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Genomic Screening in Vivo Reveals the Role Played by Vacuolar H^+ ATPase and Cytosolic Acidification in Sensitivity to DNA-Damaging Agents Such as Cisplatin

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ABSTRACT

Screening the *Saccharomyces cerevisiae* homozygous diploid deletion library against a sublethal concentration of cisplatin revealed 76 strains sensitive to the drug. As expected, the largest category of deletions, representing 40% of the sensitive strains, was composed of strains lacking genes involved in DNA replication and damage repair. Deletions lacking function of the highly conserved vacuolar H^+ translocating ATPase (V-ATPase) composed the category representing the second largest number of sensitive strains. The effect on cell death exhibited by V-ATPase mutants was found to be a general response to various DNA damaging agents as opposed to being specific to cisplatin, as evidenced by sensitivity of the mutants to hydroxyurea (a DNA-alkylating agent) and UV irradiation. Loss of V-ATPase does not affect DNA repair, because double mutants defective for V-ATPase function and DNA repair pathways were more sensitive to cisplatin than the single mutants. V-ATPase mutants are more prone to DNA damage than wild-type cells, indicated by enhanced activation of the DNA damage checkpoint. Vacuole function per se is not cisplatin-sensitive, because vacuolar morphology and vacuolar acidification were unaffected by cisplatin in wild-type cells. V-ATPase also controls cytoplasmic pH, so the enhanced sensitivity to DNA damage may be associated with the drop in pHi associated with V-ATPase mutants. The increased loss in cell viability induced by cisplatin at lower pH in V-ATPase mutants supports this hypothesis. The loss in viability seen in wild-type cells under the same conditions was far less dramatic.

Cisplatin [*cis*-diamminedichloroplatinum(II)] is one of the most widely used anticancer drugs. Platinum-based chemotherapy cures most cases of advanced testicular cancer and has high efficacy in the treatment of other solid tumors, such as ovarian and small-cell lung cancers. The principal cytotoxic mechanism associated with cisplatin is the generation of platinum-DNA adducts, the most significant DNA lesions being 1,2-intrastrand cross-links that form across adjacent guanines (Wang and Lippard, 2005).

Unfortunately, acquired resistance to cisplatin can limit therapeutic potential (Perez, 1998). There are several resistance mechanisms, including decreased intracellular drug accumulation, enhanced cellular detoxification by glutathione and metallothionein, altered DNA repair, and inhibition of apoptosis (Perez, 1998; Huang et al., 2005). However, these mechanisms do not completely account for the observed in vivo unresponsiveness of certain tumors to cisplatin (Niedner et al., 2001; Schenk et al., 2003). Therefore, novel pathways mediating cisplatin resistance exist.

Use of model organisms, such as the yeast *Saccharomyces cerevisiae*, has been instrumental in revealing the molecular basis of cisplatin toxicity. Complex systems driving signal transduction, DNA repair, and the cell cycle are all highly conserved throughout the eukaryotic lineage. The range of mechanisms that can be probed using *S. cerevisiae* are those relating to maintenance of viability at the unicellular as opposed to the multicellular level. However, conclusions drawn from studies using these organisms are unambiguous because of the ability to disrupt expression of individual genes. Not surprisingly, these studies confirm the importance of DNA repair pathways including NER, RER, and PR (Grossmann et al., 2001; Beljanski et al., 2004; Wu et al., 2004). A significant advantage conferred by the use of *S. cerevisiae* genome-wide resources has been the identification of new genes not associated with DNA repair that mediate

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ABBREVIATIONS: NER, nucleotide excision repair; RER, recombination dependent repair; PR, postreplication repair; RR, replication-dependent repair; V-ATPase, vacuolar H^+-transporting ATPase; YPD, yeast extract/peptone/dextrose; PBS, phosphate-buffered saline; HU, hydroxyurea; MMS, methyl methanesulfonate.

response to cisplatin. For example, elevated levels of phosphodiesterase 2 and the transcription factor Cin5 confer resistance to cisplatin (Burger et al., 2000; Furuchi et al., 2001). Cells lacking the serine/threonine kinase Sky1, the copper transporter Ctr1, and the nitrogen permease regulator Npr2, are also resistant to cisplatin (Ishida et al., 2002; Schenk et al., 2003, 2004). These strategies, exploiting gene overexpression from genomic libraries or transposon-mediated gene disruption, do not provide comprehensive coverage of the entire genome, because overexpression libraries rarely include all genes, and transposon insertion libraries do not disrupt genes in a random manner. Therefore, we screened the entire set of 4728 homozygous deletion strains, representing deletion of all nonessential open reading frames in *S. cerevisiae*, to identify genes that lead to sensitivity to cisplatin. A similar screen found 130 deletion strains that were sensitive to cisplatin (Wu et al., 2004). This screen, however, involved mixing all deletions followed by exposure to cisplatin and extraction of genomic DNA. Molecular barcodes identifying each deletion were amplified, and hybridized to an oligonucleotide array, enabling abundance of each deletion strain to be determined (Wu et al., 2004). Cisplatin itself is a potent DNA damaging agent. As a result, a DNA barcode associated with a strain that is sensitive to cisplatin might not be amplified, because it has sustained excessive damage. This would be the case in barcodes bearing consecutive guanines, given the nature of cisplatin-induced DNA damage. Lack of amplification is more likely in strains most sensitive to the drug. To avoid this problem, we individually assessed each deletion, as described previously in screens for strains sensitive to the DNA-alkylating agent methyl methanesulfonate and tirapazamine, a topoisomerase II inhibitor (Chang et al., 2002; Hellauer et al., 2005). Our approach was vindicated because we present identification of 49 cisplatin-sensitive deletions not identified by the screen involving amplification of molecular barcodes. Among the deletions novel to our screen were eight strains that each lacked a component of the highly conserved vacuolar H^+ translocating ATPase (V-ATPase). Work in mammalian systems correlates V-ATPase activity with the response to cisplatin. Genes encoding subunits of the V-ATPase are induced when human cell lines are treated with cisplatin and are up-regulated in cisplatin-resistant cell lines (Murakami et al., 2001; Torigoe et al., 2002). Many genes are up-regulated in tumors, although not all of the corresponding proteins play a role in tumor progression. Our data suggest that correlation between V-ATPase function and cisplatin sensitivity underpins a key relationship between the enzyme and sensitivity to the drug. Furthermore, we showed that reduction in V-ATPase activity increased susceptibility to DNA damage per se, explaining why V-ATPase inhibitors render tumors more sensitive to DNA-damaging agents in general.

Materials and Methods

Yeast Strains and Media. The homozygous diploid deletion strains and individual haploid deletion strains were made by the *Saccharomyces* Gene Deletion Project (Winzeler et al., 1999). They were obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (Frankfurt, Germany). The parental diploid strain BY4743 was used as control in the screening of the deletion library for sensitivity to cisplatin. Strain BY4742 was used as the control for work using haploid strains. Genes encoding Vma6 or Vma8 subunits of V-ATPase were deleted in the BY4742 background using a Hygromycin B resistance gene as selectable marker, as described by Goldstein and McCusker (1999). Combinations of mutants in the same strain were constructed by standard procedures (Rose et al., 1990). The genotypes of strains used in this study are listed in Table 1. Yeast was grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) prepared as described in Rose et al., 1990. Cisplatin, HU, and MMS were obtained from Sigma (St. Louis, Missouri). Cisplatin stock solutions were prepared in YPD or phosphate-buffered saline (PBS) and stored as aliquots at −20°C.

Screen for Cisplatin-Sensitive Yeast Strains. We determined that the sublethal concentration of cisplatin required to result in visibly slower growth of the parental yeast diploid strain (BY4743) was 250 μg/ml. Deletion strains were maintained by growth as arrays of 384 colonies on solid YPD. All replications were automated and were carried out using a 384-pin replicator operated by a Biomek FX Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA). Colonies were picked and resuspended in 50 μl of YPD in 384-well plates and incubated for 48 h at 30°C (to stationary phase). Each culture (5 μl) was transferred to fresh 50 μl of YPD in 384-well plates and grown to log-phase (14 h at 30°C with intermittent agitation). Replicas of these plates were made on solid YPD media with and without cisplatin (250 μg/ml), followed by incubation at 30°C. During incubation for 3 days, growth was scored by colony size compared with that of the wild-type strain BY4743 (as described in Hellauer et al., 2005). Mutants showing a significant growth defect or absence of growth after 1 day in the presence of 250 μg/ml cisplatin were scored as "×" or "××." Mutants showing a significant growth defect or absence of growth after 3 days in the presence of 250 μg/ml cisplatin were scored as "×××" or "××××."

TABLE 1
S.cerevisiae strains used in this study.

Strain	Genotype	Source
BY4742	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*	EUROSCARF
BY4743	*Mat a/α*; *his3Δ2/his3Δ2*; *leu2Δ0/leu2Δ0*; *met15Δ0/MET15 LYS2/Lys2Δ0*; *ura3Δ0/ura3Δ0*	EUROSCARF
rad52	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YML032c::kanMX4*	EUROSCARF
rad1	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YPL022w::kanMX4*	EUROSCARF
rev3	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YPL167c::kanMX4*	EUROSCARF
vma6	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YLR447c::HygB*	This study
vma8	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YEL051w::HygB*	This study
vma6/raD52	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YLR447c::HygB YML032c::kanMX4*	This study
vma6/raD1	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YLR447c::HygB YPL022w::kanMX4*	This study
vma6/rev3	*Mat α*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YLR447c::HygB*; *YPL167c::kanMX4*	This study

EUROSCARF, European *Saccharomyces cerevisiae* Archive for Functional analysis

Assessing Sensitivity or Viability of Individual Strains to Cisplatin. Cultures were grown in liquid YPD at 30°C to exponential phase and diluted to equal cell density. Six-fold serial dilutions were spotted across YPD, or YPD containing the concentration of cisplatin indicated in figure legends, followed by incubation at 30°C for 3 days. For viability assays, equal cell densities of exponential phase cultures were incubated in PBS, pH 7.4, containing the concentration of cisplatin indicated in figure legends; aliquots were removed after 1 h, diluted, and spread over YPD plates. Percentage viability was determined from the number of colonies that appeared after incubation for 3 days at 30°C, as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

Sensitivity to DNA Damaging Agents Other than Cisplatin. Cultures were grown at 30°C to exponential phase and diluted to equal cell density (1×10^7 cells/ml). Four successive 6-fold serial dilutions were spotted across YPD or YPD plus 20 mM hydroxyurea (HU) or 0.01% methyl methanesulfonate (MMS). For assessing sensitivity to UV irradiation, dilutions spotted across YPD were exposed to 40 J/m^2 UV using a UV cross-linker (Syngene, Frederick, MD). All plates were subsequently incubated for 3 days at 30°C.

Vacuole Staining. FM4-64 was obtained from Invitrogen (Carlsbad, CA) and quinacrine from Sigma (St. Louis, MO). Staining with FM4-64 was performed as described by Conibear and Stevens (2002). FM4-64 was loaded into cells at 40 μM for 15 min followed with one wash to remove free dye and a chase period for 60 min at 30°C in YPD, or YPD with 100 μg/ml cisplatin. Cells were examined by confocal laser microscopy using Texas Red filters. Staining with 200 μM quinacrine (in the presence or absence of 100 μg/ml cisplatin) was performed as described by Roberts et al. (1991). Once stained, cells were visualized within 10 min by confocal laser microscopy using fluorescein filters.

Western Blot Analysis of Rad53. Exponential phase cultures grown at 30°C were incubated for 1 h in the presence of cisplatin at concentrations indicated in the figure legends. Yeast protein extracts were prepared from TCA-treated cells, and Rad53 was detected with a rabbit polyclonal antibody (Tercero et al., 2003).

Effect of pH on Cisplatin Sensitivity. Equal cell densities of exponential phase cells were incubated for 1 h at 30°C in PBS (pH 7.4 or 5.8) containing cisplatin, at the concentrations of drug indicated in figure legends, followed by dilution and inoculation over YPD plates. Percentage viability was determined from the number of colonies that appeared after incubation for 3 days at 30°C and expressed as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

Results

Screen for Cisplatin-Sensitive Yeast Strains. Hypersensitive strains revealed by screening with a sublethal concentration of cisplatin would reveal the identities of genes that normally function in pathways sensitive to this drug. We performed a high-throughput robot-aided screen for sensitivity to 250 μg/ml cisplatin, using a collection of 4728 homozygous diploid yeast deletion mutants corresponding to nonessential yeast genes. Sensitive mutants were scored by comparison with colony size of the isogenic wild-type strain, after incubation for 1 day. The same colonies were scored again after a further 2-day incubation. Overall, 76 strains were sensitive to cisplatin (Table 2). The sensitivity of these mutants was confirmed by individually pinning serial dilutions of deletion strains on 250 μg/ml cisplatin and control plates.

Four of the seventy-six strains, however, correspond to deleted open reading frames that overlap with other genes. These are the *YLR235c*, *YGL167c*, *YOR331c*, and *YKL118w* deletions, which overlap with *TOP3*, *HUR1*, *VMA4*, and *VPH2*, respectively. Two of the overlapped genes (*TOP3* and *HUR1*) are involved in DNA repair. The other two are involved in V-ATPase structure and function. In each case, it is likely that the resistance is due to deletion of the overlapping gene (*TOP3*, *HUR1*, *VMA4*, and *VPH2*), given 1) the well established importance of DNA repair mechanisms in the processing of cisplatin-DNA adducts, and 2) the importance of V-ATPase activity in resistance to cisplatin, demonstrated in the work presented here and inferred through correlative studies by others (Murakami et al., 2001; Torigoe et al., 2002). The genes are presented in categories based on known or inferred function (Table 2).

Exposure to cisplatin leads to DNA damage, so it was not surprising to find that the largest category, representing 42% of the sensitive strains, was composed of 31 strains deleted for genes involved in DNA replication and damage repair. These fell mainly into the following categories of DNA repair: NER (six genes, including *RAD1* and *RAD2*), RER (eight genes, including *RAD51* and *RAD52*); replication-dependent repair (RR; eight genes, including *MUS81* and *POL32*), and PR (three genes, including *RAD18*). Deletions of two DNA helicases (*HPR5* and *SGS1*) and one topoisomerase (*TOP3*) were also sensitive. Twenty-three (of the 31 strains) were reported previously to be cisplatin-sensitive (Birrell et al., 2002; Wu et al., 2004); and seven were reported to be sensitive to tirapazamine, another anticancer drug that causes DNA damage (Hellauer et al., 2005). Deletion of genes in the following functional categories also gave rise to cisplatin sensitivity: cell cycle (10 genes: Table 2), cell stress, and signal transduction (*YDJ1*, *PPH3*, *SOD1*, *ZUO1*, *SEP1*, and *HAL5*), protein synthesis (*EAP1*, *MRF1*, *RPL13B*, *RPL7A*, and *SRO9*), transcription (*RPB9* and *HCM1*), and two transporters (*TPK1* and *GUP1*).

The category represented by the second largest number of cisplatin-sensitive deletions was composed of strains lacking function of the vacuolar H^+ translocating ATPase (V-ATPase). The disruption of V-ATPase function is lethal in all eukaryotic organisms except *S. cerevisiae* (Graham et al., 2003). The lethality in higher systems is connected to aspects of V-ATPase function that are more significant in such organisms than they are in a unicellular organism like yeast, such as receptor-mediated endocytosis. Yeast has one hormone receptor, the action of which is nonessential; this is in contrast to various essential receptors in membranes of complex organisms (Graham et al., 2003). This made budding yeast an ideal system to further investigate the role of V-ATPase in cisplatin resistance. We present data to explain why such mutants show more sensitivity to cisplatin as well as other DNA-damaging agents.

V-ATPase Mutants Are Sensitive to Cisplatin. The V-ATPase is composed of thirteen subunits (Graham et al., 2003). Loss of any one of eight V-ATPase subunits led to cisplatin sensitivity (Table 2). Four strains lacked a component of the catalytic (V_1) domain responsible for ATP hydrolysis, and the other four lacked a component of the proton translocating (V_o) domain. In addition, deletion of *VPS33*, a gene required for assembly of V-ATPase (though itself not a part of the enzyme) also led to cisplatin sensitivity. To confirm these results, we generated two strains, one lacking a component of the V_o domain (*vma6*Δ, Table 1), the other lacking a component of the V_1 domain (*vma8*Δ, Table 1). As expected, both *vma6*Δ and *vma8*Δ strains exhibited severely

TABLE 2

Cisplatin-sensitive deletion strains

Mutants showing a significant growth defect or absence of growth after 1 day in the presence of 250 μg/ml cisplatin scored as "× " or "× × ." Mutants showing a significant growth defect or absence of growth after 3 days in the presence of 250 μg/ml cisplatin scored as "× × × " or "× × × × ."

ORF	Gene	Sensitivity	Cellular Function and Comments
DNA replication and damage repair			
YJL092W	*HPR5*	× × × ×	DNA helicase involved in DNA repair
YMR137C	*PSO2*	× × × ×	DNA cross-link repair protein
YPL022W	*RAD1*	× × × ×	Single-stranded DNA endonuclease involved in DNA repair
YGR258C	*RAD2*	× × × ×	Single-stranded DNA endonuclease involved in DNA repair
YER162C	*RAD4*	× × × ×	NER
YML095C	*RAD10*	× × × ×	Single-stranded DNA endonuclease involved in DNA repair
YMR201C	*RAD14*	× × × ×	NER
YCR066W	*RAD18*	× × × ×	DNA PR
YNL250W	*RAD50*	× × × ×	Subunit of MRX complex involved in double-strand break repair
YER095W	*RAD51*	× × × ×	DNA recombinational repair
YML032C	*RAD52*	× × × ×	DNA double-strand break repair
YDR076W	*RAD55*	× × × ×	DNA recombinational repair
YDR004W	*RAD57*	× × × ×	DNA recombinational repair
YLR234W	*TOP3*	× × × ×	DNA topoisomerase III
YDR369C	*XRS2*	× × × ×	DNA recombinational repair
YLR235C		× × × ×	ORF overlap with TOP3
YPR135W	*CTF4*	× × × ×	Required for sister chromatid cohesion
YIL154C	*IMP2'*	× × × ×	Transcriptional activator involved in protection against DNA damage
YBR098W	*MMS4*	× × ×	DNA endonuclease
YDR386W	*MUS81*	× × ×	DNA repair and replication fork stability
YJR043C	*POL32*	× × ×	Polymerase-associated gene
YLR032W	*RAD5*	× × ×	Single-stranded DNA-dependent ATPase involved in PR
YDL059C	*RAD59*	× × ×	Involved in double-strand break repair
YIL139C	*REV7*	× × ×	Subunit of DNA polymerase ζ involved in DNA repair
YJL115W	*ASF1*	× ×	Nucleosome assembly factor
YDL013W	*HEX3*	× ×	Involved in the DNA Damage response with possible recombination role
YGL087C	*MMS2*	× ×	DNA PR
YGL175C	*SAE2*	× ×	Involved in meiotic recombination and chromosome metabolism
YMR190C	*SGS1*	× ×	Nucleolar DNA helicase of the RecQ family
YLR135W	*SLX4*	× ×	DNA repair and replication fork stability
YGL168W	*HUR1*	×	Required for hydroxyurea resistance and DNA replication
YCL060C	*MRC1*	×	Required for DNA replication and RaD53 checkpoints activation
YGL167C	*PMR1*	×	ORF overlap with HUR1
Vacuolar V-ATPase and vacuolar function			
YPR036W	*VMA13*	× × ×	Vacuolar ATPase V_1 domain subunit
YOR332W	*VMA4*	× ×	Vacuolar ATPase V_1 domain subunit
YLR447C	*VMA6*	× ×	Vacuolar ATPase V_0 domain subunit
YGR020C	*VMA7*	× ×	Vacuolar ATPase V_1 domain subunit
YEL051W	*VMA8*	× ×	Vacuolar ATPase V_1 domain subunit
YCL007C	*VMA9*	× ×	Vacuolar ATPase V_0 domain subunit
YHR039C-B	*VMA10*	× ×	Vacuolar ATPase V_0 domain subunit
YHR026W	*VMA16*	× ×	Vacuolar ATPase V_0 domain subunit
YKL119C	*VPH2*	× ×	Required for the biogenesis of a functional vacuolar ATPase
YLR396C	*VPS33*	× ×	Vacuolar sorting protein essential for vacuolar morphogenesis/function
YOR331C		× ×	ORF overlap with VAM4
YKL118W		× ×	ORF overlap with VPH2
Cell cycle			
YLR337C	*VRP1*	× × ×	Involved in cytoskeletal organization and cellular growth
YCR063W	*BUD31*	× ×	Involved in bud-site selection
YLR226W	*BUR2*	× ×	Bypass UAS requirement
YHR191C	*CTF8*	× ×	Required for sister chromatid cohesion
YMR078C	*CTF18*	× ×	Required for sister chromatid cohesion
YCL016C	*DCC1*	× ×	Required for sister chromatid cohesion
YGL240W	*DOC1*	× ×	Required for the activity of APC
YPR119W	*CLB2*	×	Involved in mitotic induction
YGR092W	*DBF2*	×	Kinase required for late nuclear Division
YGR252W	*GCN5*	×	Histone acetyltransferase, acetylates lysine 14 on histone H3
Cell stress and signal transduction			
YNL064C	*YDJ1*	× × × ×	Heat shock protein
YDR075W	*PPH3*	× × ×	Catalytic subunit of protein phosphatase 2A
YJR104C	*SOD1*	× × ×	Cu,Zn superoxide dismutase
YGR285C	*ZUO1*	× × ×	Cytosolic ribosome-associated chaperone
YLR403W	*SFP1*	× ×	Transcription factor controls expression of ribosome biogenesis genes (in response to nutrients and stress)
YJL165C	*HAL5*	×	Putative protein kinase involved in sodium and lithium tolerance
Ribosomal protein and protein synthesis			
YKL204W	*EAP1*	× ×	eIF4E-associated protein
YGL143C	*MRF1*	× ×	Mitochondrial polypeptide chain release factor
YMR142C	*RPL13B*	× ×	Component of the 60S ribosomal subunit
YGL076C	*RPL7A*	×	Component of the 60S ribosomal subunit
YCL037C	*SRO9*	×	RNA-binding protein which associates with translating ribosomes

TABLE 2—Continued

ORF	Gene	Sensitivity	Cellular Function and Comments
Transcription			
YGL070C	*RPB9*	× ×	RNA polymerase II core subunit
YCR065W	*HCM1*	×	Transcription factor involved in cell cycle specific transcription
Transporter			
YJL129C	*TRK1*	× ×	180-kDa high-affinity potassium transporter
YGL084C	*GUP1*	×	Membrane protein and putative glycerol transporter
Others			
YKR082W	*NUP133*	× × ×	Subunit of f the nuclear pore complex
YNL280C	*ERG24*	× ×	C-14 sterol reductase
YGL076C	*FYV5*	× ×	Unknown
YLR376C	*PSY3*	× ×	Unknown; deletion results in sensitivity to oxaliplatin & cisplatin
YJR121W	*ATP2*	×	F1 subunit of mitochondrial F_1F_0 ATP synthase
YDR245W	*MNN10*	×	Subunit of a Golgi mannosyltransferase complex

ORF, open reading frame; MRX, Mrell-Radso-Xrs2; UAS, upstream activation sequence; APC, anaphase promoting complex.

impaired growth in the presence of cisplatin compared with growth on media without the drug (Fig. 1A). In a cell survival assay, rapid loss of viability was exhibited by these mutants in the presence of cisplatin (Fig. 1B).

Vacuolar Morphology and Acidity Are Not Disrupted by Cisplatin. The vacuole of budding yeast is required for protein turnover, nutrient recycling, osmoregulation, storage of amino acids and inorganic phosphate, and maintenance of cytoplasmic pH (Graham et al., 2003). The vacuole (or an aspect of vacuolar function) may be a target of cisplatin, in that loss of V-ATPase function rendered cells hypersensitive to the drug. To investigate this possibility, we assessed the effect of cisplatin on vacuole morphology and vacuole acidification. In general, one to five vacuoles can be visualized in wild-type cells when stained with the fluorescent dye FM4-64 (Conibear and Stevens, 2002). Many mutants defective for vacuole function exhibit aberrant morphology of the vacuole itself (Raymond et al., 1992). Cisplatin at 100 μg/ml had no effect on the viability of wild-type cells but clearly led to loss of viability in the *vma6*Δ strain (Fig. 1B). However, under these conditions, vacuole morphology was unaltered in either strain, appearing normal in size, shape, and number (Fig. 2A). Even though cisplatin does not affect vacuolar morphology, it could disrupt vacuole acidification. The lumen of the vacuole is more acidic than the surrounding cytoplasm, so the resulting luminal pH drives numerous vacuole-associated processes (Graham et al., 2003). Cells were stained with quinacrine, a fluorescent weak base that accumulates only in the vacuolar lumen upon acidification (Roberts et al., 1991). Intensity of fluorescence is directly proportional to the degree

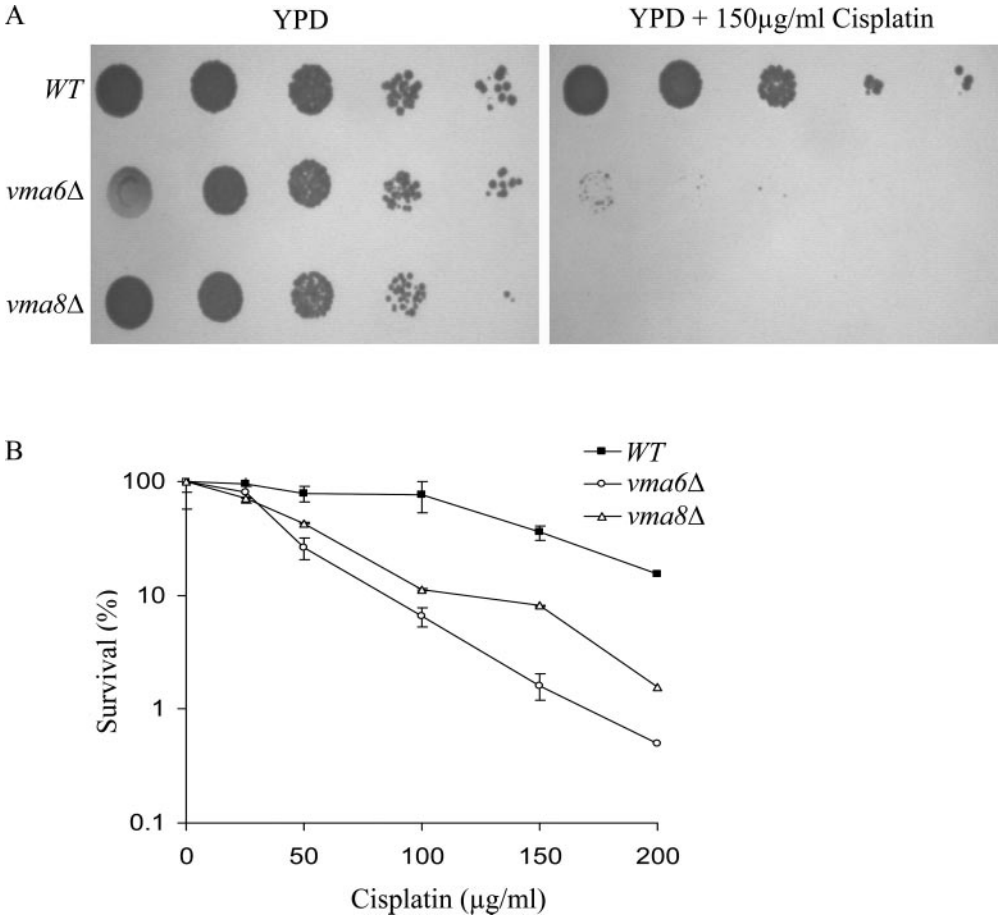

Fig. 1. Sensitivity of the V-ATPase mutants to cisplatin. A, wild-type and V-ATPase mutants were grown at 30°C to exponential phase and diluted to equal cell density. Six-fold serial dilutions were spotted across YPD or YPD containing 150 μg/ml cisplatin; plates were incubated at 30°C for 3 days. B, exponential phase wild-type and V-ATPase mutants diluted to equal cell density were incubated in PBS, pH 7.4, containing cisplatin at the concentration indicated; aliquots were removed after 1 h, diluted, and spread over YPD plates. Viability was determined from the number of colonies that appeared after incubation at 30°C for 3 days—as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

of vacuolar acidification. Wild-type cells treated with up to 200 μg/ml cisplatin accumulate the same amount of quinacrine as cells incubated without the drug (Fig. 2B). As expected, *vma6*Δ cells could not be stained with quinacrine because of the failure of V-ATPase–mediated vacuolar acidity (Fig. 2B). These data indicate that vacuoles in wild-type cells are acidified and morphologically normal in cells treated with cisplatin, suggesting that the vacuole per se is not the target of cisplatin.

V-ATPase Mutants Are Hypersensitive to Other DNA-Damaging Agents. Cisplatin hypersensitivity in V-ATPase mutants could be due to increased levels of DNA damage, or may diminish the effectiveness of DNA repair mechanisms. We assessed the response of V-ATPase mutants to various DNA-damaging agents. MMS is a DNA-alkylating agent, and HU is a DNA replication inhibitor, giving rise to stalled replication forks that are sensed by the cell as abnormal DNA structures (Tercero et al., 2003). *vma6*Δ and *vma8*Δ strains are both hypersensitive to each agent (Fig. 3). The vacuole itself can act as a detoxification mechanism by accumulation of small molecules via the endocytic machinery. The *vma6*Δ and *vma8*Δ strains, however, are both also hypersensitive to UV irradiation compared with the wild type (Fig. 3). Therefore, it is likely that the increased sensitivity to cisplatin, HU, and MMS exhibited by V-ATPase mutants is not due to a defect in a mechanism that involves vacuolar sequestration of these cytotoxic agents. Instead, DNA could be more prone to damage in these cells or repair mechanisms may be less efficient.

Assessing Sensitivity of V-ATPase/DNA Repair Double Mutants to Cisplatin. The cisplatin-induced DNA cross-linked adducts in *S. cerevisiae* are repaired mainly by three DNA repair mechanisms: the NER, RER, and PR pathways. To determine the possibility that loss of V-ATPase would diminish the effectiveness of these repair pathways, we performed epistasis analysis using V-ATPase/DNA repair double mutants. *RAD1*, encoding a single-stranded DNA endonuclease, is a classic NER gene. *REV3* encodes a subunit of DNA polymerase zeta, which is involved in PR. *RAD52* encodes a protein that stimulates strand exchange during RER. Double mutants were constructed that were defective for V-ATPase function and one DNA repair pathway. All three double mutants (*vma6*Δ*rad1*Δ, *vma6*Δ*rev3*Δ, and *vma6*Δ*rad52*Δ) were more sensitive to cisplatin than the single mutants (Fig. 4). This suggests that involvement of V-ATPase with DNA damage sensitivity is independent of DNA repair pathways. Instead, V-ATPase mutants could be more sensitive to cisplatin simply because more damage is caused by a given concentration of drug.

Activation of the DNA Damage Checkpoint in V-ATPase Mutants. When yeast cells are treated with cisplatin, the DNA damage checkpoint is activated, leading to cell cycle arrest at G_2/M. Mutants in components of the checkpoint display increased sensitivity to cisplatin (Grossmann et al., 1999). The sensitivity to cisplatin exhibited by strains lacking the S-phase checkpoint protein *MRC1* is in agreement with this (Table 2). Rad53, the effector protein kinase in this pathway, is activated via hyperphosphorylation in response to DNA damage (Tercero et al., 2003). Phosphorylated Rad53 is detected as a smear of slowly migrating forms of the protein on Western blots immunoprobed with anti-Rad53 antisera. We incubated cells in the presence of various concentrations of cisplatin for 1 h. The incubation of both wild-type and *vma6*Δ cells with 200 μg/ml cisplatin led to the appearance of hyperphosphorylated forms of Rad53,

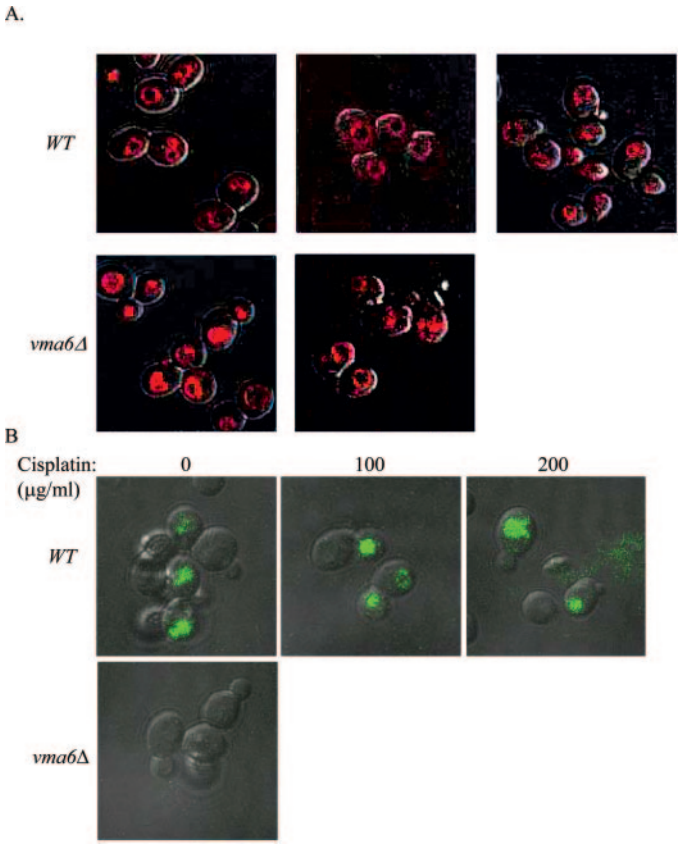

Fig. 2. Vacuolar morphology and acidity are not disrupted by cisplatin. Wild-type and V-ATPase mutants were grown at 30°C to exponential phase and diluted to equal cell density, followed by incubation with cisplatin at the concentration indicated. Cells were stained with FM4-64 for visualization of vacuolar morphology (A) or quinacrine for assessing the acidity of the vacuole (B).

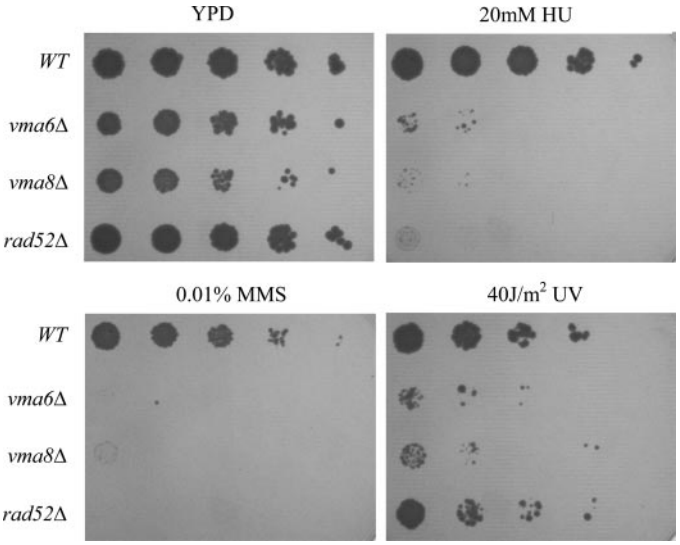

Fig. 3. Sensitivity of V-ATPase mutants to various DNA damaging agents. Wild-type and V-ATPase mutants were grown at 30°C to exponential phase and diluted to equal cell density. Six-fold serial dilutions were spotted across YPD, YPD containing 20 mM HU or 0.01% MMS, or YPD followed by exposure to 40 J/m^2 UV; plates were incubated at 30°C for 3 days.

suggesting that lack of V-ATPase activity did not impair the DNA damage checkpoint pathway (Fig. 5). A single band that indicated the nonphosphorylated form of Rad53 was detected in the absence of the drug, in both wild-type and *vma6*Δ cells (Fig. 5). In *vma6*Δ cells, however, hyperphosphorylated Rad53 was detected on incubation with 50 μg/ml cisplatin. In contrast, hyperphosphorylated Rad53 was barely detectable in wild-type cells treated in the same way, suggesting that V-ATPase mutants suffered more DNA damage than the wild-type cells.

Effect of pH on Cisplatin Sensitivity. One of the major functions of V-ATPase is maintenance of intracellular pH by translocating protons from the cytosol into the lumen of the vacuole (Graham et al., 2003). In both unicellular and multicellular organisms, cytoplasmic pH is reduced when function of the V-ATPase is compromised (Moreno et al., 1998; Murakami et al., 2001). The increased DNA damage sensitivity in the V-ATPase mutants could be due to the acidified cytoplasm. To investigate the effect of pH on cisplatin sensitivity, we followed viability of cells after incubation with cisplatin in neutral (pH 7.4) or acidic (pH 5.8) conditions. Wild-type yeast cells are efficient at maintaining a constant intracellular pH, even when they are incubated in media buffered to varying pH. This is due principally to the action of a plasma membrane H^+-translocating ATPase. This enzyme pumps H^+ out of the cell, consuming up to 40% of total cellular ATP. To remove the possibility that action of this enzyme would compensate for the effect of incubating cells at varying pH, we incubated cells in the absence of a carbon source so that cells could not make ATP to drive H^+ extrusion by PM ATPase. Intracellular pH of *S. cerevisiae* incubated under these conditions closely approaches the pH of the extracellular medium, with intracellular pH changes being due to H^+ leakage across the plasma membrane (Brett et al., 2005).

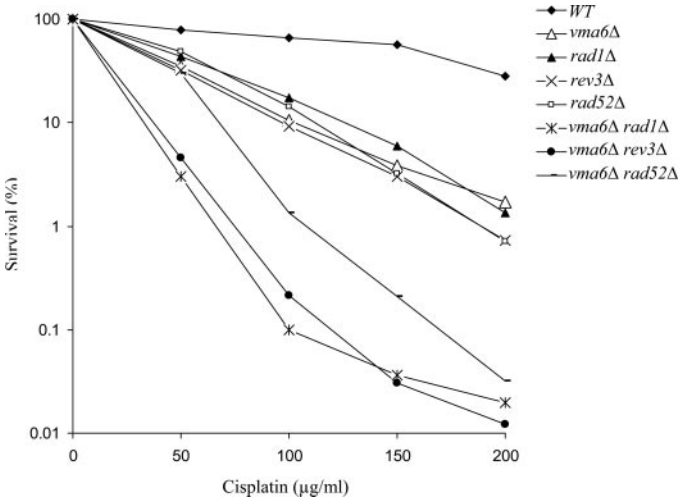

Fig. 4. Sensitivity of [V-ATPase/DNA repair] double mutants to cisplatin. Exponential phase wild type and single and double mutants were diluted to equal cell density and incubated in PBS, pH 7.4, containing cisplatin at the concentration indicated; aliquots were removed after 1 h, diluted, and spread over YPD plates. Viability was determined from the number of colonies that appeared after incubation at 30°C for 3 days and expressed as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

Both wild-type and *vma6*Δ cells were more sensitive to cisplatin when incubated at lower pH. The effect of low pH, however, was more dramatic in *vma6*Δ cells (Fig. 6). In the presence of 20 μg/ml cisplatin, the viability of *vma6*Δ cells at pH 5.8 was decreased by ~76% (by ~24.% at neutral pH), whereas the viability of the wild-type cells at pH 5.8 was decreased by ~25% (by ~10% at neutral pH). This implies that lower cytoplasmic pH increases sensitivity to DNA damage in V-ATPase mutants.

Discussion

Pathways that modulate cisplatin sensitivity could be induced in tumor cells, and development of agents to inhibit these pathways can overcome the resistance that emerges frequently during treatment. Development of new platinum drugs by substitution of ligands or chloride, leaving groups of cisplatin has not been successful in terms of overcoming drug resistance. New approaches can be developed based on discovery of mechanisms that mediate toxicity of this drug.

The budding yeast *S. cerevisiae* has been used as a powerful tool to identify and investigate pathways targeted by drugs. In the present study, we have applied a systematic approach to search for the nonessential genes in yeast that play a role in the response to cisplatin, leading to the identification of 76 deletion strains sensitive to this drug.

More than 40% of the cisplatin-sensitive strains lacked various components involved in DNA damage repair. The deleted genes were mainly involved in three DNA repair pathways: RR and PR, RER, and NER. However, none of the components involved in base excision repair or mismatch repair were identified in this screen, suggesting that PR, RR, and NER are the main mechanisms by which cells repair cisplatin-induced DNA damage, whereas base excision repair and mismatch repair are not involved. This is in agreement with previous studies (Grossmann et al., 2001; Beljanski et al., 2004; Wu et al., 2004).

A novel insight from our screen is that sister chromatid cohesion plays a role in the response to cisplatin. Dcc1, Ctf8, and Ctf18 are components of a complex required for establishment of sister chromatid cohesion. Cells lacking the genes encoding any of these three proteins are sensitive to cisplatin. Cells lacking another gene involved in cohesion (Ctf4) are affected in the same way. Recruitment of the cohesin complex to sites of DNA damage is necessary for recombination-mediated repair of double-strand breaks (Strom et al., 2004).

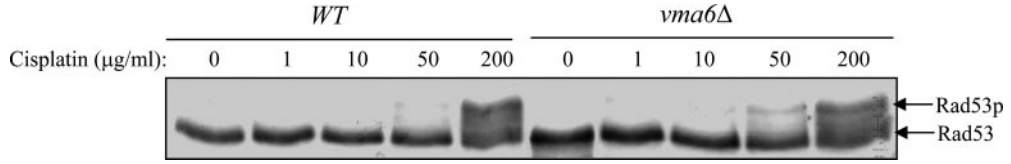

Fig. 5. Phosphorylation of the Rad53 checkpoint effector kinase. Wild-type or V-ATPase mutant cells were grown to early log phase at 30°C and then incubated for 1 h with cisplatin at the concentration indicated. Rad53 was visualized by probing Western blots with anti-Rad53 antibody. The positions of unphosphorylated Rad53 and phosphorylated Rad53 (Rad53-p) are shown on the right.

Therefore, it is likely that the Dcc1/Ctf8/Ctf18 complex and Ctf4 facilitate RER of cisplatin-induced DNA damage. In addition, two mutants (*asf1*Δ and *mrc1*Δ), known to impair the Rad53-dependent DNA damage checkpoint pathway, were sensitive to cisplatin. This is in agreement with work showing that cisplatin causes a checkpoint-dependent G_2/M arrest (Grossmann et al., 1999). To support this notion, we showed that cisplatin resulted in hyperphosphorylation of Rad53, indicating the activation of this checkpoint pathway (Fig. 5).

Several mutants that compromised cell stress tolerance and signal transduction were sensitive to cisplatin. In mammalian cells, the oxidative and osmolar stress responses protect cells from cisplatin-induced nephrotoxicity (Hanigan et al., 2005). In addition, genes involved with ribosomal function and protein synthesis were identified as cisplatin-resistant genes. In agreement with this are reports describing cisplatin-induced disruption of the translation initiation complex and overexpression of a ribosomal protein conferring resistance to cisplatin (Rosenberg and Sato, 1993; Shen et al., 2006).

It is noteworthy that a group of nine genes encoding V-ATPase subunits and an assembly factor for this enzyme were identified as cisplatin-hypersensitive strains. It is not surprising that so many genes involved in V-ATPase function were identified, given that loss of *any* V-ATPase subunit or assembly factor is known to result in loss of V-ATPase activity (Graham et al., 2003). In yeast, this enzyme is localized to the membrane of the vacuole, with a smaller population of V-ATPase complexes localized to the endosomal network. In mammalian cells, the enzyme is similarly localized to the lysosome (equivalent to the yeast vacuole) and endocytic compartments. In addition, the enzyme is localized to the membranes of specialized cells, notably the brush-border membranes of renal proximal tubules (Stevens and Forgac, 1997). In mammalian cells, one of the V-ATPase subunits is induced by cisplatin (Torigoe et al., 2002). Furthermore, several genes encoding V-ATPase subunits are up-regulated in drug-resistant tumor cell lines (Martinez-Zaguilan et al., 1999; Murakami et al., 2001). This increase in levels of V-ATPase in cisplatin-resistant cells is correlative, and could be a consequence of drug treatment rather than a cause of resistance. The data we present, however, points toward V-ATPase activity directly contributing to drug tolerance.

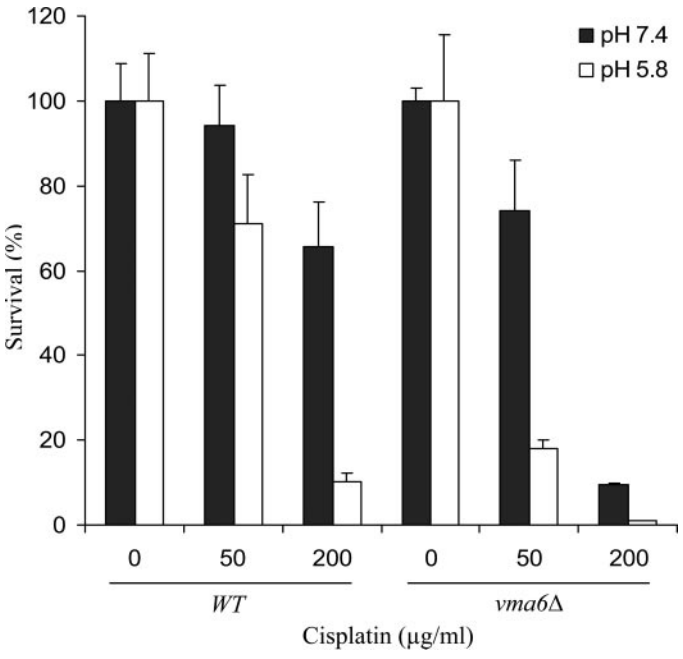

Fig. 6. Effect of pH on cisplatin sensitivity. Exponential phase cultures diluted to equal cell density were incubated in PBS, pH 7.4 or 5.8, with cisplatin at the concentration indicated; aliquots were removed after 1 h, diluted, and spread over YPD plates. Percentage viability was determined from the number of colonies that appeared after incubation at 25°C for 3 days, expressed as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

V-ATPase translocates H^+ from the cytoplasm to the vacuole. Two consequences of this are regulation of cytoplasmic pH and acidification of the vacuole; the latter is crucial for maintenance of processes associated with the vacuole lumen. We showed that cisplatin did not affect the morphology of vacuoles in wild-type cells or a V-ATPase mutant. Furthermore, acidification of vacuoles in wild-type cells was also unaffected. This implied that sensitivity to cisplatin exhibited by V-ATPase mutants was associated with an effect on processes outside the vacuole, such as DNA repair, or the extent to which cisplatin damages DNA in the first place. V-ATPase mutants were also sensitive to the DNA-alkylating agent MMS, the DNA replication inhibitor HU, and UV irradiation. This suggested that the activity of the V-ATPase was required for limiting the effects of DNA damaging agents in general. This concept is supported by a recent report describing the sensitivity of yeast V-ATPase mutants to tirapazamine, an anticancer drug that targets topoisomerase II (Hellauer et al., 2005).

V-ATPase mutants may be defective in DNA damage repair mechanisms or may lead to enhanced DNA damage. PR, RR, and NER are the mechanisms that repair damage induced by cisplatin. Epistasis analysis ruled out the possibility that a defect in V-ATPase diminished the effect of repair pathways, because [V-ATPase/repair pathway] double mutants were far more sensitive to cisplatin than the single mutants (Fig. 4). Activation of the DNA damage checkpoint by low concentrations of cisplatin was enhanced in V-ATPase mutants. This suggested that loss of the V-ATPase function facilitates the DNA damage caused by cisplatin.

V-ATPase is a regulator of cytoplasmic pH. Consequently, loss of V-ATPase activity leads to intracellular acidification, which may lead to greater levels of cisplatin-mediated DNA damage. At lower pH, a greater proportion of hydrolyzed cisplatin has an aqua ligand, rather than a hydroxo- ligand. This enhances the reactivity of cisplatin because the aquated form is more labile. Chemical activity of cisplatin in vitro is greater at lower pH, promoting DNA platination (Murakami et al., 2001). The increased sensitivity to cisplatin in a V-ATPase mutant incubated at lower pH supports this hypothesis. At 50 μg/ml cisplatin, it is notable that cell viability of the wild type in pH 5.8 buffer, is similar to cell viability of *vma6*Δ at pH 7.4. This may well reflect a similar intracellular pH in these cells.

The role played by lower pH, however, must be more complex than straightforward increases in chemical reactivity of drugs, because V-ATPase mutants were also hypersensitive to UV irradiation. Low pH can change DNA conformation (Robinson et al., 1992). Therefore, sensitivity to DNA damaging drugs or UV irradiation in V-ATPase mutants may be associated with altered DNA conformation at low cytosolic

pH, rendering DNA more prone to damage. This may explain why use of V-ATPase inhibitors in human cell lines renders them more sensitive to cisplatin (Laurencot et al., 1995; Murakami et al., 2001; Luciani et al., 2004).

In mammalian cells, cellular acidosis is an early event in apoptosis. Limiting the drop in cytoplasmic pH represses apoptosis, this being frequently associated with an up-regulation of V-ATPase subunits in tumors (Torigoe et al., 2002; Izumi et al., 2003). Accordingly, enhanced cell death in yeast V-ATPase mutants incubated with cisplatin could be due, in part, to activation of apoptosis caused by lowering of intracellular pH. Some controversy does surround the concept of yeast apoptosis. However, yeast demonstrates several markers typical of apoptosis including DNA fragmentation, phosphatidylserine externalization, chromatin condensation, and histone H2B phosphorylation (Madeo et al., 2004; Ahn et al., 2006). Furthermore, a growing list of genes that regulate apoptosis in mammalian cells have been identified in yeast, including the Yca1 caspase and the apoptosis-inducing factor Aif1 (Madeo et al., 2004).

We also demonstrated that loss of four genes associated with ribosome function and protein synthesis rendered cells sensitive to cisplatin. Accordingly, we predict that enhancing protein synthesis would confer drug resistance. This is in agreement with recent work involving use of a human epidermoid carcinoma cell line that showed cisplatin resistance is induced by overexpression of the ribosomal protein gene *RPL36* (Shen et al., 2006). Another study revealed that inactivation of the Sky1 kinase in *S. cerevisiae* leads to cisplatin resistance. Monitoring levels of the orthologous protein in testicular tumors revealed that expression of the kinase in cisplatin resistant cells are lower than in tumors from patients who were responding to platinum drug-based therapy, so levels of the protein could predict the response to the drug (Schenk et al., 2004). Such work clearly demonstrates that conclusions drawn from use of the *S. cerevisiae* model system are of direct relevance to mammalian systems. This indicates that other genes identified in our screen are worthy of further investigation, because they may be important for predicting the responsiveness of tumors to cisplatin.

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