Genome-Wide Screen Identifies Genes Whose Inactivation Confer Resistance to Cisplatin in *Saccharomyces cerevisiae*

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Abstract

To identify novel genes that mediate cellular resistance to cisplatin, we have screened the collection of *Saccharomyces cerevisiae* deletion strains. We have found reproducibly 22 genes/open reading frames (ORF), which when deleted, confer resistance to cisplatin at a concentration that is lethal to wild-type cells. Complementation of individual deletion strains with the corresponding wild-type gene abolished cisplatin resistance, confirming that specific gene deletions caused the resistance. Twenty of the genes/ORFs identified have not been previously linked to cisplatin resistance and belong to several distinct functional groups. Major functional groups encode proteins involved in nucleotide metabolism, mRNA catabolism, RNA-polymerase-II–dependent gene regulation and vacuolar transport systems. In addition, proteins that function in ubiquitination, sphingolipid biogenesis, cyclic AMP–dependent signaling, DNA repair, and genome stability are also associated with cisplatin resistance. More than half of the identified genes are known to have sequences or functional homology to mammalian counterparts. Some deletion strains are cross-resistant to selected cytotoxic agents whereas hypersensitive to others. The sensitivity of certain resistant strains to other cytotoxic agents suggests that our findings may point to particular drug combinations that can overcome resistance caused by inactivation of specific genes. (Cancer Res 2005; 65(13): 5890-7)

Introduction

Cisplatin is one of the most widely used anticancer drugs. The basis for the therapeutic effectiveness of cisplatin is not fully clear, but it is believed that cisplatin exerts its cytotoxic action by the formation of platinum-DNA adducts (1). Intrinsic and acquired resistance to cisplatin treatment is a major obstacle for the clinical use of this drug. This is particularly true for colorectal cancer, with fewer than 20% clinical responses when used alone or in combination with other agents (2, 3). Multiple mechanisms have been identified *in vitro* through biochemical studies (1, 4) to account for the development of resistance to cisplatin. These include decreased intracellular drug accumulation, inactivation by glutathione or metallothioneins, increased DNA repair, enhanced tolerance, enhanced replicative bypass, and defects in pathways modulating cell death. These *in vitro* mechanisms of cisplatin resistance, however, do not fully account for the observed clinical unresponsiveness of particular tumors to platinum-based chemotherapy (1). This may be due to the genomic instability of tumors, which gives rise to mutations or defects in multiple molecular pathways. Both gain-of-function and loss-of-function mutations can confer resistance to platinum compounds. The best known examples are the loss of DNA mismatch repair (MMR) genes, hMLH1, hMSH2, or hPMS2 (1, 4). MMR proteins function in recognition of damaged DNA adducts. Previous studies indicate that mutation or methylation-mediated silencing of these genes results in failure to recognize the adduct and propagate a signal to the apoptotic machinery, thereby producing low-level resistance to cisplatin (5). In addition, cisplatin treatment can enrich for malignant populations of cells that have lost DNA mismatch repair both *in vitro* and *in vivo* (4).

Gene knockout studies in *Saccharomyces cerevisiae, Dictyostelium discoideum*, and mammalian cells have identified several genes whose disruption results in resistance to cisplatin (4, 6–10). Some of the genes are conserved among different organisms. In addition to the MMR genes, defects in the steady-state levels of intracellular second messengers such as Ca^{2+}, cyclic AMP (cAMP), cyclic guanosine 3′,5′-monophosphate, sphingosine 1-phosphate/ceramide, and inositol polyphosphates may contribute to resistance (4). These studies have broadened our knowledge to include previously unsuspected mechanisms that control cisplatin sensitivity; however, a full description of the biochemical pathways that mediate the cisplatin-resistant phenotype is still needed. It is our goal to identify and characterize novel genes and/or molecular pathways that may contribute to cisplatin resistance.

Thus far, five of the MMR genes and six other genes (*IXR1, PHR1, SKY1, MAC1, CTR1*, and *NPR2*) have been identified as genes which, when deleted, contribute to cisplatin resistance in *S. cerevisiae* (7–10). The *MAC1, CTR1* (9), and the *NPR2* (10) genes were identified quite recently from two different transposon-insertional libraries, suggesting that additional genes remain to be found. The complete set of 4,637 viable gene deletion mutants generated by the *Saccharomyces* Gene Deletion Project (11) has been successfully used in several genome-scale studies to identify many new genes/pathways important for survival to various cellular insults (12–15). In this study, we screened the pool of deletion strains to identify additional genes that may contribute to cisplatin resistance. We have identified 20 genes not previously reported to modulate cisplatin resistance, as well as two previously reported to be involved in this process.

Materials and Methods

Yeast strains and media. Homozygous diploid deletion pool strains and individual haploid deletion strains (*MATa met15-1 his3-11, 15 leu2-3, 112 lys1-1 ura3-1*) generated by the *Saccharomyces* Gene Deletion Project (11) were obtained from Invitrogen (Carlsbad, CA) or EUROSCARF (Frankfurt, Germany). Standard yeast media and growth conditions were used (16). Yeast cultures were seeded from single colonies grown on yeast extract-peptone-dextrose (YPD) plates or synthetic defined yeast nitrogen

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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base medium (SDM) supplemented with appropriate amino acids for the strain background at 30°C.

Chemicals. Yeast nitrogen base, yeast extract, peptone, and dextrose were purchased from Difco Laboratories (Detroit, MI). Cisplatin, doxorubicin, 5-fluorouracil (5-FU), camptothecin, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) were obtained from Sigma-Aldrich (St. Louis, MO) or LKB laboratory (St. Paul, MN). Stock solutions were prepared as follows. Cisplatin was prepared in DMSO (330 mmol/L), stored as aliquots at −20°C, and used within 2 weeks. This was further diluted in 0.9% NaCl (3.3 mmol/L) before adding to the medium. Doxorubin (10 mg/mL), camptothecin (15 mmol/L), and MNNG (10 μg/mL) were in DMSO and 5-FU (10 mg/mL) in water and were stored at −20°C. All plates were made in SDM and stored in the dark and used within 24 hours.

High throughput cisplatin resistance screen. A one-step selection method was used to screen the pool of deletion strains to isolate mutants that grew on SDM plates containing high concentrations of cisplatin. A lethal concentration of cisplatin to the parental strain, BY4743, was first determined as 160 μmol/L using a colony formation assay on SDM plates. Subsequently, 1×10^5 cells in the pool of deletion strains were screened twice. Resistant colonies with various sizes were observed after 3 days of growth at 30°C.

Confirmation of cisplatin resistance by semiquantitative spot assay. Putative cisplatin-resistant colonies were picked and grown overnight in liquid YPD at 30°C. Cultures were then diluted to a concentration of 5×10^6 cells/mL, and additional 5-fold dilutions were made. One microliter of each dilution was spotted onto SDM plates containing no cisplatin or 80 to 160 μmol/L of cisplatin and grown at 30°C for 3 days. The spot intensity for each strain was determined using densitometry (Alpha imager, Alpha Innotech, Sanleandro, CA) and was divided by the spot intensity of the corresponding untreated strain to determine the percent survival. The fold resistance relative to the wild-type parental cells was calculated using the percent cell survival in each spot at a drug concentration that the wild-type cells retained 30% survival. Colonies that exhibited at least 1.5-fold resistance to cisplatin were subjected to clone identification. Two to three independent spot assays were done.

Clone identification. Genomic DNA from the candidate colonies that passed the retest were isolated and subjected to sequencing for their barcode identity. To obtain barcode sequences, the region containing the UPTAG and a portion of the KanMX cassette was amplified by PCR using the common primer U1 and a KanMX primer. The identity of each clone was identified by matching to the open reading frame (ORF) deletion primer sequences from the *Saccharomyces* Genome Deletion Project.[1]

Complementation test. The low-copy yeast vector pRS416 containing centromere and the *URA3* auxotrophic marker was used to carry the wild-type genes to complement the deletion strains. The region of the chromosome containing the gene and an upstream region (~500 bp) including the endogenous promoter were PCR amplified from BY4743 genomic DNA. The sequences of the primers used will be made available upon request. The PCR products were cloned into pRS416 vector using the primer restriction sites and sequenced to ensure correct gene insertion. The constructs and the empty vector were introduced into the yeast deletion strains using the EZ transformation kit (Epicenter, Madison, WI) and plated on SDM plates without uracil. To test for cisplatin sensitivity, transformants were grown overnight in SDM without uracil and serial dilutions were spotted on plates with or without cisplatin.

Cross-sensitivity to cytotoxic agents. The approximate concentrations of each drug to obtain 30% survival relative to untreated cells was determined for wild-type strain, BY4741, and used for comparison with the haploid deletion strains. Diluted cultures were spotted in duplicate on plates with or without drugs as described above. Percentage cell survival for each drug is expressed relative to untreated cells (100%).

[1] http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html.

Results and Discussion

Identification of novel yeast genes that, when deleted, confer resistance to cisplatin. To identify novel genes that may contribute to cisplatin resistance, we did two genome-wide screens of the pooled set of 4637 *Saccharomyces cerevisiae* diploid gene deletion mutants. We used a one-step selection method, which selected for strains that grew on plates containing a concentration of cisplatin that is lethal to wild-type cells. Colonies of various sizes were observed and arbitrary grouped into large, medium, and small; 133 and 137 of the large and medium size colonies were recovered in the first and second screens, respectively. In addition, >200 small colonies were also found in each of the screens. All of the large, medium, and 60 of the small colonies from each screen were retested for resistance to cisplatin by spotting diluted cells onto medium containing 80 to 160 μmol/L cisplatin. The relative survival of each clone compared with that of the wild-type strain was determined (Material and Methods). Figure 1 shows an example of a plate in which most colonies passed the retest (i.e., R2-R7). We found that 353 of 390 colonies retested were at least 1.5-fold resistant to cisplatin compared with wild-type cells. A cutoff of 1.5-fold was chosen because 1.5- to 3-fold resistance is frequently seen clinically and is sufficient to reduce tumor responsiveness to cisplatin *in vivo* (17).

The identities of these colonies were then determined by sequencing the PCR-amplified UPTAG using the universal primers that flank the barcodes (see Materials and Methods). Twenty-three genes/ORFs were identified, all in both screen one and screen two. Only 22 of them are listed in Table 1 because one of the original deletion strains turned out not to be resistant to cisplatin (see below). Most of the genes were identified multiple times in each screen (Table 1). In particular, approximately one third of the sequenced colonies were deletions of the *FCY2* gene. In addition, a total of 117 genes/ORFs were identified in one or the other of the two screens, with 61 in the first and 56 in the second (Supplementary Table S1). Because colonies with deletions of these genes/ORFs also passed the retest, we considered that these might also play some role in cisplatin resistance. Of those genes/ORFs identified in both screens, two (*IXR1* and *SKY1*) have been previously reported (4, 6, 8) and 20 have not previously been shown to mediate cisplatin cytotoxicity in yeast.

To rule out the possibility that the resistant phenotype of these colonies is a result of cisplatin-induced mutations during the screening process, the individual deletion strains from the mutant collection were obtained and tested for cisplatin resistance using the spot assay. This test was done using the haploid deletion strains because they require a lower drug concentration to achieve cytotoxicity with cisplatin and other drugs. We tested all the mutants that were identified in both screens (Table 1), and 18 mutants, which were identified in only one of the two screens. Strains with deletion of the *UPF1* gene or the *SKI8* gene, which belong to the mRNA catabolism pathway, were also included in the test. Figure 2*A* shows the results obtained with selected strains. Most of these individual deletion strains had a growth rate similar to that of the wild-type cells, as indicated in the plates without cisplatin. Quantitative measurements of the spots in Fig. 2*A* and doubling time analysis showed that only *Δsnf6* and *Δbul1* grew significantly slower than the wild-type cells (data not shown). The percentage survival rates upon cisplatin treatment indicated that most of these strains were ~2- to 40-fold resistant to cisplatin at a concentration (140 μmol/L) that the wild-type cells retained 5% survival (WT, Fig. 2*B*). Of the 23 strains that were

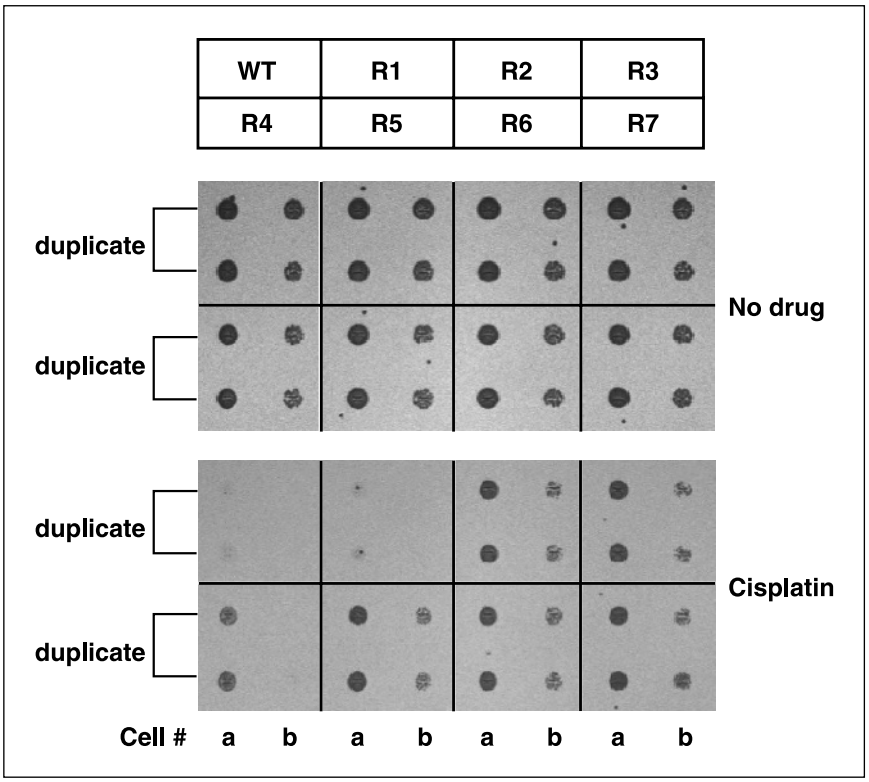

Figure 1. Retest of cisplatin sensitivity. Parental strain (WT, BY4743) and several putative cisplatin resistant colonies (R1-R7) were grown in YPD overnight at 30°C. Dilutions of the cultures (*a*, 1×10^4 and *b*, 1×10^3) were spotted in duplicate (*bracket*) on SDM plates with or without 160 μmol/L of cisplatin and incubated at 30°C. Map of the strains corresponding to the spots on the plates. Photographs were taken 3 days after spotting. The test was repeated twice for each colony.

identified in both initial screens, only one (*Δesc2*) did not exhibit significantly more resistance than the wild-type cells. Of the 18 mutants tested that were identified in only one of the two initial screens, 15 of them exhibited significant resistance to cisplatin (Fig. 2*B*; Supplementary Table S1), suggesting that many of these genes also mediate a certain degree of cisplatin resistance.

Complementation of the resistance phenotype of deletion strains by wild-type genes. To confirm that the cisplatin-resistant phenotype of the deletion strains is due to the specific disruption of genes and is not the result of unrelated mutations acquired during the screening procedure, a complementation test with the wild-type genes was done in selected strains. The region of the chromosome containing the gene along with an upstream region containing the endogenous promoter were cloned into a low-copy, centrometric, *URA3*-containing vector and transformed into the corresponding deletion strain. The *Δfcy2, Δhpt1, Δnmd2*, and *Δbul1* strains carrying the plasmids containing the corresponding amplified wild-type genes exhibit sensitivity to cisplatin similar to that of wild-type cells, in contrast to strains transformed with the empty vector (Fig. 3). The introduction of the wild-type genes or the vector into these deletion strains did not affect their growth rate as determined by the doubling time (data not shown) and as shown on the plates without cisplatin. These results show that the resistance phenotype of these strains was indeed caused by deletion of specific genes.

Cross-sensitivity of cisplatin-resistant mutants to other cytotoxic agents. Cisplatin is frequently used in combination with other anticancer agents in chemotherapy. In addition, some of the gene deletions identified here have been reported to be associated with sensitivity to other DNA damaging agents such as MMS, HU, UV, ionizing radiation, and bleomycin (14, 15, 18). To determine the specificity of the resistance phenotype, we examined some cisplatin-resistant mutants for sensitivity to various agents, including carboplatin (cisplatin analogue), 5-FU (antimetabolite), doxorubicin (topoisomerase II inhibitor), camptothecin (topoisomerase I inhibitor), and MNNG (alkylating agent) using the spot assay. Diluted cell cultures were spotted on SDM plates containing different drugs at concentrations in which the wild-type cells retained ~30% survival. The results are summarized in Table 2. We found that these strains did not exhibit uniform sensitivity to the agents tested; some strains showed selective cross-resistance to certain agents whereas exhibiting hypersensitivity to others or exhibiting sensitivity similar to that of wild-type cells (Table 2). For examples, strains with deletion of *FCY2, HPT1*, and *SOK1* genes showed cross-resistance to 5-FU, whereas deletions of the *ITR1, SKY1, BUL1, SKI3*, and *SKI8* genes, and YJL175W ORF were all hypersensitive to 5-FU. Whereas 10 cisplatin-resistant mutants (deletion of *FCY2, UPF1, NMD2, UPF2, YGL214W, ITR1, ELG1, SKY1*, *UBI4*, and *ELG1* genes) displayed weak resistance to doxorubicin, six (deletion of *NOT3, STP1, VPS29, BUL1, UBP13*, and YJL175W) exhibited hypersensitivity to this drug. It is interesting to note that deletions of several of the genes in the mRNA catabolism pathway (*UPF1*, *NMD2*, *SKI3*, *SKI8*, and YGL214W) also showed weak cross-resistance to the topoisomerase I inhibitor, camptothecin. We were not able to detect any significant difference in sensitivity to carboplatin between the deletion strains and the wild-type cells, because the highest concentration in its maximal solubility for this drug only reached 20% killing of the wild-type cells in the spotting assay (data not shown). Finally, deletions of the identified genes did not cause cross-resistance to MNNG. Instead, most of them showed hypersensitivity to this agent. The results suggest that in only a few cases does resistance to these agents operate by a similar pathway. These data also suggest that sensitivity to cisplatin damage requires functions different from those of other cytotoxic agents tested and that particular drug combinations may be able to overcome resistance caused by specific gene defects.

Novel genes mediate cisplatin resistance. The identified genes fall into several distinct functional groups according to the ontology categories in the SGD (19). Multiple genes were found in four major pathways: nucleotide metabolism, mRNA catabolism, RNA Pol-II-dependent gene regulation, and vacuolar and membrane transport system (Table 1). A database search revealed that 16 of the 22 reproducible genes encode proteins that share either sequence or functional homology with human counterparts (Table 1).

Among the genes identified, deletion of the *FCY2* gene, a purine-cytosine permease, which also transports protons through the plasma membrane (19), was found most frequently. A related gene, *HPT1*, encoding the hypoxanthine guanine phosphoribosyl transferase (19) was also identified multiple times (Table 1). Both Fcy2p and Hpt1p function in nucleotide metabolism, which involves *de novo* biosynthesis and the salvage of intracellular and extracellular nucleobases or nucleosides. The reasons that deletion of *FCY2* or *HPT1* genes confers cellular resistance to cisplatin are not readily apparent. It has been shown that mutation in the *FCY2* gene results in resistance to purine and cytosine analogues and this was attributed to a defect in analogue transport. It is tantalizing to suggest that cisplatin resistance in *Δfcy2* may result from a defect in cisplatin transport. Alternatively, the cisplatin resistant phenotype of *Δfcy2* and *Δhpt1* cells may be a result of deregulation of the *de novo* AMP biosynthesis pathway.

Another major group of genes identified comprises pathways involved in mRNA catabolism. These include the *NMD2/UPF2* and *UPF3* genes in the nonsense mRNA decay (NMD) pathway which degrades transcripts harboring premature signals for translation termination (20) and the *SKI3* gene in a family of exosome-associated proteins which degrades mRNAs without translation termination codons (nonstop decay; ref. 21). Deletion of other genes (*UPF1* and *SKI8*) in this pathway also confers resistance to cisplatin (Fig. 2). mRNA degradation controls an important aspect of gene expression and often serves as a surveillance mechanism that eliminates aberrant mRNAs and deleterious proteins in all

Table 1. Yeast genes identified in cisplatin resistance screen

Gene/ORF	Function*	Fold^{R}	Frequency† (first/second)	Mammalian orthologues‡
Nucleotide metabolism				
FCY2	purine-cytosine permease	++++	47/60	nucleoside transporters
HPT1	enzyme involved in purine biosynthesis	+++	4/5	HPRT1
mRNA catabolism				
NMD2	nonsense mRNA decay	++	2/2	hUPF2
UPF3	nonsense mRNA decay	++	5/4	hUPF3A or hUPF3B
SKI3	translation repressor activity	++	3/2	exosome
RNA Pol II-dependent gene regulation				
NOT3	exoribonuclease activity, Poly(A) shortening	++	2/2	hNOT3
SNF6§	chromatin remodeling transcription factor	++	4/3	Snf/Swi complex
STP1	specific RNA polymerase II transcription factor	++	2/2	ZNFN1A4
IXR1$^{\|}$	DNA binding, DNA repair, transcription	++	2/1	hMGB2
SOK1	cAMP-dependent signaling	+	2/2	hMST4
Vacuolar and membrane transport				
SKY1$^{\|}$	SRPK1-like kinase, RNA splicing factor	+++	4/3	SRPK1
SEM1	regulator of exocytosis	++	4/3	DSS1
VPS29	endosome to Golgi transport	+	2/1	VPS29
ITR1	myo-inositol transporter	+	3/2	SLC2A13
Others				
ECM30	cell wall organization	++	2/2	
BUL1§	mitochondria, monoubiquitination	++	4/6	NEDD4L
ELG1	recombination, genome stability, DNA repair	+	2/2	alternative RF-C
Uncharacterized ORFs				
YGL214W	dubious, overlap with SKI8 (translation repressor)	++	3/3	
YJL175W	dubious, within SWI3 (transcription factor)	++	3/3	
YJL135W	dubious, overlap with LCB3 (sphingosine-1-P phosphatase)	++	2/2	
YLR352W	uncharacterized	+	2/2	
YDL173W	uncharacterized	+	3/2	
	Total colonies analyzed	193/197		

NOTE: Fold^{R}, fold resistance relative to the wild-type parental cells was determined at a concentration approximating the 30% survival (IC_{70}) for the wild-type cells.

*Cellular role as indicted in Saccharomyces Genome Database (SGD) and Munich Information Center for Protein Sequences (Mips, https://mips.gsf.de/).

†Number of colonies found in two independent screens (first/second).

‡Potential human orthologue genes or functional counterparts found in SGD, NCBI Entrez Gene, and Genbank databases.

§Strains that exhibit slow growth.

‖Genes previously characterized which, when deleted, confer cisplatin resistance.

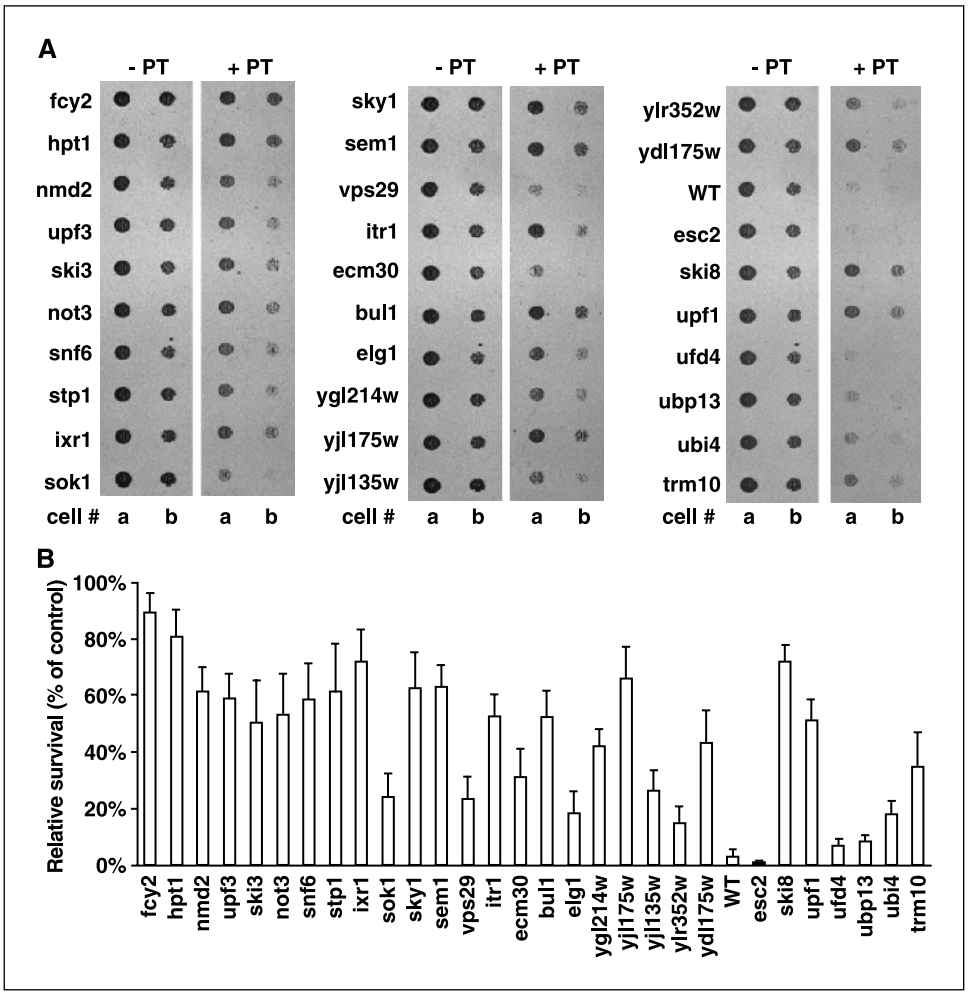

Figure 2. Confirmation of cisplatin resistance of individual deletion strains. *A,* exponentially growing deletion strains were diluted to an $A_{600\ nm}$ of 0.25. Cells were then serially diluted, and ~5,000 (*cell # a*) and 1,000 (*cell # b*) cells were spotted onto SDM plates without or with 100 to 160 μmol/L of cisplatin. A total of 42 deletion mutants and the parental wild-type strain (WT, BY4741) were tested. Plates were photographed after 48 hours of incubation at 30°C. Representative pictures from plates with 140 μmol/L of cisplatin. *B,* percentage survival (%) of strains shown in (*A*) is expressed relative to untreated cells. *Columns,* densitometry measurements of the cell # a and averages of at least three independent experiments; *bars,* SD.

eukaryotic cells (22). These genes also regulate normal transcripts (23, 24). It should be interesting to investigate how accumulated nonsense or nonstop transcripts and/or altered regulation of normal transcripts in these deletion strains contribute to their cisplatin resistant phenotypes.

Several of the genes identified encode proteins belonging to a network of transcription factors (YIL038C/*NOT3*, YDR463W/*STP1*, YHL025W/*SNF6*, YDR006C/*SOK1*, and YJL175W). The Not3p is a subunit of the CCR4-NOT complex (19), which is a global transcriptional regulator with roles in transcription initiation and elongation and in mRNA degradation. *STP1* encodes a transcription factor (19), which is activated by proteolytic processing in response to signals from the plasma membrane sensor SPS (*SSY1, PTR3*, and *SSY5*) system for external amino acids (25). It regulates amino acid permease genes and may have a role in tRNA processing (26). *SNF6* is involved in global regulation of transcription and is part of the SNF/SWI chromatin remodeling complex (19). YJL175W is a dubious ORF that overlaps the *SWI3* gene which interact with *SNF6*. In addition, *SPT20* (Supplementary Table S1) is a member of the SAGA (Spt/Ada/Gcn5 acetyltransferase) complex (19). These transcriptional complexes form a network in which some members of each complex interact genetically or physically in many cellular processes. *SOK1* is a suppressor of protein kinase A, functioning in cAMP-dependent signaling (19). Protein kinase A activity has been related to cisplatin cytotoxicity in human cells (27). Interestingly, knockout of the RegA cAMP-phosphodiesterase in *D. discoideum* (28) has been found to result in cisplatin resistance. Another 13 genes involved in RNA processing, mainly of RNA-polymerase-II-mediated transcripts, were also found to contribute weakly to cisplatin resistance (Supplementary Table S1). Transcription factors such as SNF/SWI chromatin modifiers have been shown to facilitate DNA accessibility and repair in different pathways leading to the maintenance of genome integrity (29). It is possible that defects in genes involved in RNA-polymerase-II transcription result in deregulation of repairing the cisplatin-DNA adducts and consequently lead to increased genome instability and altered drug sensitivity.

The largest group of genes identified is involved in vacuolar and membrane transport (Table 1; Supplementary Table S1). These include YDR497C/*ITR1*, YHR012W/*VPS29*, YDR363W-A/*SEM1*, and YMR216C/*SKY1*. Transcriptional regulation of *ITR1*, a member of the sugar permease family, depends on the *INO4* gene (30), which also confers resistance to cisplatin when deleted (Supplementary Table S1). *VPS29* functions in vacuolar protein sorting and is involved in retrograde transport of proteins from endosomes to the trans-Golgi network (31). *SEM1* encodes a regulator of exocytosis which also functions as a subunit of the 26 S proteasome (32). In addition, deletions of another 28 genes involved in vacuolar transport or membrane-trafficking pathways also exhibited low levels of resistance to cisplatin (Supplementary Table S1). These

data suggest that both the Golgi-to-endosome and endosome-to-vacuole stages of transport play major roles in cisplatin cytotoxicity. Indeed, it has been shown that cisplatin-accumulating vesicles are associated with the Golgi apparatus (33, 34). Consistent with our findings, deletion of Golvesin, a Golgi-associated protein that putatively functions in vesicular membrane trafficking, results in cisplatin resistance in *D. discoideum* (28). The previously identified copper transporter *CTR1* gene (9, 35) was not identified under our conditions, but one of the copper transporters, YLR214W/*FRE1*, encoding ferric reductase was detected in one of our screens (Supplementary Table S1). We have also found that deletion of genes encoding for regulators of various transporters confers resistance to cisplatin. These include the *SKY1, PTK2*, and *NPR2* genes (Table 1; Supplementary Table S1). Sky1p and Ptk2p are members of two different kinase families and are involved in regulating polyamine transport (36). Other studies have shown that disruptions of the *SKY1* and *NPR2* (nitrogen permease regulator) genes confer resistance to cisplatin and doxorubicin (10, 37), and resistance to both drugs was confirmed here for the *SKY1* mutant. However, it was shown that neither Sky1p nor Npr2p is directly involved in the accumulation of cisplatin and doxorubicin (10, 37). It is possible that these regulators of transporters mediate intracellular drug transport to DNA.

Genes from several other pathways, such as ubiquitination (YMR275C/*BUL1*), cell wall biogenesis and architecture (YLR436C/*ECM30*), and DNA repair (*IXR1*) and genome stability (YOR144C/*ELG1*) also contribute to cisplatin resistance when deleted. Although only one gene from each of these pathways was found repeatedly in both screens, at least one other gene in the same pathway was identified in one of the screens. For example, *BUL1*, encoding a protein involved in mono-ubiquitination (19), was identified in both screens, whereas *UBP13, UMP1*, and *UBI4*, which also function in ubiquitination, were found in one of the screens (Supplementary Table S1). In addition, five ORFs with unknown function were also found in both screens and some of these ORFs reside within or overlap genes with known function. The cisplatin resistant phenotype of these mutants could be derived from the mutations of the known genes. For example, YGL214W overlaps with the *SKI8* gene (19), which functions in mRNA catabolism, and deletion of *SKI8* gene also confers cisplatin resistance (Fig. 2).

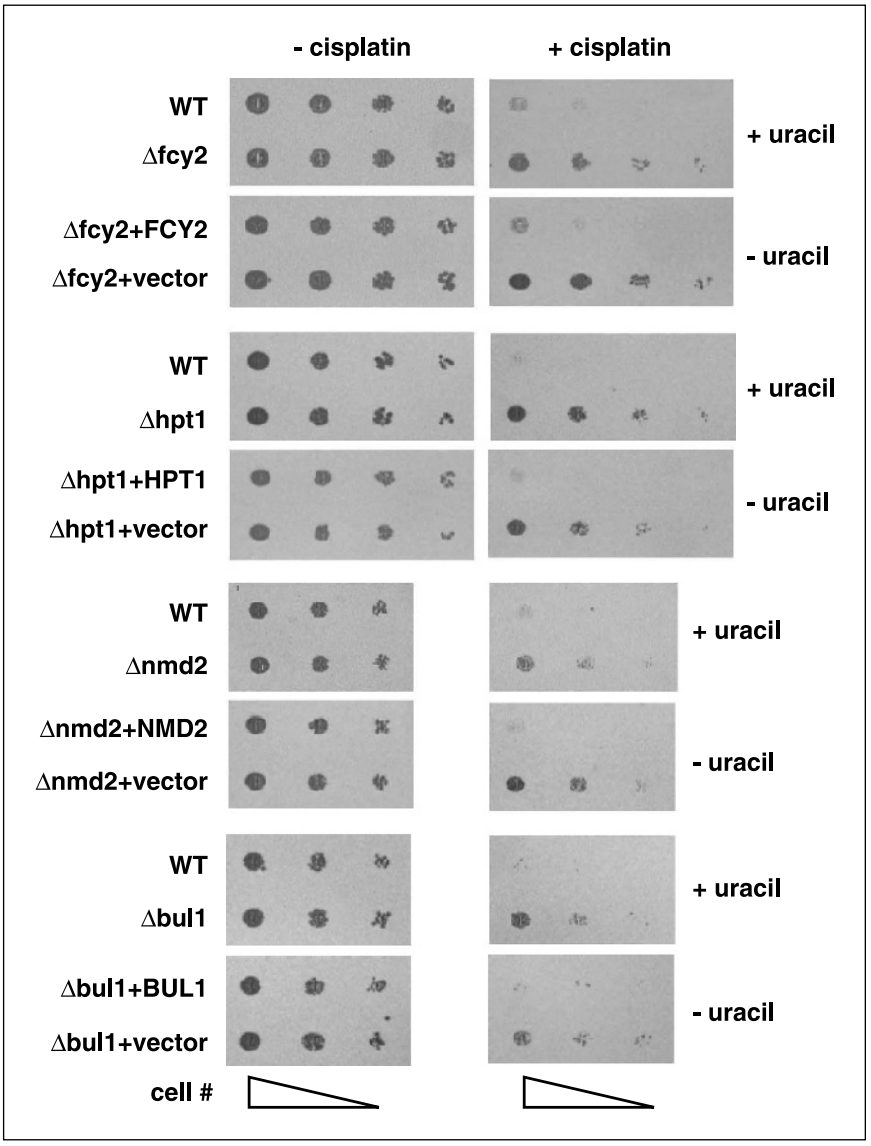

Figure 3. Complementation of selected deletion strains resistant to cisplatin. The haploid deletion strains were transformed with pRS416 carrying the corresponding wild-type gene or with empty pRS416 vector. Five-fold serial dilutions of $\sim 6 \times 10^4$ log-phase cells were spotted in duplicate on SDM plates without (− cisplatin) or with (+ cisplatin) 140 μmol/L of cisplatin. For expressing the wild-type genes in the deletion mutants, uracil was omitted in plates for selection of the *URA3* gene in pRS416 (− *uracil*). Plates were incubated at 30°C for 48 hours. Representative of three independent tests.

Table 2. Cross-sensitivity of cisplatin-resistant deletion strains to other cytotoxic agents

Gene/ORF	5-FU	Doxorubicin	Camptothecin	MNNG
FCY2	+5	+1.5	0	0
HPT1	+2.5	0	0	0
UPF1	0	+2	+1.5	−1.5
NMD2	0	+2	+1.5	−1.5
UPF3	0	+1.5	+1.5	−2
SKI3	−3	+1.5	+1.5	−2
YGL214W	−2	+2	+1.5	−4
SKI8	−2	+1.5	+1.5	−3
NOT3	0	−2	0	−3
SOK1	+2	0	−1.5	0
STP1	−1.5	−4	−1.5	−2
YJL175W	−3	−2	+2	0
SKY1	−5	+2	0	−4
ITR1	−1.5	+2	−1.5	−10
VPS29	−1.5	−2	−2	−3
BUL1	−4	−2	0	0
UBP13	−1.5	−4	0	−2
YLR352W	0	0	0	−1.5
ECM30	0	0	0	0
ELG1	0	+4	−2	−10

NOTE: Relative sensitivity to each drug was determined in comparison with the wild-type cells by semiquantitative spot assay. Fold resistance (+) or fold sensitivity (−) relative to the wild-type parental cells was calculated using the percent survival from duplicate spots at a drug concentration which the wild-type cells retained ~30% survival. 0 represents strains that have similar sensitivity to that of the wild-type cells to the drugs. The values were derived from average of two to three independent experiments. Concentrations of the drugs are 5-FU, 25 μg/mL; doxorubicin, 20 μg/mL; camptothecin, 20 mmol/L; MNNG, 1 μg/mL.

In addition, YJL135W overlaps with YJL134W/*LCB3*, which functions in sphingolipid metabolism. Six other genes involved in lipid biogenesis were also identified in one of the two screens (Supplementary Table S1) suggesting that sphingolipid metabolism also plays an important role in cisplatin resistance. Studies in *D. discoideum* also found that disruption of sphingosine-1 phosphate (S1P) lyase confers resistance to cisplatin (28). Finally, the YDL173W protein has been found in the yeast two hybrid assay to interact with proteins, Sec17p and Sec4p, which function in vesicular transport (19). Deletion of the *SEC4* gene was also found in one of the cisplatin resistance screens (Supplementary Table S1).

Whether the other gene products in these functional groups are also involved in cisplatin cytotoxicity remains to be determined. We expect that many more genes can be identified because more than two thirds of the small colonies found in the screens have not been tested. It is also possible that only a subset of genes in each pathway is required for cisplatin sensitivity. Very recently, Wu et al. (38) have used the oligonucleotide array approach to screen the same set of deletion mutants and found that deletions of 130 genes involved in diverse DNA repair pathways result in hypersensitivity to cisplatin. The same study also reported 100 strains that seemed resistant to cisplatin (Supplementary Data in ref. 38); however, the resistance of the individual strains has not been confirmed. Of these 100 deletion strains, only two were identified in our study. Different screening methods and cisplatin treatment procedures likely account for the discrepancy. Our method uses drug at a lethal concentration for the wild-type cells and screens for gene deletion strains that show enhanced resistance to the drug. Whereas this method can only, and maybe better, be used to identify genes whose deletion confers resistance to drugs, the oligonucleotide-array approach was optimized to identify sensitive strains. Our approach is straightforward and does not rely on the availability of the oligonucleotide arrays and special software for the analysis. Others have also used robot-aided screen of arrays of individual deletion strains to identify both bleomycin-hypersensitive and bleomycin-resistant mutants (39); however, this requires expensive instrumentation and resources.

Conclusions

It is well known that tumors may harbor many mutations or deletions. These alterations may result in low-level resistance where the survival and expansion of the malignant cell population occur during treatment. We have shown that a cisplatin resistance phenotype can be produced from defects in many different genes, some of which are quite specific for cisplatin among the agents tested, others of which also exhibit resistance to other agents. The genes identified here constitute novel components of a cisplatin resistance phenotype. Although some of the genes (or genes with related function) whose disruption have been found to mediate cisplatin resistance in *D. discoideum*, most of these have not been previously reported to mediate cisplatin resistance in yeast or humans. With RNA interference technology, it should be possible to determine whether knockdown of any of the human functional orthologues confers cisplatin resistance. In addition, future studies to investigate whether these genes are mutated or down-regulated in cisplatin-resistant tumor cells may also provide indications regarding the role that these genes play in cisplatin cytotoxicity in human cancers.

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