, and defects in the regulation of ribosomal assembly can have serious deleterious effects. For instance, complete loss-of-function mutations in most assembly factors and r-proteins are lethal in yeast and higher organisms, and several defects in ribosomal biogenesis are linked to various diseases in humans, such as cancer and aging (64, 119). In the present study, several genes associated with ribosomal biogenesis including RNA and nuclear RNA processing (five genes), RNA polymerase I subunits (three genes), ATP-dependent RNA helicase (two genes), ribosomal SSU and LSU biogenesis, and other related processes (13 genes) were significantly downregulated in \( S.\ cerevisiae \) upon exposure to high inhibitory concentration of NP for 120 min (Supplemental Table S4). On the other hand, several different genes associated with ribosomal SSU (five genes) and LSU (two genes) were also downregulated in \( S.\ cerevisiae \) in response to a high inhibitory concentration of NP for 180 min (Supplemental Table S5). Although these findings conflict with our previous findings on BPA exposure (10), several previous studies observed a significant downregulation of ribosomal biogenesis and assembly genes in yeast in response to different stresses (105, 122).

**Exposure to high inhibitory concentration of NP suppresses mitochondrial biogenesis.** Mitochondria are essential organelles for energy production, metabolism, cellular proliferation, differentiation, and cell death. To carry out such processes, the morphology, numbers, and distributions of mitochondria vary in different cell types. The morphological organization of mitochondria is maintained and protected by regular cell fusion.
and division rates under normal conditions (49, 125). Interestingly, exposure to a high inhibitory concentration of NP for 120 min affected the expression of mitochondrial distribution and morphology genes. Specifically, MDM20 and MDM36, involved in mitochondrial inheritance, morphology, and actin assembly, were significantly downregulated (Supplemental Table S4).

Moreover, several genes associated with mitochondrial biogenesis were significantly affected by exposure to a high inhibitory concentration of NP for 180 min (Supplemental Table S5). Similar to our previous results obtained from BPA exposure (10), TIM21 and IMP2, encoding subunits of the inner membrane TIM23 and peptidase complexes, respectively, were downregulated (71, 114). Additionally, PET54, OAC1, and ARH1 were downregulated. PET4 encodes a mitochondrial inner membrane protein that binds to the 5'UTR of COX3 mRNA to activate its translation together with Pet122p and Pet494p (23). OAC1 is a mitochondrial inner membrane transporter that transports oxaloacetate, sulfate, thiosulfate, and mitochondrial iron homeostasis (66). Moreover, one mitochondrial GTP/GDP transporter encoding gene (GGC1), one mitochondrial translational activator of the COB mRNA encoding gene (CBS1), one mitochondrial elongation factor encoding gene (MEF2), one protein subunit of mitochondrial RNase P encoding gene (RPMP2), one mitochondrial intermediate peptidase encoding gene (OCT1), one diveral metal ion transporter encoding gene (SMF1), one mitochondrial C1-tetrahydrofolate synthase encoding gene (MIS1), one mitochondrial serine hydroxymethyltransferase encoding gene (SHM1), one mitochondrial NADP-specific isocitrate dehydrogenase encoding gene (IDP1), one mitochondrial ribosomal protein of the small subunit encoding gene (MRPS35), and one mitochondrial ribosomal protein of the large subunit encoding gene (RML2) were significantly downregulated. Based on these findings, we believe that NP may affect mitochondrial biogenesis as evidenced by the downregulation of essential components that mediate various functions in the mitochondrion subcompartments.

Exposure to high inhibitory concentration of NP attenuates the expression of genes involved in oxidative phosphorylation. Several genes associated with the oxidative phosphorylation (OXPHOS) system, which includes five enzyme complexes (complex I to complex V) and two electron carriers (quinonecoenzyme Q and cytochrome C) (34), were significantly downregulated in *S. cerevisiae* in response to a high inhibitory concentration of NP for 180 min (Table 3). Substrates produced via glycolysis and β-oxidation are converted into energy (ATP) by the OXPHOS system, and mitochondria play a crucial role in death and survival signaling pathways. Moreover, several mitochondrial and neurodegenerative diseases, including Parkinson’s, Alzheimer’s, and Huntington’s disease, are connected to mitochondrial defects (30). In the present study, one gene (SDH2) associated with mitochondrial complex II [i.e., succinate dehydrogenase (SDH)]; four genes (QCR2, QCR7, QCR8, and QCR10) associated with mitochondrial complex III, referred to as ubiquinol cytochrome-c reductase or cytochrome bc1 complex; six genes (COX4, Cox6, COX7, COX12, COX13, and COX15) associated with mitochondrial respiratory chain complex IV, known as cytochrome c oxidase (COX); and two genes (ATP17 and ATP25) associated with complex V, called as F0F1-ATP synthase were repressed. In addition, CYC1-cytochrome c isoform I related to the electron carrier of the mitochondrial inner membrane (39), and AFG1, which may act as a chaperone in the degradation of misfolded or unassembled cytochrome c oxidase (59) subunits were also reduced in response to the high inhibitory concentration of NP for 180 min. This finding is consistent with our previous study on BPA toxicity (10), and several studies have shown that mutations and defects in various subunits downregulated in the present study may severely or completely inhibit the complex activity (16, 25, 48, 63) and may cause various neuromuscular disorders in humans (62). Moreover, the human homolog of the CYC1 gene has been associated with insulin-responsive hyperglycemia (39).

**NP inhibits the de novo biosynthesis of NAD and pyrimidines.** Two genes, BNA1 and BNA4, required for the de novo biosynthesis of NAD from tryptophan via kynurenine were significantly downregulated upon exposure to a high inhibitory concentration of NP for 180 min (Table 3), consistent with our previous BPA study (10). NAD is used as a cofactor for energy metabolism and as a substrate in several biological processes, such as signaling pathways, transcriptional regulation, DNA repair, age-associated diseases, and lifespan extension (67).

A previous study demonstrates that de novo pyrimidine nucleotide biosynthesis is required for growth-related processes. For instance, the uracil and cytosine nucleotides are necessary for the synthesis of RNA, DNA, phospholipids, UDP sugars, and glycogen. Moreover, some synthetic pyrimidine enzymes have higher activities in tumor cells, and several studies on pyrimidine synthesis inhibitors have identified useful drugs for the treatment of leukemia and solid cancers (52). Three genes, *URA1*, *URA4*, and *URA5*, associated with de novo pyrimidine biosynthesis were significantly repressed in *S. cerevisiae* in response to a high inhibitory concentration of NP after 180 min (Table 3). *URA1* encodes dihydroorotic acid dehydrogenase, which catalyzes the fourth enzymatic step in the de novo pyrimidine biosynthesis (115). Furthermore, *URA1* expression is repressed by chemicals, DMSO, and lithium (14, 124). *URA4* encodes dihydroorotase, which catalyzes the third step in pyrimidine biosynthesis (47), while *URA5* encodes orotate phosphoribosyltransferase isozyme that catalyzes the fifth enzymatic step in this pathway (27). Notably, mutations in the human homolog of this gene result in orotic aciduria (108). In addition to these genes, two genes, *FU11* and *FUR4*, encode uridine permease and uracil permease, respectively, were also repressed (Table 3).

**High inhibitory concentration of NP downregulates genes associated with N-linked glycosylation.** Three genes, *ALG5*, *ALG7*, and *ALG13*, associated with the dolichol pathway of protein asparagine-linked glycosylation were significantly downregulated upon exposure to NP for 180 min (Supplemental Table S5). Oligosaccharide synthesis via N-linked protein glycosylation occurs in several steps carried out in the endoplasmic reticulum and Golgi. For this, high-mannose core oligosaccharides are assembled on the dolichyl pyrophosphate with two molecules of N-acetylglucosamine (GlcNAc), nine molecules of mannose, and three molecules of glucose. Then, these oligosaccharide cores are transferred to asparagine residues in the endoplasmic reticulum. Subsequently, the oligo-
saccharide cores are modified in the Golgi apparatus to generate various glycosylated proteins (7). In this process, ALG5 encodes dolichyl-phosphate glucosyltransferase involved in asparagine-linked glycosylation in the endoplasmic reticulum (106), while ALG7 encodes UDP-N-acetyl-glucosamine-1-P transferase involved in the first step in the synthesis of lipid-linked oligosaccharides and catalyzes the addition of the first N-acetylglucosamine to dolichyl phosphate on the cytosolic side of the endoplasmic reticulum (7). Moreover, ALG13 encodes a catalytic component of UDP-GlcNAc transferase required for the second step of dolichyl-linked oligosaccharide synthesis (40).

In the meantime, most of the genes upregulated in response to the high inhibitory concentration of NP were associated with response to chemicals, carbohydrate metabolism, transcription from RNA polymerase II promoter, cell wall organization or biogenesis, and transmembrane and ion transport in both exposure times (Table 4).

**Table 4. Selected biological processes were significantly enriched by the genes upregulated in Saccharomyces cerevisiae cells exposed to 5 mg/l nonylphenol for 120 and 180 min**

<table>
<thead>
<tr>
<th>GOID</th>
<th>GO Term</th>
<th>Frequency</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42221</td>
<td>response to chemical</td>
<td>48/510</td>
<td>PDR3, UGA2, NRG2, HSP30, FIG2, RP4, HBT1, UBC1, CTAL, PDR15, TXA2, MFA1, FRD1, HAC1, AGA2, MIG2, STF2, MTL1, NQM1, SLJ1, VMR1, BAR1, VHR1, XBP1, IML2, SIP4, GPI1, HSP104, FET3, GAD1, SIP18, CRZ1, NCE103, MFA2, PDR16, BXI1, DTR2, TMC1, GYC1, GDR5, PDR10, POS5, USV1, RDS5, YDL124W, YJL144W</td>
</tr>
<tr>
<td>5975</td>
<td>carbohydrate metabolic process</td>
<td>37/510</td>
<td>GAL7, GAL10, TPS1, GI7, GLK1, CT12, NDE2, TPS2, GLC3, UBC8, GIP2, IGD1, PYC1, AMS1, TO53, FIG2, SGAI, GUT2, IMA3, SIP4, INO1, FBP26, PCK1, HSP104, YXL2, GAL2, FBP1, TSL1, SRT1, PG2, CRZ1, MLS1, GYC1, GAC1, TVE1, YIG1, GDB1</td>
</tr>
<tr>
<td>6366</td>
<td>transcription from RNA polymerase II promoter</td>
<td>24/510</td>
<td>PAU8, PDR3, NRG2, RP4N, REF2, CRF1, MET32, YAP6, EM2, HAC1, HSP1, SGI2, RFT1, DTD3, VHR1, XBP1, SIP4, TAF11, CRZ1, GAL11, SKM1, USV1, CSR2, YHR177W</td>
</tr>
<tr>
<td>55085</td>
<td>transmembrane transport</td>
<td>22/510</td>
<td>PEX22, RTC2, FTH1, SUL1, PHO89, VBA3, ADY2, GPD1, CCA2, ATO3, SIT1, HXT13, SSA4, FTR1, PEX1, MFC3, FET3, TOM7, SKM1, PDR10, YFL040W, YOL162W</td>
</tr>
<tr>
<td>71554</td>
<td>cell wall organization or biogenesis</td>
<td>22/510</td>
<td>TIP1, FMP45, DIT1, SPS2, SPS1, SHE10, MTL1, SPS100, SDP1, YPS6, PIR3, PIR1, OSW2, CRZ1, YGPI, LDS2, GAS4, HPF1, PTP2, OSW1, CSR2, YLR194C</td>
</tr>
<tr>
<td>6811</td>
<td>ion transport</td>
<td>22/510</td>
<td>RTC2, FTH1, SUL1, PHO89, VBA3, ADY2, CCA2, ATO3, FTR1, BTN2, MFC3, SFC1, GAP1, CTR3, FET3, CRZ1, PDR16, ENB1, RSBI, COT1, DIP5, CTR1</td>
</tr>
<tr>
<td>42221</td>
<td>response to chemical</td>
<td>50/473</td>
<td>PDR3, UGA2, NRG2, HSP30, FIG2, RP4, HBT1, SNO2, UBC1, CTAL, PDR15, TXA2, MFA1, FRD1, MIG3, UBC6, HSP12, GAT1, AGA2, MIG2, MTL1, NQM1, SLJ1, VMR1, YHK8, SCH9, BAR1, VHR1, XBP1, IML2, SIP4, IXR1, HSP104, AFB1, YAPI, FET3, SIP18, CRZ1, NCE103, MFL3, MFA2, PDR16, GAG1, DRR2, PDR5, PDR10, USV1, RDS5, YDL124W, YJL144W</td>
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<tr>
<td>6366</td>
<td>transcription from RNA polymerase II promoter</td>
<td>44/473</td>
<td>PAU8, PDR3, TDO6, NRG2, RP4N, REF2, MET32, EM2, MIG3, ACA1, RPH1, GAT1, PHO4, AFT1, HSF1, MIG2, RFT1, TF126, DTD3, SIC9, VHR1, XPB1, SIP4, MEL1, IXR1, ABF1, YAPI, TAF11, MCM1, RGM1, CRZ1, MKS1, RAPI, NRD1, GAL11, SDH5, RLM1, USV1, CSR2, ROX1, YHR177W, YOR335W</td>
</tr>
<tr>
<td>5975</td>
<td>carbohydrate metabolic process</td>
<td>27/473</td>
<td>GAL7, VID24, GI7D, NDE2, PMT5, UBC8, IDGI, AMS1, TO53, CRH1, SGA1, GUT2, SIP4, INO1, PCK1, XYL2, GAL2, FBP1, SRT1, CRZ1, RAPI, GPD2, GAC1, TVE1, HSP104, YIG1, GDB1</td>
</tr>
<tr>
<td>6811</td>
<td>ion transport</td>
<td>26/473</td>
<td>RTC2, FTH1, PHO89, VBA3, ADY2, CCA2, ATO3, FTR1, FET3, AFT1, BTN2, MFC3, SFC1, GAP1, FET3, CRZ1, PDR16, FET7, ENB1, RSBI, MCHS, COT1, DIP5, CTR1</td>
</tr>
<tr>
<td>71554</td>
<td>cell wall organization or biogenesis</td>
<td>23/473</td>
<td>TIP1, FMP45, PST1, DIT2, SPS1, SHE10, MTL1, CRH1, SPS100, IRC18, HSP150, PIR3, PIR1, YLR194C, CRRI, CCR14, CRZ1, YGPI, LDS2, HPF1, PTP2, CSR2</td>
</tr>
<tr>
<td>55085</td>
<td>transmembrane transport</td>
<td>20/473</td>
<td>PEX22, RTC2, FTH1, PHO89, VBA3, ADY2, CCA2, ATO3, SIT1, HXT13, SSA4, FTR1, TOM20, PEX21, MFC3, HOT13, AGY2, FET3, YOL162W, PDR10</td>
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</table>

**High inhibitory concentration of NP upregulates genes encoding heat shock proteins and ubiquitin-conjugating enzymes.** Several genes encoding heat shock proteins (HSPs) with chaperone activity were significantly upregulated in *S. cerevisiae* in response to a high inhibitory concentration of NP for both 120 and 180 min exposure times (Table 4, Supplemental Tables S4 and S5). HSPs are conserved proteins involved in the response to various environmental stresses. Interestingly, several HSPs exhibit structural similarities but are functional different between species. In addition, HSPs are essential under normal conditions, particularly in protein biogenesis and function from first-chain synthesis through multimeric complex formation. As such, these factors are known as molecular chaperones (9, 24). In the case of unfolded and/or nonfunctional proteins during abnormal cellular processes, molecular chaperones may aid in protein refolding or proteolysis (24). Seven genes, HSP12, HSP30, HSP42, HSP78, HSP104, HSF1, and SSA4, were induced at both exposure times. Moreover, SSE2, which...
is a member of the Hsp110 subclass of the heat shock protein 70 (HSP70) family, was only induced upon exposure for 120 min. Notably, it serves as a nucleotide exchange factor that loads ATP onto the SSA class of cytosolic Hsp70s known to serve as molecular chaperones (9). On the other hand, HSP150 and MDJ1 were another two genes induced upon exposure for 180 min. HSP150 encodes O-mannosylated HSP required for cell wall stability (77), while MDJ1 encodes chaperone that stimulates HSP70 protein Sec1p ATPase activity and involved in protein folding/refolding in the mitochondrial matrix (116).

Several studies show that HSP genes are upregulated in response to various stresses. For instance, Alexandre et al. (2001) (3) found that HSP genes were upregulated in response to ethanol stress, while HSP70 and HSP90 were significantly induced in C. japonica crabs exposed to different inhibitory concentrations of NP (86, 87). Furthermore, several stress genes, including HSP12 and HSP26, were significantly upregulated in yeast cells exposed to cadmium (76).

We also detected significant upregulation of several genes encoding ubiquitin-conjugating enzymes (Table 4, Supplemental Tables S4 and S5), which are conserved among eukaryotes and play central roles in the cellular stress response and degradation of short-lived and abnormal proteins (50, 54). Notably, UBC1, UBC6, UBC8, UBI4, and PSH1 were induced upon exposure to a high inhibitory concentration of NP for 180 min. Of these, UBC1, UBC8, and UBI4 were also upregulated after the 120 min exposure time. Increased UBI4 expression has been observed in response to a wide variety of stress treatments including heat shock, DNA-damaging agents, such as 4-nitroquinoline-1-oxide and methyl methane sulphonate, and inhibitors of DNA replication, such as hydroxurea, amino-acid analogs, and heavy metals (35, 102, 112). Moreover, it was indicated that environmental stresses in S. cerevisiae cells result in protein degradation and/or misfolding that cause growth inhibition and cell death (50). Taking these data together, we speculated that increased expression of molecular chaperones and ubiquitin-conjugating enzymes, as well as the observed growth inhibition, indicate that NP exposure may cause cellular stress that leads to abnormal and/or nonfunctional proteins.

High inhibitory concentration of NP promotes the expression of genes involved in iron and copper acquisition. Similar to the results obtained for the low inhibitory concentration of NP, several genes associated with iron and copper acquisition were significantly induced following exposure to a high inhibitory concentration of NP at either time durations (Table 4). FET3, FTH1, FTR1, and SIT1 were common upregulated genes associated with iron acquisition, while CTR1, CTR3, and CCC2 were common genes involved in copper acquisition at both exposure times. In addition, two genes associated with iron uptake (FRE1 and FRE7), one ferric enterobactin transporting gene (ENB1), and two genes (AFT1 and FET5) involved in the transcriptional regulation of ferric reductase were induced after 180 min of exposure. These findings suggest that NP may prevent iron and copper uptake by cells or interfere with these ions indirectly, thereby reducing nutrient allocation to the appropriate compartments as previously described (10). Moreover, these findings may also explain the repression of genes associated with the OXPHOS system, as iron and copper are essential for electron transfer and as a cofactor for cytochrome oxidase, respectively (96).

High inhibitory concentration of NP upregulates chemical response genes. As observed in S. cerevisiae cells treated with BPA (10) and exposed to a low inhibitory concentration of NP for 120 min, several ABC superfamily transporters were significantly induced upon exposure to a high inhibitory concentration of NP for 120 and 180 min. For instance, PDR3, PDR5, PDR10, PDR15, PDR16, SNQ, and VMR1 were upregulated at both exposure times in response to a high inhibitory concentration of NP (Table 4). These findings indicate that both low and high inhibitory concentrations of NP may activate the PDR network in yeast cells, suggesting that certain gene products induced in the present study may protect yeast cells by pumping NP molecules out of the cytoplasm as previously found with other toxins (10, 44, 97).

High inhibitory concentration of NP upregulates genes involved in sugar and glycogen metabolism. Sugar metabolism appeared to be significantly affected by exposure to high inhibitory concentration of NP, as evidenced by upregulation of several hexose transporter genes. HXT1, HXT2, HXT5, and HXT13 were induced upon exposure for both 120 and 180 min (Supplemental Tables S4 and S5). Of these, HXT2 and HXT13 encode high-affinity hexose transporters, while HXT5 encodes a moderate hexose transporter (84). Additionally, HXT9, HXT11, and HXT14 were upregulated in S. cerevisiae in response to a high inhibitory concentration of NP after 120 min (Supplemental Table S4). Of these, HXT9 and HXT11 are MFS-related genes and HXT14 encodes a high-affinity hexose transporter (84). Moreover, four of these genes, HXT1, HXT11, HXT13, and HXT15, were downregulated upon exposure to a low inhibitory concentration of NP (Supplemental Tables S2 and S3), suggesting that increasing the NP concentration may change gene regulation in yeast cells based on available glucose concentrations. Furthermore, genes involved in galactose metabolism were also affected in yeast cells exposed to a high inhibitory concentration of NP. In particular, GAL2, GAL7, GAL10, and GAL11 were induced upon exposure to NP for 120 min. Of these, GAL2, GAL7, and GAL11 were also induced after 180 min of exposure to NP (Table 4). GAL2 encodes a high-affinity galactose permease that is an integral plasma membrane protein required for galactose utilization and glucose transport (113). GAL7 encodes Gal7p galactose-1-phosphate uridylyltransferase that catalyzes the conversion of galactose-1-phosphate to UDP-galactose, and mutations in human ortholog GALT have been linked to lethal disease galactosemia (22). Similarly, GAL10 encodes a bifunctional enzyme with mutarotase and UDP galactose 4-epimerase activities, and the human homolog GALE is also implicated in galactosemia (73, 75).

As observed for exposure to the low inhibitory concentration, several genes involved in glycogen metabolic process were also upregulated following exposure to a high inhibitory concentration. Six genes, GIP2, PIG2, DB1, GAC1, IG1, and GLC3, were significantly affected upon exposure for 120 min. of which three, DB1, GAC1, and IG1, were also induced after 180 min of exposure (Table 4).

NP induces autophagy-related gene expression. As observed with the low inhibitory concentration NP treatment, several autophagy-related genes were significantly upregulated upon exposure to a high inhibitory concentration of NP (Supplemental Tables S4 and S5). In particular, ATG8, ATG10, and ATG41 were upregulated after exposure for both 120 and 180 min time
High inhibitory concentration of NP may promote cell wall biogenesis. Several genes encoding cell wall proteins were significantly affected upon exposure to a high inhibitory concentration of NP (Supplemental Tables S4 and S5). Six genes, TIP1, FIG2, PAU24, YGP1, PIR1, and PIR3, were induced after both exposure times (Table 4). TIP1 encodes a major cell wall mannoprotein induced by heat and cold shock (61), while PAU24 encodes cell wall mannoprotein expressed under anaerobic conditions and repressed during aerobic growth (72). Moreover, FIG2 encodes a cell wall adhesion protein required for cell wall integrity during mating (123), while YGP1 encodes a cell wall-related secretory glycoprotein upregulated by nutrient deprivation-associated growth arrest (26). PIR1 and PIR3 encode O-glycosylated, covalently bound cell wall proteins required for cell wall stability (79). Additionally, NCW2, encoding a structural constituent of the cell wall attached to the plasma membrane by a GPI anchor, was induced after 120 min of exposure to NP. NCW2 plays a role in cell wall biogenesis and is induced in response to cell wall stress (33, 111). On the other hand, PST1 and CCW14 were upregulated after 180 min of exposure. PST1 encodes a cell wall protein and is induced on cell wall damage (111), while CCW14 encodes a covalently linked cell wall glycoprotein and is found in the inner layer of the cell wall (78). Moreover, various genes associated with the cell wall were induced in response to a high inhibitory concentration of NP. BAG7 and ARC18 were affected after both 120 and 180 min exposure times. BAG7 acts as a Rho GTPase-activating protein that plays a central role in regulating of cell wall synthesis and organization of the actin cytoskeleton. BAG7 upregulation suppresses the lethality of RHO1 hyperactivation in response to cell wall damage (99). ARC18 encodes a subunit of the ARP2/3 complex, and the null mutant of this gene cannot localize actin patches to growing buds and shows defect in cell growth and viability (118). Moreover, YPK2, which was induced after 120 min of exposure to NP, encodes a protein kinase participating in a signaling pathway required for optimal cell wall integrity (93). In addition, CRH1 encodes a glycosylphosphatidylinositol-cell wall protein involved in adaptation of cell wall stress and was significantly induced upon exposure to NP for 180 min (11). Agarwal et al. (2003) (2) reported that yeast cells respond to amphotericin B and caspofungin by inducing several genes involved in cell wall maintenance and integrity and suggested that the increased expression of these genes generates a compensatory reaction to cell wall damage and increases cell wall stability. Taken together, these data suggest that a high inhibitory concentration of NP may damage the cell wall, and yeast cells upregulate many genes encoding cell wall proteins and functioning in cell wall biosynthesis to overcome potential NP-induced toxicity.

High inhibitory concentration of NP upregulates genes associated with ribonucleotide reductase. Ribonucleotide reductase (RNR) is a tetrameric protein complex that catalyzes the conversion of ribonucleotides to deoxyribonucleotides during de novo DNA synthesis. In addition, RNR has a central role in regulating the overall and balance concentrations of dNTPs for accurate genome duplication and functions in high-fidelity DNA replication and DNA repair processes (51, 90). The RNR complex is composed of two small subunits (Rnr2p and Rnr4p) and two large subunits (Rnr1p and Rnr4p). In case of S phase deficiency and/or DNA damage during cell cycle progression, transcripts encoding the Rnr2p:Rnr4p subcomplex are highly induced and subsequently bind the Rnr1p homodimer to form an active RNR (51, 121). However, RNR may be a good target for cancer therapy, because RNR assembly and RNR2 expression may lead to increased resistance to DNA damage agents and protect cells from chemotherapeutic drugs (1). Moreover, the small subunit of the RNR complex is found as a site of action for antitumor agents in mammalian cells (92). Consistently, several genes related to the RNR complex were induced following exposure to a high inhibitory concentration of NP, including RNR2, RNR3, RNR4, and HUG1 after 120 min of exposure (Supplemental Tables S4 and S5). Of these, RNR2, RNR3, and HUG1 were also upregulated after 180 min of exposure. On the basis of these results, we speculate that NP may cause DNA damage and yeast cells respond to this effect by upregulating RNR complex related genes.

High inhibitory concentration of NP upregulates genes involved in trehalose biosynthesis. Trehalose biosynthesis occurs in two steps. In the first step, glucose 6-phosphate and UDP-glucose are converted to α,α-trehalose 6-phosphate by trehalose-6-phosphate synthase (TPS) encoded by TPS1. In the second step, trehalose-6-phosphate phosphatase encoded by TPS2 converts α,α-trehalose 6-phosphate into trehalose and phosphate (38). Four genes associated with trehalose biosynthesis, TPS1, TPS2, TSL1, and PGM2, were significantly induced upon exposure to a high inhibitory concentration of NP for 120 min (Table 4), while none of these genes or any genes related to trehalose were affected after 180 min. In yeast cells, trehalose is the primary storage carbohydrate in response to various stresses, such as thermal, osmotic, oxidative, and ethanol (89). Moreover, it was reported that co-induction of genes involved in trehalose synthesis and HSP genes in response to ethanol stress supports a strong connection between these functions, consistent with a previous report (3). In addition, Liu and Sung (2011) (68) showed that stress-related TPS expression was significantly upregulated in aquatic crustaceans in response to NP.

High inhibitory concentration of NP promotes the expression of genes involved ribosomal biogenesis and RNA processing. Several genes involved in ribosomal biogenesis and RNA processing were significantly upregulated after 180 min of exposure to a high inhibitory concentration of NP (Supplemental Table S5). Eight genes, MRD1, IMP3, IMP4, KRI1, RCLI1, SRP40, HCA4, and TS4R, involved in ribosomal SSU, six genes, MAK16, CIC1, NOP16, NOG1, RRS1, and RRP36, associated with ribosomal LSU, two genes, TGD6 and MTR4, associated with RNA processing were affected. Interestingly, as indicated above, we also found that several genes associated with ribosomal biogenesis were downregulated upon exposure to a high inhibitory concentration of NP. In analyzing the process, we observed that both the repressed and induced genes are involved in similar compartments. Taking these results together, we speculated that ribosomal production of yeast cells may be affected by NP exposure, and the cells then try to balance the production by downregulating and upregulating different genes in the pathway.
Comparing the gene expression data of the low and high inhibitory concentrations of NP. Clustering analyses showed that several processes/pathways were significantly affected by both inhibitory concentrations of NP. After treatment for 120 min, highly repressed genes in the low inhibitory concentration of NP group were associated with cellular amino acid and monocarboxylic acid metabolic processes, ion transport, transmembrane transport, and transcription from RNA polymerase II promoter. Of these, monocarboxylic acid metabolic processes and transcription from RNA polymerase II promoter were not affected in the high inhibitory concentration group. On the other hand, exposure to a high inhibitory concentration of NP resulted in repression of genes associated with rRNA processing, mitotic cell cycle, ribosomal small subunit biogenesis, transmembrane transport, cellular amino acid metabolic process, and ion transport, which were not affected by the low inhibitory concentration of NP. Among these affected processes, rRNA processing was the most repressed biological process with 16 genes downregulated upon exposure to a high inhibitory concentration of NP, while monocarboxylic acid metabolic process was the least affected process with four genes downregulated in the low inhibitory concentration of NP group (Fig. 3). Moreover, functional annotations show that genes involved in sugar transport, amino acid biosynthesis, zinc regulation, and phosphate metabolism were significantly affected in the low inhibitory concentration group, while 12-spanner drug: H+ antiporter DHA1 family, O-linked glycosylation, cell cycle progression, and ribosomal biogenesis genes were repressed upon exposure to a high inhibitory concentration of NP.

Conversely, the genes induced upon exposure to a low inhibitory concentration of NP were primarily associated with carbohydrate, monocarboxylic acid, and lipid metabolism, generation of precursor metabolites and energy, response to chemicals, and ion transport. Of these, monocarboxylic acid and lipid metabolism and generation of precursor metabolites and energy were not significantly affected by the high inhibitory concentration of NP. On the other hand, response to chemicals, carbohydrate metabolism, transcription from RNA polymerase II promoter, transmembrane transport, cell wall organization or biogenesis, and ion transport were the most induced processes upon exposure to a high inhibitory concentration of NP. Subsequent heat map analysis showed that cell wall organization or biogenesis, transmembrane transport, and transcription from RNA polymerase II promoter were markedly affected in the high inhibitory concentration group. Within these processes, ion transport was the least induced process with nine genes upregulated upon exposure to the low inhibitory concentration of NP, and response to chemicals was the most induced biological process with 48 genes upregulated in the high inhibitory concentration of NP.
inhibitory concentration group (Fig. 3). Moreover, functional annotation for pathway analysis showed that genes involved in both inhibitory concentrations, while fatty acid metabolism, glycogen metabolic process, and drug resistance genes were affected upon exposure to the low inhibitory concentration of NP. On the other hand, genes encoding molecular chaperones, cell wall biogenesis, ribonucleotide reductases, and trehalose biosynthesis were upregulated upon the high inhibitory concentration exposure.

After the 180 min treatment, the most prominently repressed genes in the low inhibitory concentration of NP group are involved in cellular amino acid and monocarboxylic acid metabolic processes, and ion, transmembrane, and carbohydrate transport. Within these processes, carbohydrate transport and monocarboxylic acid metabolism are specific to the low inhibitory concentration group. On the other hand, exposure to the high inhibitory concentration of NP downregulated genes associated with mitochondrion organization, transmembrane and ion transport, nucleobase-containing small molecule and cellular amino acid metabolism, and protein complex biogenesis. Of these, mitochondrion organization, nucleobase-containing small molecule, and protein complex biogenesis were not significantly affected in the low inhibitory concentration of NP group. Among these affected processes, mitochondrion organization is the most repressed biological process with 19 genes downregulated upon exposure to the high inhibitory concentration of NP, whereas carbohydrate transport was the least affected process with three genes downregulated in the low inhibitory concentration group (Fig. 3). According to functional annotations, genes involved in sugar transport, aromatic amino acid biosynthesis, zinc regulation, and phosphate metabolism were significantly downregulated in the low inhibitory concentration group, while genes related to O-linked and N-linked glycosylation, mitochondrial biogenesis, OXPHOS, de novo NAD and pyrimidine biosynthesis, RNA polymerase II-mediated transcription, and ribosomal activity were prominently induced in the high inhibitory concentration group. Furthermore, genes associated with the 12-spanner drug:H+ antiporter DHA1 family were affected within both groups.

Of the upregulated genes, ion transport, response to chemicals, generation of precursor metabolites and energy, organelle fission, cellular amino acid metabolism, and carbohydrate metabolic process were most processes affected upon exposure to a low inhibitory concentration of NP. Of these, cellular amino acid metabolism, organelle fission, and generation of precursor metabolites and energy were significantly affected processes in the low inhibitory concentration group, whereas exposure to the high inhibitory concentration of NP induced genes involved in response to chemicals, transcription from RNA polymerase II promoter, carbohydrate metabolic process, ion transport, cell wall organization or biogenesis, and transmembrane transport. Of these, transcription from RNA polymerase II promoter, cell wall organization or biogenesis, and transmembrane transport were significantly affected processes in the high inhibitory concentration of NP group compared with the low inhibitory concentration group. Among all these processes, carbohydrate metabolic process was the least induced process with three genes upregulated upon exposure to the low inhibitory concentration of NP, and response to chemicals was the most induced biological process with 50 genes upregulated in the high inhibitory concentration group (Fig. 3). Moreover, iron and copper acquisition was the common pathway affected, while arginine catabolism was affected upon exposure to a low inhibitory concentration of NP, whereas heat shock proteins, ubiquitin-conjugating enzymes, cell wall biogenesis, drug resistance genes, ribosomal biogenesis, and RNA processing were affected in the high level group.

These results identified several processes prominently affected by NP exposure depending on the concentration, although certain processes were affected by both inhibitory concentrations, including ion transport, response to chemical, transmembrane transport, and cellular amino acid and carbohydrate metabolism. Moreover, the number and magnitude of the affected genes were also distinct for each inhibitory concentration. After exposure to the high inhibitory concentration of NP, there were severe effects on various processes and several genes with substantial changes in expression. Meanwhile, exposure to the low inhibitory concentration of NP resulted in more modest changes in expression of a small number of genes. Moreover, considering the 120 min exposure time, there were only 15 common downregulated genes between the low and high inhibitory concentrations, while 63 common upregulated genes were found (Fig. 4). Some of these genes were clustered within common biological processes, but most were unrelated. On the other hand, after 180 min of exposure, 16 common genes were downregulated by exposure to both low and high inhibitory concentrations, while only 31 genes were upregulated in both the low and high inhibitory concentration groups. Similar to the 120 min treatment, most of these genes were unrelated, but a small number were found to be involved in common biological processes.

**Validation of microarray data by quantitative real-time RT-PCR analysis.** qRT-PCR analysis was performed with RNA samples used in the microarray experiments to validate the transcriptomic analyses results. For this purpose, 10 *S. cerevisiae* genes that were repressed or induced upon exposure to the high inhibitory concentration of NP for 180 min were selected. These genes were selected for inclusion in a statistically significantly overrepresented biological process and/or to exhibit substantial changes in expression based on the microarray data. Of these 10 genes, three were downregulated (QCR2, QCR7, and COX4, involved in the OXPHOS system), and seven were upregulated (PDR5, PDR16, and SNQ2, ABC superfamily; FRO1 and CTR3, iron and copper acquisition; SUE1, protein involved in degradation of unstable forms of cytochrome c; and PHO89, plasma membrane Na+/Pi cotransporter) in response to the high inhibitory concentration of NP. Of these genes, SUE1 and PHO89 did not meet the first criterion but were chosen because of their significant increase in expression upon exposure to NP. CCW12 was selected as an internal control in this study. No changes in the mRNA expression of this gene were observed in our microarray data in response to NP exposure. As a result, the quantitative real-time RT-PCR data and microarray data were highly consistent with a Pearson correlation of 0.935 for the compared genes (Fig. 5). These observations confirm that our microarray results accurately reflect the gene expression changes in *S. cerevisiae* upon exposure to NP.

In conclusion, the present study analyzed genome-wide transcriptional profiles of *S. cerevisiae* upon exposure to two different inhibitory concentrations of NP. In addition, several
common and unique processes/pathways were affected in response to NP depending on the inhibitory concentration. Considering high production rates, wide use of NP in several industrial applications worldwide, and the potential risks of exposure and bioaccumulation of this compound, future studies should be conducted to analyze the long-term effects of NP at much lower concentrations, which simulate realistic contamination scenarios, although the results ob-

Fig. 4. Common downregulated and upregulated genes upon exposure to low or high inhibitory concentrations of NP for 120 and 180 min.

Fig. 5. Correlation of gene expression from quantitative real-time RT-PCR and microarray data.


Nonylphenol exposure and yeast transcriptomic changes


