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Large-scale analysis of genes that alter sensitivity to the anti-cancer drug tirapazamine in *Saccharomyces cerevisiae.*

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Running title:

Genes that alter sensitivity to the anti-cancer drug TPZ

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Non-standard abbreviations:

TPZ, tirapazamine

ORF, open reading frame

YEPD, yeast extract peptone dextrose

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ABSTRACT

Tirapazamine (TPZ) is an anti-cancer drug that targets topoisomerase II. TPZ is preferentially active under hypoxic conditions. The drug itself is not harmful to cells, rather it is reduced to a toxic radical species by a NADPH cytochrome P450 oxidoreductase. Under aerobic conditions, the toxic compound reacts with oxygen to revert back to TPZ and a much less toxic radical species. We have used yeast (*Saccharomyces cerevisiae*) as a model to better understand the mechanism of action of TPZ. Overexpression of *NCP1*, encoding the yeast orthologue of the human P450 oxidoreductase, results in greatly increased sensitivity to TPZ. Similarly, overexpression of *TOP2* (encoding topoisomerase II) leads to hypersensitivity to TPZ suggesting that topoisomerase II is also a target of TPZ in yeast. Thus, our data show that yeast mimics human cells in terms of TPZ sensitivity. We have performed robot-aided screens for altered sensitivity to TPZ using a collection of approximately 4,600 haploid yeast deletion strains. We have identified 117 and 73 genes whose deletion results in increased or decreased resistance to TPZ, respectively. For example, cells lacking various DNA repair genes are hypersensitive to TPZ. In contrast, deletion of genes encoding some amino acid permeases results in cells that are resistant to TPZ. This suggests that permeases may be involved in intracellular uptake of TPZ. Our discoveries in yeast may help to better understand TPZ biology in humans.

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INTRODUCTION

Nonsurgical treatment of cancer includes radiotherapy and chemotherapy. A major drawback of these treatments is that they do not specifically target cancer cells. Approaches under current study include the use of hypoxic-selective drugs (reviewed by Brown and Giaccia, 1998; Rooseboom et al., 2004; Seddon et al., 2004). The approach is based on the fact that oxygen levels are generally lower in the center of a tumour because of poor vascularization (Brown and William, 2004). Since these hypoxic cells are generally more resistant to radiation and conventional chemotherapy (Gatenby et al., 1988; Hockel et al., 1993; Nordsmark et al., 1996; Okunieff et al., 1993; Teicher, 1994), drugs specifically active under low oxygen levels are of great interest for cancer treatment (Brown, 1999). The best prototype is probably 3-amino-1,2,4-benzotriazine 1,4 dioxide (also called tirapazamine or SR4233; hereafter referred to as TPZ). Phase II and III clinical trials have shown the efficacy of TPZ when used in combination with radiotherapy or chemotherapy (Bedikian et al., 1999; Craighead et al., 2000; Rischin et al., 2001; von Pawel et al., 2000).

The hypoxic toxicity of TPZ is thought to be due to the addition of one electron to TPZ by enzymatic reductases, yielding a radical species that causes single- and double strand DNA breaks leading to chromosome aberration and cell death (reviewed by Patterson et al., 1998). The radical species is unstable and, under normal oxygen levels, reacts with oxygen to revert back to TPZ and a much less toxic radical species (Lloyd et al., 1991). The exact mechanism of TPZ's action is not known. Under hypoxic conditions, a protonated neutral form of a TPZ nitroxide radical is formed, but there is no formal proof that this compound is responsible for the toxicity

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(Patterson et al., 1998). The TPZ nitroxide is unstable and reacts with biomolecules such as DNA to form a non-toxic two-electron product called SR4317 (Lloyd et al., 1991). Interestingly, only a fraction (30-70%) of TPZ is converted to SR4317. This may explain why the rate of formation of SR4317 does not always correlate with toxicity (Siim et al., 1996). Recently, it was shown that TPZ inhibits DNA replication (Peters et al., 2001) and that it mediates its effect through topoisomerase II (Peters and Brown, 2002). Topoisomerase II unwinds DNA by introducing transient double stranded breaks. Therefore, TPZ treatment likely leads to covalent binding of the topoisomerase II α subunit to DNA, stabilizing topoisomerase II-induced double strand breaks and resulting in cell toxicity (Peters and Brown, 2002).

Under hypoxia, there is good evidence that NADPH cytochrome P450 oxidoreductase (E.C. 1.6.2.4) is involved in the metabolism of TPZ to a toxic compound (Chinje et al., 1999; Patterson et al., 1997; Saunders et al., 2000). Hypoxic sensitivity of human breast cancer cell lines to TPZ correlates with the expression of P450 oxidoreductase (Patterson et al., 1995). Furthermore, stable transfection of an expression vector encoding P450 oxidoreductase results in increased sensitivity to TPZ in human breast and lung cancer cell lines (Patterson et al., 1997; Saunders et al., 2000). In addition to P450 oxidoreductase, a nuclear enzyme is probably involved in the conversion of TPZ to a toxic molecule (Evans et al., 1998). Using a human lung cancer cell line, nuclei were found to be responsible for only 20% of the TPZ metabolism, but DNA damage was similar to what was observed for whole cells. These results suggest that an enzyme(s), other than the P450 oxidoreductase, is responsible for conversion of TPZ to a toxic compound. Thus, the relevant enzyme(s) appear to be nuclear unlike the oxidoreductase which is located at the membrane of the endoplasmic reticulum. In addition, other enzymes such as

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cytochrome P450 and DT-diaphorase, can also metabolize TPZ (reviewed in (Brown and Giaccia, 1998; Patterson et al., 1998)).

Saccharomyces cerevisiae (referred to as yeast hereafter) has been a useful model organism to study various drugs (reviewed by Barret and Hill, 1998). In keeping with these results, our study shows that TPZ targets topoisomerase II and that overexpression of the *NCP1* gene (encoding an orthologue of the human P450 oxidoreductase) results in increased TPZ sensitivity in yeast cells. Screening of a panel of yeast deletion strains has allowed the identification of many genes that confer resistance or sensitivity to TPZ, including genes involved in DNA repair and amino acid transport.

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MATERIAL AND METHODS

Yeast strains

Wild-type strains used were BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al., 1998) and a derivative of BY4741, R1158 (Hughes et al., 2000) (MATa *his3Δ1 leu2Δ0 met15Δ0 URA3*::CMV-tTA). Strains yTH-NCP1 and yTH-TOP2 were obtained from Openbiosystems (Huntsville, AL, U.S.A.). Haploid deletion strains are derived from BY4741 (Winzeler et al., 1999) and were arrayed on sixteen 768-format plates (Tong et al., 2001).

Media and drug assays

Media were prepared according to Adams et al. (Adams et al., 1997). YEPD (yeast extract peptone dextrose) contained 1% yeast extract, 2% peptone, 2% glucose. TPZ was obtained from Sigma Chemical Co. (St.Louis, MO, U.S.A.) or Sanofi-Synthélabo (Malvern, PA, U.S.A.) and dissolved in 50% methanol or 50% ethanol. Anaerobic conditions were obtained using an anaerobic jar (Becton Dickinson) and gas pack (BBL GasPak Plus, Becton Dickinson). Anaerobic conditions were verified by using an anaerobic indicator (BBL, Becton Dickinson) and monitoring growth of the strict anaerobe *Clostridium tetanomorphum* (see *Supplementary Fig. S3*). Growth assays were all performed at 30^0C.

Ncp1 and Top2 overexpression

Haploid wild-type strain R1158 and strains carrying a doxycycline repressible promoter integrated at the *NCP1* or the *TOP2* loci were grown overnight in YEPD in the absence or the

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presence of doxycycline (20 μg/ml; Sigma Chemical Co., St.Louis, MO, U.S.A.). Cells were serially diluted and spotted on YEPD plates containing various concentrations of TPZ and 20 μg/ml doxycycline for cells grown overnight in the presence of the antibiotic.

Western blot analysis of Top2

Extracts were prepared as described (Akache et al., 2004) and proteins were run on a 7.5% polyacrylamide gel. Western blot analysis was performed with a polyclonal antibody against *S. cerevisiae* Top2 (cat #2014,TopoGEN inc., Port Orange, Florida U.S.A.)

Screen for altered sensitivity to TPZ

Deletion strains were propagated on standard YEPD or YEPD supplemented with 200 μg/ml G418 (Invitrogen) using a colony picker (BioRad). Hypersensitive mutants were screened by pinning the deletion collection on YEPD supplemented with and without 300 μM TPZ, and then scoring the colony size after a 3.5 day incubation. Resistant mutants were screened by pinning the deletion collection on YEPD and then on YEPD supplemented with 750 μM TPZ. After 48 h, plates were replicated on fresh YEPD containing 750 μM TPZ and growth was scored after a 48 h incubation. Out of two screens for hypersensitive and a single screen for resistant mutants, 256 and 263 mutants were identified, respectively.

The sensitivity of these mutants was confirmed by the following spotting procedure: cells were grown in liquid YEPD to log-phase, diluted to an OD_{600} of 0.5, serially diluted 10-fold four times, and 5 μl were spotted on YEPD plates supplemented with and without 200 μM and 500 μM TPZ. After 2 days of incubation, growth of mutants in the presence or absence of TPZ was

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scored and compared to that of the wild-type BY4741 strain. Mutants showing significant growth defect or absence of growth in the presence of 200 μM TPZ were scored as "- -" or "- - -", respectively. Mutants showing similar or more vigorous growth than the *fre1*Δ mutant in the presence of 500 μM TPZ were scored as "++" or "+++", respectively. Finally, 73 and 117 mutants exhibited hypersensitivity and resistance to TPZ, respectively.

Search for human proteins with yeast homologues involved in modulating TPZ sensitivity

A list of approximately 34,000 human protein sequences was obtained from Ensembl database (accessible from http://www.ensembl.org) and used as query in a search for homologues against the yeast proteome (approximately 6,000 protein sequences accessible from http://www.yeastgenome.org). We found about 26,000 human proteins matching a yeast protein sequence (E-value $\leq$ 0.001). Of this set, 614 human peptides showed significant homology to yeast product of genes involved in sensitivity or resistance to TPZ (data not shown). A partial list of these genes can be found in Tables 3 and 4.

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RESULTS

To determine if yeast can be used as a model for studying the mode of action of the anti-cancer drug TPZ, wild-type yeast cells were grown overnight under aerobic conditions, serially diluted, and spotted on plates containing increasing concentrations of TPZ. Cells were then grown under anaerobic or aerobic conditions for about 24 h (aerobia) or 48 h (anaerobia) (Fig. 1). Interestingly, TPZ was somewhat more toxic to cells grown under anaerobic conditions. For example, with 200 μM TPZ, growth was almost completely abolished under anaerobia while only a moderate effect was observed in the presence of oxygen (Fig. 1, panel E). Similar growth was observed in the absence of TPZ (panel A). However, the difference in TPZ toxicity of cells grown under aerobic and hypoxic conditions is much more pronounced in human tumour cell lines. For example, equal cell killing for human tumour cells grown under aerobic conditions requires approximately 300-times higher TPZ concentration when compared to hypoxic cells (Brown, 1993). The basis for this species difference is unknown but it may be related to the fact that yeast is a facultative aerobe (see below).

There is good evidence that the human NADPH oxidoreductase (EC 1.6.2.4) is responsible for metabolizing TPZ to a toxic compound (Chinje et al., 1999; Patterson et al., 1997; Saunders et al., 2000). We were interested in determining if a related enzyme would perform a similar function in yeast. The essential gene *NCP1* encodes the yeast orthologue of human P450 oxidoreductase. To study the involvement of *NCP1* in TPZ toxicity in yeast, the *NCP1* promoter of a haploid strain was replaced with a doxycycline repressible promoter

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(Mnaimneh et al., 2004). Use of this promoter results in overexpression of the targeted gene in the absence of doxycycline and reduced expression in the presence of the antibiotic.

Overexpression of *NCP1* did not affect growth under aerobic or anaerobic conditions as compared to a wild-type strain (Fig. 1, panel A), while reduced expression of *NCP1* impaired growth only under anaerobic conditions (Fig. 1, panel B). The nearly normal aerobic growth under repressible conditions is probably due to leaky expression of *NCP1*, as observed for some other genes (Mnaimneh et al., 2004). Overexpression of *NCP1* was highly toxic to cells grown in the presence of TPZ (Fig. 1, panels C to F). This suggests that, as observed in human cells, high levels of P450 oxidoreductase result in increased production of a toxic metabolite (Patterson et al., 1997; Saunders et al., 2000). This provides further evidence that yeast NADPH oxidoreductase, as its human counterpart, is responsible, at least in part, to the conversion of TPZ to a toxic compound. Thus, yeast mimics human cells with regard to TPZ toxicity.

Since a recent study in animal cells shows that TPZ targets topoisomerase II (Peters and Brown, 2002), we tested if this enzyme also mediates TPZ toxicity in yeast cells. Overexpression of topoisomerase II results in hypersensitivity of yeast to some anti-cancer drugs (Nitiss et al., 1992) when tested in a DNA repair-deficient *rad52*Δ background. Since yeast topoisomerase II is encoded by the essential gene *TOP2* (Wang, 1996), we altered *TOP2* expression by using a doxycycline repressible promoter as described above for *NCP1*. Using this system, greatly increased expression of *TOP2* was observed with cells grown aerobically in the absence of doxycycline as compared to cells treated with doxycycline or a wild-type strain (Fig. 2A, left panel). Surprisingly, expression of *TOP2* in wild-type cells was greatly increased under

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anaerobic conditions while the overexpression system gave only a modest increase in *TOP2* levels when compared to wild-type cells (Fig. 2A, right panel). Growth of these strains was similar when assayed under aerobic and anaerobic conditions in the presence or absence of doxycycline (Fig. 2B, panels A and B). Addition of doxycycline is likely not to lead to full repression of the promoter driving *TOP2* expression since *TOP2* is an essential gene (as suggested by the Western blot analysis). Under aerobic conditions, overexpression of *TOP2* in a wild-type background resulted in increased cell sensitivity to TPZ while cells with reduced expression of *TOP2* behaved as wild-type cells (Fig. 2B, compare panels C to F). This effect was less apparent under anaerobic conditions in agreement with the Western blot analysis of *TOP2* expression. These results suggest that topoisomerase II is a target of TPZ in yeast cells. Thus, our data show that yeast mimics human cells in terms of TPZ sensitivity.

Genome-wide screen for altered sensitivity to TPZ

The identification of yeast mutants (other than *NCP1* and *TOP2*) showing an altered sensitivity to TPZ should give insights into the mode of TPZ action and tools to design more effective drug treatments. As stated above, the difference of TPZ toxicity with regard to oxygen levels is much less pronounced in yeast than in human cells. It is well established that growth of yeast under anaerobic conditions results in global changes in gene expression (Becerra et al., 2002). For example, anaerobia results in cell wall and membrane remodeling (Aguilar-Uscanga and Francois, 2003). Altered TPZ entry into the cells may explain the relative weak sensitivity of yeast cells grown under anaerobic conditions. Anaerobicity also results in more rapid response to osmotic shock {Krantz, 2004 #3861] and in altered expression of genes encoding *NCP1* and cytochrome P450. Since oxygen levels have only a minor effect on TPZ sensitivity of yeast and

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for easier manipulation of a large number of strains, we decided to perform a large-scale screen under aerobic conditions.

We performed robot-aided screens for altered sensitivity to TPZ using a collection of ~ 4,600 haploid deletion mutants corresponding to most non-essential yeast genes. Phenotypes were confirmed by individually spotting serial dilutions of deletion strains on TPZ and control plates (see *Supplementary Fig. S1*). Fig. 3 shows examples of strains that are resistant or sensitive to TPZ. In all, 73 strains were sensitive to the drug (Table 1) while 117 strains showed increased resistance to TPZ (see Table 2 for a list of the strongest resistance phenotypes and *Supplementary Table S1* for weaker resistance). Genes were grouped in categories according to their known or inferred function and are discussed accordingly. It should be stressed that we do not know what mechanism of TPZ action renders some deletion mutants sensitive to the drug. For example, the effect could be mediated by topoisomerase II or, alternatively, by DNA damage produced by a TPZ metabolite.

DNA repair and genome stability

Given that exposure to TPZ results in DNA damage, it was not unexpected that cells lacking various DNA repair genes would be hypersensitive to the drug. These genes encode members of the *RAD52* epistasis group (*RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*), subunits of the MRX complex (*MRE11*, *RAD50* and *XRS2*), topoisomerase III (*TOP3*), factors involved in the repair of replication-dependent DNA damage (*ASF1*, *MMS1*, *MMS4*, *MMS22*, *MUS81*, *RAD5*, *RTT101*, *RTT107* and *UBC13*) and subunits of the nucleotide excision repairosome (*RAD10* and *RAD16*). In addition, four poorly characterized genes (*NCE4*, *RTT109*,

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WSS1 and *YBR094W*), whose deletion leads to TPZ hypersensitivity, were included in this category because they show synthetic lethality with genes involved in DNA repair or genome stability. For example, a double deletion of *NCE4* and *TOP1* (encoding topoisomerase I) is lethal while *WSS1* and *YBR094W* show synthetic lethality with *SGS1*, a gene encoding a nucleolar DNA helicase involved in maintenance of genome integrity {Tong, 2004 #3825}. In contrast, deletion of the DNA repair genes *RAD18* or *DNL4* resulted in increased resistance to TPZ (Table 2). We do not know the reason for these observed resistance phenotypes.

Transporters

Interestingly, a number of resistant strains lack amino acid permeases such as Agp3 (Schreve and Garrett, 2004), Alp1 (Regenberg et al., 1999) or the choline permease, Hnm1. These results suggest that uptake of TPZ within the cell could be mediated by permeases (see discussion). In keeping with these results, genetic interactions suggest that Asi3 is a regulator of permease gene expression (Forsberg et al., 2001). Expression of putative permeases involved in TPZ uptake would be reduced in cells lacking Asi3 resulting in increased resistance to the drug.

Reductases and related proteins

As stated above, Ncp1 is very likely to be responsible for metabolizing TPZ to a toxic compound in yeast, as observed in mammalian cells. Interestingly, deletion of other reductase genes leads to resistance to TPZ. For example, cells lacking Fre1 are resistant to the drug. *FRE1* encodes a ferric and cupric reductase necessary for uptake of environmental $Cu2^{+}$ and $Fe3^{+}$ (Eide, 1998). Reduced copper is a substrate for the high affinity transporter Ctr1 and related transporters. Although Fre1 and Ctr1 are functionally linked, deletion of *CTR1* does not result in

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resistance to TPZ, in contrast to what was observed for the anticancer drug cisplatin (Ishida et al., 2002; Lin et al., 2002; Nitiss, 2002). In addition, a strain lacking Utr1 shows increased resistance to TPZ. *UTR1* encodes a NAD kinase that enhances the activity of Fre1 (Lesuisse et al., 1996), an observation that may explain the phenotype of an *utr1*Δ strain. The other reductase identified in our screen is His4, a multifunctional enzyme bearing dehydrogenase activity and involved in histidine biosynthesis (Alifano et al., 1996).

Cell stress signaling and signal transduction

Deletion of genes required for resistance to oxidative stress such as *LYS7*, *SOD1* and *SOD2* leads to hypersensitivity. *SOD1* and *SOD2* encode superoxide dismutases and *LYS7* encodes a copper chaperone required for Sod1 activity. Hypersensitivity of strains lacking these stress genes is likely to be explained by the fact that metabolism of TPZ leads to the formation of a superoxide radical toxic to cells (Lloyd et al., 1991). In addition, mutants defective in the protein kinase C MAP-kinase pathway (*bck1* and *slt2*) or affected in signaling through multiple MAP-kinase pathways (*sit4*) show an increased TPZ sensitivity. In contrast, deletion of *HSP104* or *WSC2* leads to TPZ resistance. Wsc2 is a putative integral membrane protein and a stress response component required for cell wall integrity (Verna et al., 1997).

Vesicular transport

Deletion of genes involved in protein recycling to the endosomal compartment increases TPZ sensitivity. Included here are members of the ESCRT-I (*VPS26*), ESCRT-II (*SNF8* and *VPS25*) ECSRT-III (*SNF7*) complexes, which are involved in ubiquitin-mediated protein sorting to the vacuole, factors involved in protein sorting from the late-Golgi to the vacuole through AP-

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3 transport vesicles (*VAM3* and *VPS41*), and components of the endosome-to-Golgi recycling pathway (*RIC1* and *WHI6*). In contrast, removal of three genes involved in ER-to-Golgi transport (*ERV41*, *SPO20*, *YOS9*) conferred resistance to TPZ.

Other categories

A set of deletion strains hypersensitive to a range of inhibitory compounds has been identified (Parsons et al., 2004). A number of these mutants also show hypersensitivity to TPZ. A first group is involved in the function of the vacuolar H^+-ATPase (*PPA1*, *TFP3*, *VMA4*, *VMA7* and *VMA10*). A second group of genes is involved in ergosterol biosynthesis (*ERG2*, *ERG3* and *ERG4*). The increased sensitivity to TPZ of the second group of deleted genes is likely due to altered plasma membrane fluidity. Similarly, other genes involved in lipid, fatty acid or sterol metabolism (*DPL1*, *EKI1*, *FAA3*, *PDR17*) resulted in resistance to TPZ when deleted. Removal of these genes may also alter plasma membrane fluidity and integrity, thereby restricting entry of TPZ into the cells. Other genes that modulate TPZ sensitivity are associated with transcription or RNA processing. For example, deletion of the RNA polymerase II subunit *RPB9*, components of the RNA-polymerase II mediator complex (*GAL11*, *PGD1*, *ROX3* and *SRB2*), subunit of the CCR4-NOT1 complex *POP2*, or transcriptional regulators (*DBF2*, *SPT10*, *SPT20* and *SWI4*) confers hypersensitivity to TPZ. These genes may be required for the transcription of TPZ resistance gene(s).

Relevance to human TPZ biology

We were interested to determine if the genes identified in our screen have human counterparts. A selected set of 30 human proteins showing significant homology to products of

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yeast genes whose deletion leads to resistance to TPZ is shown in Table 3. Some of these human proteins have a role in cell proliferation (e.g. CLK1), cell morphogenesis (DAAM1 and 2) and signal transduction (e.g. HRAS). Others have been reported to exhibit altered expression in cancer cells. For example, OS-9 is amplified in sarcomas (Su et al., 1996). OS-9 is involved in oxygen-dependent degradation of the hypoxia inducible factor (Baek et al., 2005) and is associated with the ER membrane (Litovchick et al., 2002). Other human proteins, such as the KIST kinase or the XPR1 (Battini et al., 1999) may be involved in signaling and could play a role in TPZ sensing. Table 4 lists a selection of 40 human proteins sharing significant homology with products of yeast genes whose deletion confers TPZ hypersensitivity. These proteins may be important for resistance to TPZ in human cells. For example, removal of the manganese superoxide dismutase leads to TPZ hypersensitivity in both human (Wouters et al., 2001) and yeast cells (Table 1). Inhibition of processes such as microtubule cytoskeleton assembly, nuclear transport, protein synthesis, transport to the endosome, proton transport through V-type ATPase is likely to be synergistic with TPZ treatment in human cells as observed in yeast.

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DISCUSSION

Yeast has been a useful model organism in better understanding the mode of action of various drugs (reviewed in (Barret and Hill, 1998)). In this report, we show that yeast can also be used to study the anti-cancer drug TPZ. Yeast mimics animal cells with regard to TPZ toxicity. Firstly, overexpression of the *NCP1* gene (encoding a P450 oxidoreductase) results in a marked increased sensitivity to TPZ (Fig. 1), in analogy to human cells where overexpression of the P450 oxidoreductase results in increased TPZ toxicity (Chinje et al., 1999; Patterson et al., 1997; Saunders et al., 2000). Secondly, we provide good evidence that topoisomerase II is a target of TPZ in yeast (Fig. 2) as observed in animal cells (Peters and Brown, 2002). Thus, these observations reinforce the view that yeast can be used as a model to gain insights into the mechanism of action of TPZ.

We took advantage of the yeast system to perform a large-scale screen of non-essential genes that modulate sensitivity to TPZ (Tables 1 and 2, see also *Supplementary Table 1*). As other similar studies, our screen was not totally comprehensive; for example, essential genes were not tested and some non-essential genes modulating TPZ sensitivity may have not been identified in this study. However, our work led to the identification of one hundred and ninety deletion strains that showed an altered growth in the presence of TPZ., For example, a major class of mutants is related to DNA repair or genome stability, in agreement with the model of TPZ action. Similar results were obtained for screens with other anti-cancer agents such as cisplatin, oxaliplatin, mitomycin, and bleomycin (Aouida et al., 2004; Wu et al., 2004). *RAD1* and *RAD10* gene products form a complex and deletion of either gene results in similar

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phenotypes (Prakash and Prakash, 2000). Surprisingly, a *rad1* deletion strain was not recovered in our screen while a *rad10* strain showed sensitivity to the drug. We manually spotted the *rad1* mutant and found it to be similar to that of a wild-type strain. Thus, the phenotype of a *rad10* mutant does not always appear to match that of a *rad1* mutant.

Besides Ncp1, two other reductases were found to confer resistance to TPZ when removed: Fre1 and His4. Both enzymes may metabolize TPZ to a toxic compound in analogy to Ncp1. To our knowledge, there is no human homologue of His4 ruling out the possibility that a human His4-like protein would be responsible for TPZ metabolism. However, various human proteins have domains that show similarity to Fre1 (Lambeth et al., 2000) and may modulate sensitivity to the drug.

Our screen identified three transporters encoding genes whose deletion enhances TPZ resistance: the choline permease gene *HNM1* and the amino-acid permease genes *AGP3* and *ALP1*. This suggests a role for these genes in TPZ uptake within the cells. Such membrane permeases have been previously shown to mediate the uptake and toxicity of other compounds. For example, Hnm1 is involved in the uptake of the alkylating agent nitrogen mustard, and an *hnm1*Δ mutant is resistant to this drug (Li and Brendel, 1994). Bleomycin action was found to be modulated by the level of the L-carnitine transporter Agp2. Drug uptake and toxicity were decreased and increased upon deletion and overexpression of *AGP2*, respectively (Aouida et al., 2004). Similarly, the copper transporter Ctr1 mediates cisplatin uptake in yeast and human cells (Ishida et al., 2002; Lin et al., 2002; Nitiss, 2002). Thus, it appears that TPZ, as other anti-cancer drugs, uses membrane transporters to enter the cells. Since related amino acid transporters are

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found in humans (e.g. SLC7A2, Table 3), it will be interesting to determine if these transporters are involved in mediating uptake of TPZ in human cells.

This hypothesis is reinforced by our findings that deletion of a number of genes involved in ubiquitin-regulated protein trafficking alters the resistance to TPZ. Indeed, ubiquitination is known to regulate the transport of the general amino acid permease Gap1 (Soetens et al., 2001) and may regulate the transport of other amino acid permeases as well. According to this hypothesis, mutations affecting this ubiquitin-regulated endocytosis pathway (such as *snf7*, *snf8*, *vps25* or *vps26*) would perturb the turnover of permeases resulting in their accumulation at the plasma membrane. The TPZ hypersensitivity of these mutants may be explained by the resulting increased TPZ uptake into the cells. Conversely, a defect in forward permease trafficking (for example, *erv41* or *yos9*) would result in a decreased efficiency of TPZ entry into cells and, as a result, in an increased resistance to TPZ. In summary, we have shown that yeast can be used as a model to study the anti-cancer drug TPZ. This allowed the identification of many yeast genes that modulate sensitivity to the drug. These observations will be invaluable to further increase our understanding of the mode of action of TPZ in human cells. Moreover, our results suggest that yeast could be used to design derivatives of TPZ and related bioreductive drugs.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1

Overexpression of Ncp1 increases sensitivity to TPZ.

Wild-type strain R1158 ("WT") and yTH-NCP1 ("Tet-NCP1") were grown overnight under aerobic conditions in rich medium in the presence or absence of doxycycline to allow control of the expression of *NCP1*. Cells were serially diluted (left to right: approximately $1.25X10^4$, $2.5X10^3$, $5X10^2$ and $1X10^2$ cells) and spotted on rich plates containing ("+ DOX") or lacking ("- DOX") doxycycline. Concentrations of TPZ are indicated on the right part of the figure. Cells were grown aerobically (left panel) for about 24 h or anaerobically (right panel) for about 48 h.

Figure 2

Overexpression of topoisomerase II increases sensitivity to TPZ.

A) Wild-type strain R1158 ("WT") and yTH-TOP2 ("Tet-TOP2") were grown under aerobic or anaerobic conditions in the presence ("+ DOX") or absence ("- DOX") of doxycycline to an $O.D._{600}$ of 0.8-1.0. Total extracts were analyzed by immunoblotting with an anti-Top2 polyclonal antibody.

B) Wild-type strain R1158 ("WT") and yTH-TOP2 ("Tet-TOP2") were grown overnight under aerobic conditions in rich medium in the presence or absence of doxycycline to allow control of the expression of *TOP2*. Cells were serially diluted (left to right: approximately $1.25X10^4$, $2.5X10^3$, $5X10^2$ and $1X10^2$ cells) and spotted on rich plates containing ("+ DOX") or lacking ("-

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DOX") doxycycline. Concentrations of TPZ are indicated on the right part of the figure. Cells were grown aerobically (left panel) for about 24 h or anaerobically (right panel) for about 48 h.

Figure 3

Examples of deletion strains exhibiting increased or decreased resistance to TPZ.

Wild-type strain (BY4741) and various deletion strains were grown overnight under aerobic conditions in YEPD to log-phase. Cells were serially diluted (left to right approximately $1.2X10^4$, $1.2X10^3$, $1.2X10^2$ and $1.2X10^1$ cells) and spotted on YEPD plates supplemented with and without 200 μM and 500 μM TPZ (as indicated in the top part of the Fig.). After a 48 h incubation, growth was scored as indicated on the right part of the Fig. ("ND": not determined).

Table 1

Genes whose deletion confers hypersensitivity to TPZ.

Genes whose deletion results in hypersensitivity to TPZ are listed along with their systematic names ("ORF") and their cellular function (if known). Scores for strain sensitivities are also given:"---", hypersensitive strain; "- -", sensitive strain. See Fig. 3 for examples of relative sensitivities.

Gene	ORF	Score	Cellular function and Comment
			DNA REPAIR AND GENOME STABILITY
ASF1	*YJL115W*	---	Target of the Rad53-dependent DNA damage response
MMS1	*YPR164W*	---	Required for repair of replication-dependent DNA damage
MMS4	*YBR098W*	---	Required with Mus81 for repair of DNA damage by MMS
MMS22	*YLR320W*	---	Acts in a DNA repair pathway with Mms1
MRE11	*YMR224C*	---	Single-stranded endonuclease and double-stranded exonuclease required for double strand break repair
MUS81	*YDR386W*	---	Part of a complex with Rad54 and Mms4
NCE4	*YPL024W*	--	Synthetic interaction pattern suggests a role in DNA repair

RAD5	*YLR032W*	---	Single-stranded DNA-dependent ATPase involved in error-free DNA repair
RAD10	*YML095C*	---	Component of the nucleotide excision repairosome
RAD16	*YBR114W*	--	DNA helicase, subunit of the nucleotide excision repair factor *NEF4*
RAD50	*YNL250W*	---	Coiled-coil protein required for resection at double-stranded breaks and for DNA repair
RAD51	*YER095W*	---	Stimulates pairing and strand-exchange between homologous single-stranded and double-stranded DNA
RAD52	*YML032C*	---	Required for recombination and repair of X-ray damage
RAD54	*YGL163C*	---	DNA dependent ATPase required for X-ray damage repair
RAD55	*YDR076W*	---	With Rad57 promotes DNA strand exchange by Rad51 recombinase
RAD57	*YDR004W*	---	With Rad55 promotes DNA strand exchange by Rad51 recombinase
RAD59	*YDL059C*	---	Homologue of Rad52 involved in homologous recombination and DNA repair
RTT101	*YJL047C*	---	Ubiquitin protein ligase possibly involved in genomic stability
RTT107	*YHR154W*	--	Functions in DNA synthesis after DNA damage during S phase
RTT109	*YLL002W*	---	Involved in resistance to mutagens such as diepoxybutane and mitomycin C
TOP3	*YLR234W*	---	DNA topoisomerase III
UBC13	*YDR092W*	---	Ubiquitin-conjugating (E2) enzyme involved in Rad6-dependent post-replicative repair
WSS1	*YHR134W*	--	Involved in sensitivity to UV irradiation.
XRS2	*YDR369C*	---	Required for DNA-repair and meiotic recombination

	YBR094W	---	Synthetic interaction pattern suggests a role in DNA repair
			REDUCTASES AND RELATED PROTEINS
PRO2	*YOR323C*	---	Gamma-glutamyl phosphate reductase (phosphoglutamate dehydrogenase)
			CELL STRESS AND SIGNAL TRANSDUCTION
BCK1	*YJL095W*	---	Bypass requirement for protein kinase C homologue; MEKK
LYS7	*YMR038C*	---	Copper chaperone for superoxide dismutase Sod1
SIT4	*YDL047W*	---	Protein phosphatase of the PP2A family
SLT2	*YHR030C*	---	Protein kinase of MAP kinase family
SOD1	*YJR104C*	---	Cu, Zn superoxide dismutase
SOD2	*YHR008C*	--	Manganese superoxide dismutase, mitochondrial
			LIPID, FATTY ACID AND STEROL METABOLISM
ERG2	*YMR202W*	---	C-8 sterol isomerase, ergosterol biosynthesis enzyme
ERG3	*YLR056W*	--	C-5 sterol desaturase, ergosterol biosynthesis enzyme

ERG4	*YGL012W*	--	C-4(28) sterol reductase, ergosterol biosynthesis enzyme
			VESICULAR TRANSPORT
RIC1	*YLR039C*	---	In complex with Rgp1 to form as a guanyl-nucleotide exchange factor for Ypt6
SNF7	*YLR025W*	---	ESCRT-III subunit, functions in protein sorting to the pre-vacuolar endosome
SNF8	*YPL002C*	--	ESCRT-II subunit, functions in protein sorting to the pre-vacuolar endosome
VAM3	*YOR106W*	---	Syntaxin homologue (t-SNARE), required for vacuolar assembly
VPS25	*YJR102C*	--	ESCRT-II subunit, functions in protein sorting to the pre-vacuolar endosome
VPS28	*YPL065W*	--	Required for traffic to the vacuole through the endocytic and biosynthetic pathways
VPS41	*YDR080W*	---	Required for formation of AP-3 transport vesicles
WHI6	*YKR020W*	---	Class B vacuolar sorting protein
			VACUOLE
PPA1	*YHR026W*	--	Component of the V0 subcomplex of the vacuolar H+-ATPase
TFP3	*YPL234C*	---	Component of the V0 subcomplex of the vacuolar H+-ATPase
VMA4	*YOR332W*	---	Component of the V1 subcomplex of the vacuolar H+-ATPase

VMA7	*YGR020C*	---	Component of the V0 subcomplex of the vacuolar H+-ATPase
VMA10	*YHR039C-A*	---	Component of the V1 subcomplex of the vacuolar H+-ATPase

PROTEIN SYNTHESIS AND DEGRADATION

RPS4A	*YJR145C*	---	Ribosomal protein S4A
ZUO1	*YGR285C*	---	Zuotin, associates with Ssz1 to form the ribosome-associated complex

TRANSCRIPTION, RNA PROCESSING

DBF2	*YGR092W*	--	Serine/threonine protein kinase of the CCR4-NOT transcriptional complex
GAL11	*YOL051W*	---	Component of RNA polymerase II holoenzyme and Kornberg's mediator complex
PGD1	*YGL025C*	---	Component of RNA polymerase II holoenzyme and mediator subcomplex
POP2	*YNR052C*	---	Component of the CCR4 complex
ROX3	*YBL093C*	---	Component of RNA polymerase II holoenzyme and mediator subcomplex
RPB9	*YGL070C*	---	Non-essential subunit of RNA polymerase II
RSC2	*YLR357W*	--	Component of the abundant RSC complex involved in chromatin remodeling
SPT10	*YJL127C*	---	Amplifies the magnitude of transcriptional regulation at various loci

SPT20	*YOL148C*	---	Component of the histone acetyltransferase SAGA complex
SRB2	*YHR041C*	--	Component of RNA polymerase II holoenzyme and Kornberg's mediator complex
SWI4	*YER111C*	---	Transcription factor involved in cell cycle dependent gene expression
UAF30	*YOR295W*	--	Upstream activation factor complex component; synthetic lethal with *top1* mutation

OTHER FUNCTIONS

AKR1	*YDR264C*	---	Ankyrin repeat-containing protein, inhibitor of signaling in the pheromone pathway
ALF1	*YNL148C*	---	Alpha-tubulin folding cofactor B, assists in formation of the α-β-tubulin heterodimer
BEM1	*YBR200W*	---	SH3-domain protein maintaining Cdc42-Cdc24 at the bud tip
BEM4	*YPL161C*	---	Bud emergence protein that activates Cdc42
BUD20	*YLR074C*	--	Putative nuclear pore protein
CIK1	*YMR198W*	---	Spindle pole body associated protein
MDM20	*YOL076W*	---	Required for N-terminal acetylation of Tpm1 necessary for actin cable organization
MOG1	*YJR074W*	--	Involved in nuclear protein import, interacts with Gsp1
NUP188	*YML103C*	---	Nucleoporin
SLA1	*YBL007C*	---	Cytoskeleton assembly control protein

YNL171C	---	Overlaps with 3' of the essential gene *APC1/YNL172W*

Table 2

Genes whose deletion enhances resistance to TPZ.

Genes whose deletion results in marked resistance to TPZ are listed along with their systematic names (‘ORF”) and their cellular function (if known). All strains listed were scored as “+++” for resistance. See Fig. 3 for examples of relative resistances. For a list of less resistant deletion strains, see *Supplementary Table 1*.

Gene	ORF	Cellular function and Comment
		DNA REPAIR AND GENOME STABILITY
DNL4	*YOR005C*	DNA ligase involved in non-homologous DNA end joining
RAD18	*YCR066W*	Zn finger protein, putative ATPase
		TRANSPORTERS
AGP3	*YFL055W*	Amino acid permease
ALP1	*YNL270C*	Arginine permease
ASI3	*YNL008C*	Involved regulation of amino acid permease gene expression

HNM1	*YGL077C*	Choline permease

REDUCTASES AND RELATED PROTEINS

FRE1	*YLR214W*	Ferric and cupric reductase
HIS4	*YCL030C*	Histidine biosynthesis enzyme
UTR1	*YJR049C*	NAD kinase enhances the activity of ferric/cupric reductase Fre1

CELL STRESS AND SIGNAL TRANSDUCTION

DIG1	*YPL049C*	MAP kinase-associated protein involved in regulation of invasive growth
HSP104	*YLL026W*	Heat shock protein
RAS1	*YOR101W*	GTP-binding protein involved in regulation of cAMP pathway
WSC2	*YNL283C*	Protein required for maintenance of cell wall integrity

LIPID, FATTY ACID AND STEROL METABOLISM

DPL1	*YDR294C*	Dihydrosphingosine-1-phosphate lyase
EKI1	*YDR147W*	Ethanolamine kinase I

FAA3	*YIL009W*	Acyl-CoA synthase
PDR17	*YNL264C*	Phosphatidylinositol transfer protein

VESICULAR TRANSPORT

ERV41	*YML067C*	COPII-coated vesicle component involved in ER to Golgi transport
SPO20	*YMR017W*	Subunit of the t-SNARE complex, required during sporulation
YOS9	*YDR057W*	Involved in ER to Golgi trafficking of GPI-anchored proteins

PROTEIN SYNTHESIS AND DEGRADATION

FYV10	*YIL097W*	Protein involved in the degradation of fructose-1,6-bisphosphatase
HRD1	*YOL013C*	E3 ubiquitin ligase required for degradation of misfolded proteins
RPS12	*YOR369C*	Ribosomal protein S12
UMP1	*YBR173C*	Proteasome maturation factor involved in proteasome assembly

TRANSCRIPTION AND RNA PROCESSING

CAF40	*YNL288W*	Strong similarity to *C. elegans* hypothetical protein

CTK2	*YJL006C*	RNA polymerase II C-terminal domain kinase beta subunit
HAA1	*YPR008W*	Transcription activator
MGA2	*YIR033W*	ER membrane protein involved in regulation of *OLE1* transcription
NTC20	*YBR188C*	Splicing factor
PAF1	*YBR279W*	Protein associated with RNA polymerase II
PUS4	*YNL292W*	Pseudouridine synthase
RNH1	*YMR234W*	Ribonuclease H, endonuclease that degrades RNA in RNA-DNA hybrids
RSE1	*YML049C*	U2 snRNP-associated protein involved in pre-mRNA splicing

OTHER FUNCTIONS

BDH1	*YAL060W*	Stereospecific (2R, 3R)-2,3-butanediol dehydrogenase
BNR1	*YIL159W*	Regulates reorganization of the actin cytoskeleton
DAL3	*YIR032C*	Ureidoglycolate hydrolase
ECM1	*YAL059W*	Protein involved in ribosome assembly
HOS2	*YGL194C*	Component of Set3 histone deacetylase
IBD2	*YNL164C*	Component of the *BUB2*-dependent spindle checkpoint pathway

KGD1	*YIL125W*	component of the E1 alpha-ketoglutarate dehydrogenase complex
MAM33	*YIL070C*	Mitochondrial protein required for normal respiratory growth
RNR3	*YIL066C*	Ribonucleotide reductase
SPO1	*YNL012W*	Meiosis-specific protein with similarity to phospholipase B enzymes
SYG1	*YIL047C*	Involved in G-protein coupled receptor signal transduction
TIR3	*YIL011W*	Member of the seripauperin and TIP1 family
	YBL083C	Overlaps with 3' part of *RHK1/YBL082C*
	YER049W	Component of NuA3 histone acetyltransferase complex

UNKNOWN AND POORLY CHARACTERIZED FUNCTIONS

AKL1	*YBR059C*	Serine/threonine protein kinase of unknown function
DOS2	*YDR068W*	Protein containing a BSD domain, may be involved in protein degradation
KNS1	*YLL019C*	Putative serine/threonine protein kinase
PHM8	*YER037W*	Protein of unknown function
RSM25	*YIL093C*	Protein of unknown function
UIP3	*YAR027W*	Protein with high similarity to *S. cerevisiae* Mst27, which binds COPI and

		COPII complexes, member of the duplication (DUP) family
SMY2	*YBR172C*	Protein of unknown function, suppresses myo2-66, sec22, bet1, sec16-3, spt15, and yrb1-51 mutants when overexpressed, may be involved in RNA splicing
	YCL023C	Protein of unknown function
	YDL156W	Protein containing three WD domains (WD-40 repeat), which may mediate protein-protein interactions, has moderate similarity to uncharacterized C. albicans Ipf2218
	YGR290W	Protein of unknown function
	YHR131C	Protein containing a pleckstrin homology (PH) domain, which mediate protein-protein and protein-lipid interactions, has low similarity to uncharacterized *S. cerevisiae YNL144*
	YIL161W	Protein of unknown function
	YJL163C	Hypothetical protein
	YJL218W	Protein with similarity to *E. coli* galactoside O-acetyltransferase
	YJR018W	Protein of unknown function
	YJR038C	Protein of unknown function

YJR056C	Protein of unknown function
YKL161C	Serine/threonine protein kinase with similarity to MAP kinases
YKR096W	Protein of unknown function, has high similarity to *S. cerevisiae YIL151*
YML050W	Protein of unknown function
YMR253C	Protein of unknown function, likely membrane protein
YNL144C	Protein of unknown function, has low similarity to uncharacterized *S. cerevisiae YHR131*
YNR024W	Protein of unknown function
YOL163W	Protein of unknown function
YOR044W	Protein of unknown function
YPR022C	Predicted transcription factor with two tandem C2H2-type zinc fingers, contains Q/N-rich regions which may mediate prion-like aggregation
YAL065C	Protein of unknown function, has high similarity to a region of flocculin (*S. cerevisiae FLO1*), which is a cell wall protein involved in flocculation

Table 3

Selected human gene products with yeast homologues whose gene deletion enhances resistance to TPZ.

Human gene products are listed with their yeast homologues. E values (identical proteins would have an E value of zero) and the percentage of identities are also given.

Human gene product	Human gene product description	Yeast gene	E value	Identity (%)
	CELL DIFFERENTIATION, MORPHOGENESIS, SIGNALING			
DAAM1	Formin homolog involved in morphogenesis	*BNR1*	2E-18	24
DAAM2	Formin homolog involved in morphogenesis	*BNR1*	2E-18	23
MAEA	Anti-apoptotic factor mediating erythroblast attachment to macrophage	*FYV10*	1E-12	22
CLK1	Protein kinase involved in cell proliferation	*KNS1*	2E-68	40
HRAS	Transforming protein p21/H-Ras-1	*RAS1*	7E-49	63
MUC15	Mucin family member	*WSC2*	2E-07	22

TRANSPORT				
SLC7A2	Low-affinity cationic amino acid transporter	*AGP3*	1E-11	25
SLC7A1	High-affinity cationic amino acid transporter	*AGP3*	4E-08	24
SLC7A3	Cationic amino acid transporter	*AGP3*	1E-06	25
CDA14	Gene downregulated in prostate tumors	*ERV41*	1E-27	30
OS-9	Gene amplified in sarcomas	*YOS9*	8E-04	25
C2orf30		*YOS9*	3E-07	29
DNA REPAIR				
RQCD1	Transcription factor	*CAF40*	2E-89	61
LIG4	DNA ligase IV	*DNL4*	1E-75	25
TRUB1	TruB pseudouridine synthase homolog 1	*PUS4*	3E-23	33
TRUB2	TruB pseudouridine synthase homolog 2	*PUS4*	1E-06	27
RAD18	Postreplication repair protein RAD18	*RAD18*	1E-15	23
RNASEH1	Ribonuclease H1	*RNH1*	4E-09	25
RRM1	Ribonucleoside-diphosphate reductase M1 chain	*RNR3*	0	66

	OTHER			
SGPL1	Sphingosine-1-phosphate lyase 1, involved in cisplatin sensitivity in *D. discoideum*	*DPL1*	4E-108	41
ETNK1	Ethanolamine kinase	*EKI1*	5E-18	24
NP_061961	PAF domain containg protein	*PAF1*	1E-07	22
RPS12	40S ribosomal protein S12.	*RPS12*	3E-28	55
SF3B3	Splicing factor 3B subunit 3	*RSE1*	5E-61	27
XPR1	Xenotropic and polytropic retrovirus receptor	*SYG1*	3E-37	26
C13orf12		*UMP1*	8E-04	24
NP_079184		*YDL156W*	3E-21	24
NP_060703		*YER049W*	6E-20	32
KIST	Protein kinase interacting with stathmin	*YKL161C*	1E-13	29

Table 4

Selected human proteins sharing homology to yeast proteins whose gene deletion confers TPZ hypersensitivity.

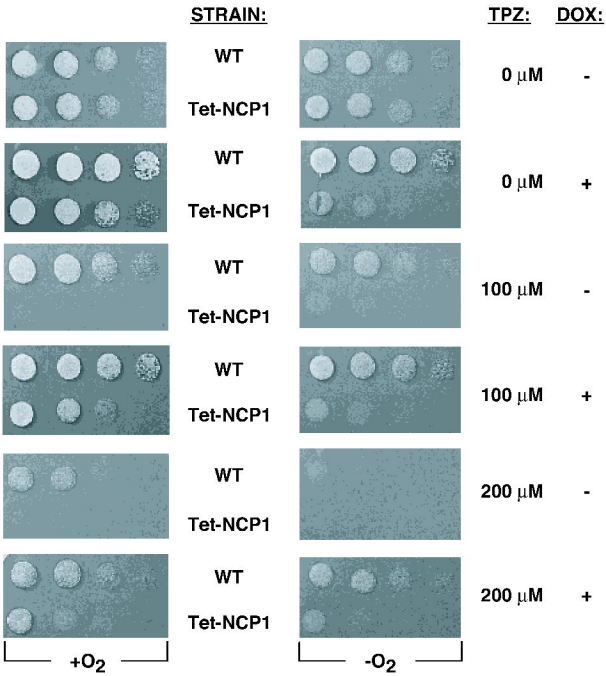
Human gene products are listed with their yeast homologues. E values (identical proteins would have an E value of zero) and the percentage of identities are also given.

Human gene product	Human gene product description	Yeast gene	E value	Identity (%)
	CYTOSKELTON			
CKAP1	Tubulin-specific chaperone B	*ALF1*	4E-12	29
TTL	Tubulin-tyrosine ligase	*YBR094W*	4E-13	26
	NUCLEAR TRANSPORT, TRANSCRIPTION			
NP_057576	RAN guanine nucleotide release factor	*MOG1*	4E-12	29
NUP188	Nucloeoporin	*NUP188*	1E-04	22
POLR2I	RNA polymerase II subunit	*RPB9*	2E-26	45

SMARCD1	SWI/SNF complex 60 kDa subunit	*UAF30*	2E-07	36
	PROTEIN SYNTHESIS			
RPS4Y1	40S ribosomal protein S4, Y isoform 1	*RPS4A*	9E-109	71
RPS4Y2	40S ribosomal protein S4, Y isoform 2	*RPS4A*	7E-104	69
ZRF1	M-phase phosphoprotein	*ZUO1*	3E-39	43
	REDOX, SIGNALING			
ALDH18A1	γ-1-pyrroline-5-carboxylate synthetase	*PRO2*	2E-78	39
PPP6C	Serine/threonine protein phosphatase	*SIT4*	8E-115	65
MAPK7	Mitogen-activated protein kinase 7	*SLT2*	3E-93	46
SOD1	Cu/Zn superoxide dismutase	*SOD1*	5E-43	55
SOD2	mitochondrial Mn superoxide dismutase	*SOD2*	3E-49	46
	TRANSPORT			
C20orf178	Snf7 homologue	*SNF7*	2E-19	41
NP_689497	Alix3 interacting protein	*SNF7*	2E-18	41

HSPC134	Alix 2 interacting protein	*SNF7*	3E-17	38
EAP30	EAP30 subunit of ELL complex	*SNF8*	7E-37	37
NP_115729	VPS25 homolog	*VPS25*	5E-19	31
VPS28	Endosomal sorting protein	*VPS28*	2E-27	30
VPS41	Golgi to endosome sorting protein	*VPS41*	1E-63	25
	VACUOLE			
ATP6V0B	V-type H^+-ATPase V0 subunit	*PPA1*	1E-44	55
ATP6V1G1	V-type H^+-ATPase V1 subunit G1	*VMA10*	1E-07	38
ATP6V1G3	V-type H^+-ATPase V1 subunit G3	*VMA10*	7E-07	36
ATP6V1E2	V-type H^+-ATPase V1 subunit E2	*VMA4*	7E-19	35
ATP6V1E1	V-type H^+-ATPase V1 subunit E	*VMA4*	1E-18	33
ATP6V1F	V-type H^+-ATPase V1 subunit F	*VMA7*	8E-27	53
	DNA REPAIR			
ASF1B	Chromatin assembly factor	*ASF1*	5E-51	59
ASF1A	Chromatin assembly factor	*ASF1*	1E-40	61

MRE11A	Double-strand break repair protein	*MRE11*	2E-108	41
MUS81	Crossover junction endonuclease	*MUS81*	2E-19	29
CNOT8	CCR4-NOT complex subunit 8	*POP2*	2E-51	37
ERCC1	DNA excision repair protein	*RAD10*	4E-13	30
RAG1	V(D)J recombination activating protein 1	*RAD16*	7E-04	31
SMARCA3	Helicase/ATPase of the SWI/SNF family	*RAD5*	2E-76	33
RAD50	RAD50 homolog isoform 1	*RAD50*	4E-149	28
RAD51	DNA repair protein RAD51 homolog 1	*RAD51*	5E-126	67
RAD52	DNA repair protein RAD52	*RAD52*	1E-40	49
RAD54L	DNA repair protein RAD54	*RAD54*	2E-165	50
TOP3A	DNA topoisomerase III alpha	*TOP3*	7E-125	42
UBE2N	E2 ubiquitin-conjugating enzyme	*UBC13*	6E-56	70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Tet-NCP1
100 μM
-
D
WT
Tet-NCP1
100 μM
+
E
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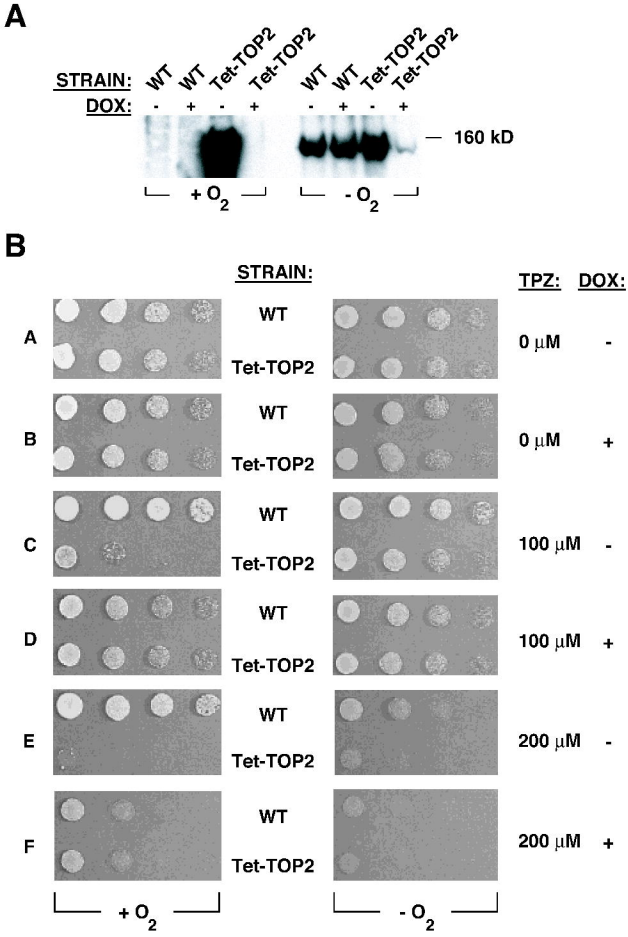
200 μM
+
+O2
-O2

Fig. 1

Fig. 2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+ O2
- O2
B
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DOX:
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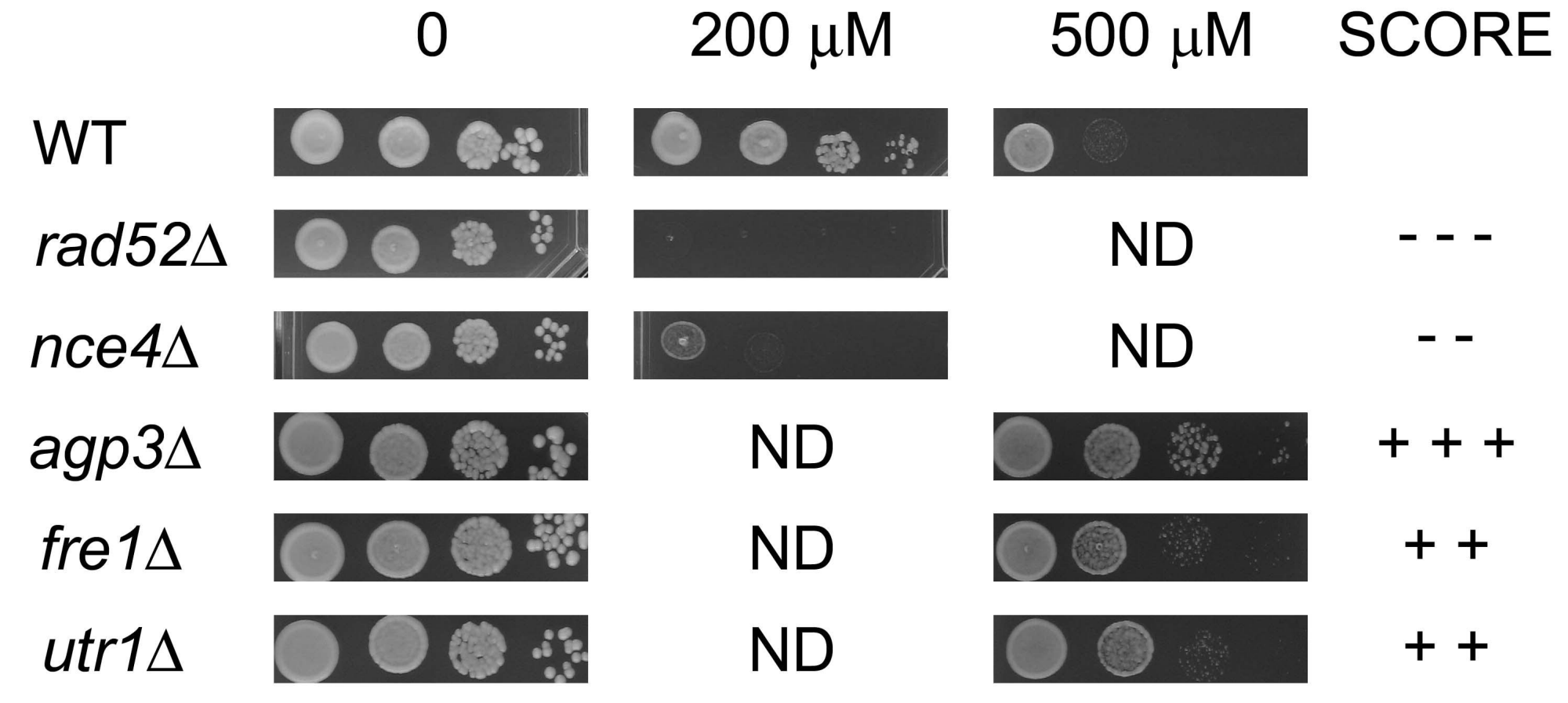
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fre1Δ
ND
+ +
utr1Δ
ND
+ +

Supplementary Table S1.

Gene	ORF	Cellular function and Comment
		TRANSPORTERS
QDR2	*YIL121W*	Member of the multidrug-resistance 12-spanner (DHA12) family of the major facilitator superfamily (MFS-MDR)
		CELL STRESS
GPX2	*YBR244W*	Glutathione peroxidase, has a role in mitochondrial morphology and provides protection against lipid and non-lipid hydroperoxides
GRX4	*YER174C*	Glutaredoxin, has similarity to Grx3 and Grx5
		VESICULAR TRANSPORT
VPS53	*YJL029C*	Subunit of the VFT complex involved in protein sorting in the late Golgi
		PROTEIN SYNTHESIS AND DEGRADATION
GID7	*YCL039W*	Involved in the degradation of fructose-1,6-bisphosphatase
PNG1	*YPL096W*	Peptide N-glycanase
RPS10B	*YMR230W*	Ribosomal protein S10B
UBX5	*YDR330W*	Involved in proteasome function
		TRANSCRIPTION, RNA PROCESSING

IMP2'	*YIL154C*	Transcription factor involved in nucleo-mitochondrial control of maltose, galactose and raffinose utilization
MET28	*YIR017C*	Transcriptional activator regulating sulfur amino acid metabolism that functions with Met4 and Cbf1, member of the basic leucine zipper (bZIP) family
RPA12	*YJR063W*	RNA polymerase I subunit A12.2
YAP5	*YIR018W*	Transcription factor of the basic leucine zipper (bZIP) type, one of eight members of a novel fungal-specific family of bZIP proteins
	YOR302W	Arginine attenuator peptide, mediates translational regulation of a downstream gene; encoded by the 5'-leader of the carbamyl-phosphate synthetase small subunit (CPA1) mRNA

SIGNAL TRANSDUCTION

PPZ1	*YML016C*	Serine/threonine phosphatase required for normal osmoregulation

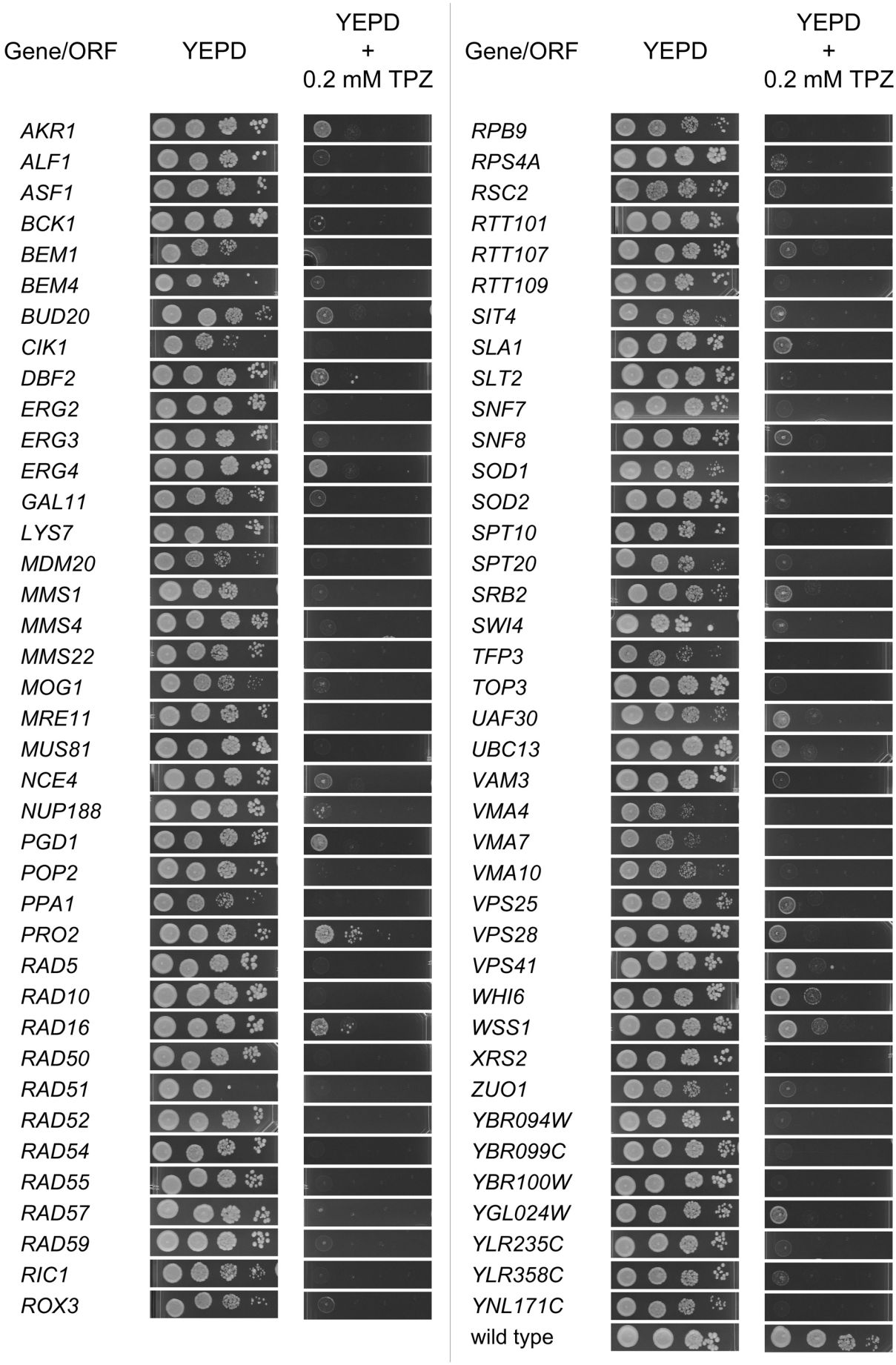
OTHER FUNCTIONS

AAC3	*YBR085W*	Mitochondrial ADP/ATP transporter
ADE8	*YDR408C*	Phosphoribosylglycinamide formyltransferase
FSH2	*YMR222C*	Cytoplasmic protein with similarity to *S. pombe* Dfr1 dihydrofolate reductase
IAH1	*YOR126C*	Isoamyl acetate-hydrolyzing esterase enzyme
PHO3	*YBR092C*	Constitutive acid phosphatase
SHM1	*YBR263W*	Mitochondrial serine/glycine hydroxymethyltransferase

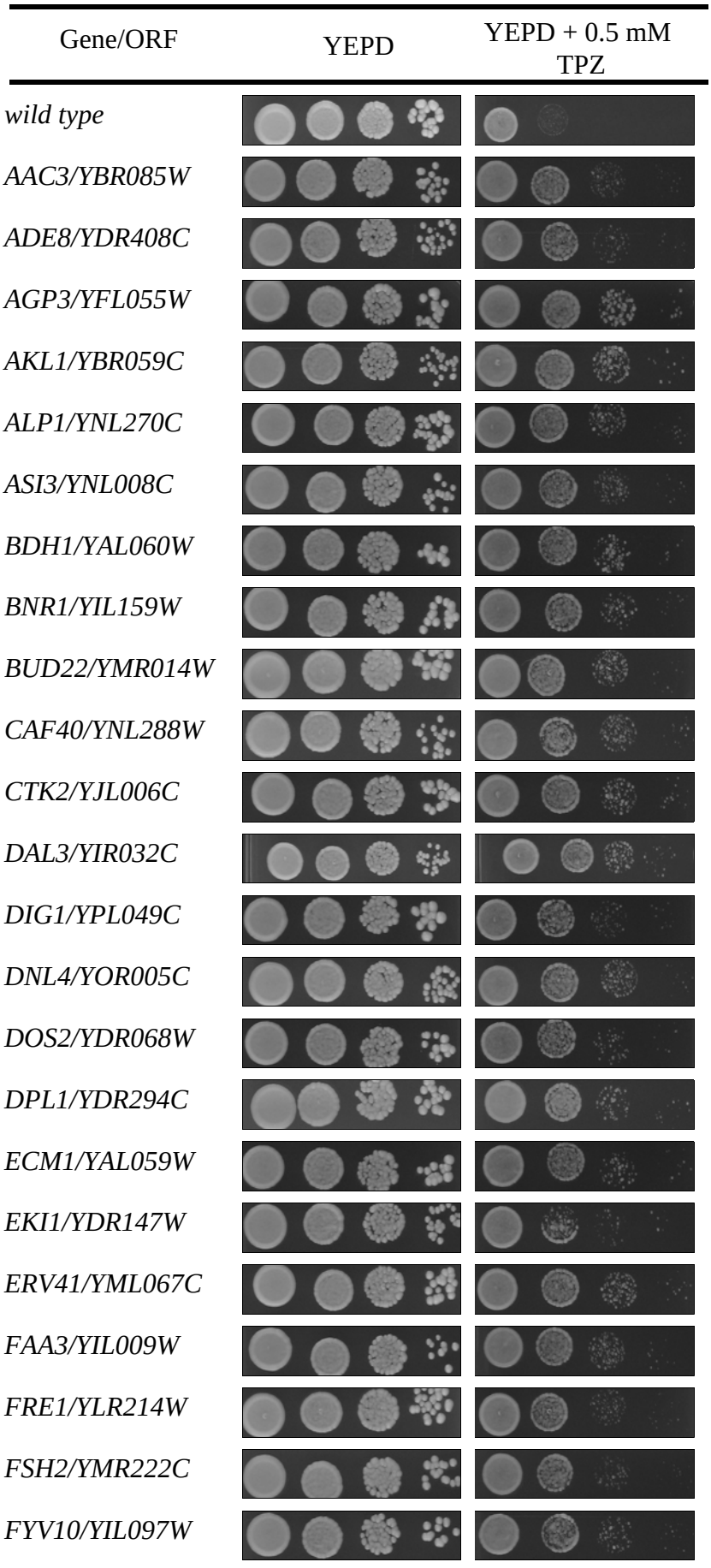
UNKNOWN, POORLY CHARACTERIZED FUNCTIONS

BUD22	*YMR014W*	Possible role in bud site polarity
FYV2	*YIL054W*	Protein of unknown function
SLX1	*YBR228W*	Subunit of Slx1-Slx4 complex
	YAR023C	Has moderate similarity to S. cerevisiae Mst27
	YBL096C	Protein of unknown function
	YBR203W	Contains an F-box domain
	YDR387C	Member of the sugar transporter family
	YDR514C	Has moderate similarity to uncharacterized *S. cerevisiae GFD2*
	YIL025C	Protein of unknown function
	YIL096C	Protein of unknown function
	YIL141W	unknown
	YIL165C	unknown
	YJL028W	unknown
	YJR054W	Protein of unknown function
	YJR087W	Protein of unknown function
	YJR149W	Member of the 2-nitropropane dioxygenase family
	YKL202W	Protein of unknown function
	YLR112W	Protein of unknown function
	YLR187W	Contains a pleckstrin homology (PH) domain
	YNL046W	Protein of unknown function
	YNL156C	Has moderate similarity to uncharacterized S. cerevisiae Yhr133
	YPL067C	Protein of unknown function
	YPL206C	Member of the glycerophosphoryl diester phosphodiesterase family

Supplementary Figure S1.



Supplementary Figure S2.



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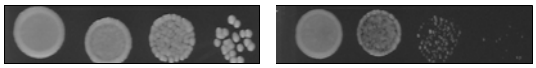
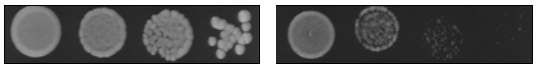
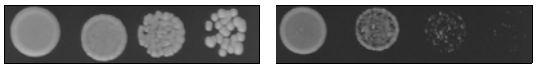
FYV2/YIL054W		
GPX2/YBR244W		
GRX4/YER174C		
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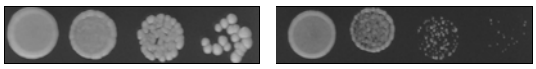
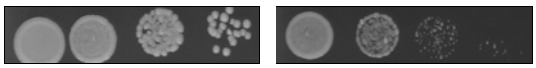
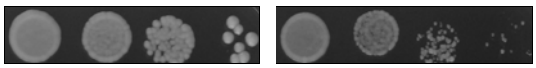
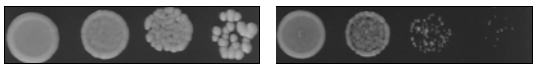
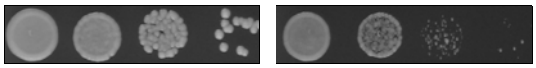
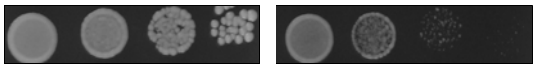
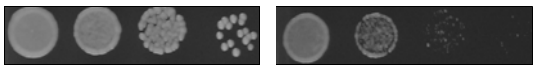
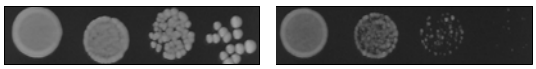
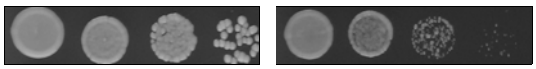
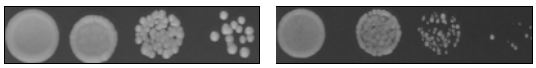
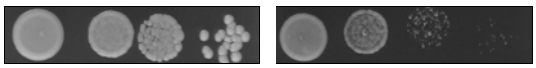
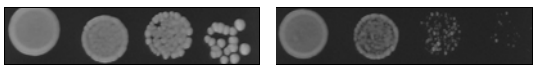
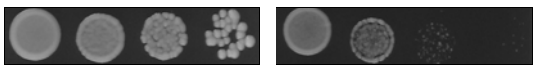
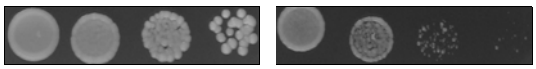
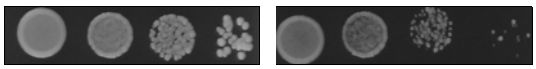
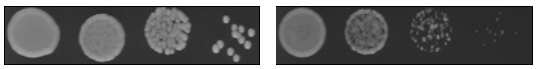
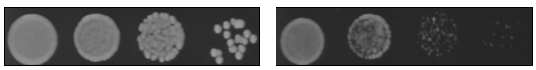
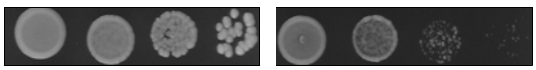
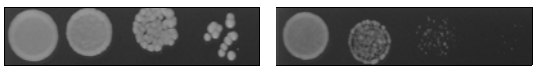
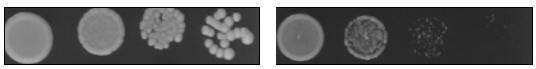
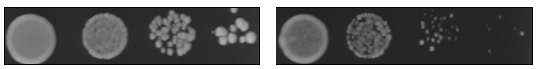
HIS4/YCL030C		
HNM1/YGL077C		
HOS2/YGL194C		
HRD1/YOL013C		
HSP104/YLL026W		
IAH1/YOR126C		
IBD2/YNL164C		
IMP2'/YIL154C		
KGD1/YIL125W		
KNS1/YLL019C		
MAL13/YGR288W		
MAM33/YIL070C		
MET28/YIR017C		
MGA2/YIR033W		
NTC20/YBR188C		
PAF1/YBR279W		
PDR17/YNL264C		
PHM8/YER037W		
PHO3/YBR092C		
PNG1/YPL096W		
PPZ1/YML016C		

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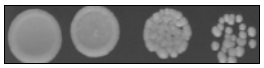
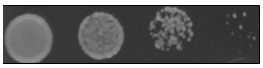
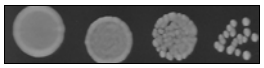
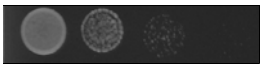
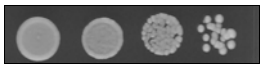
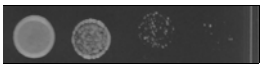
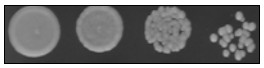
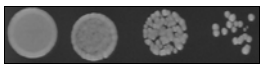
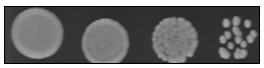
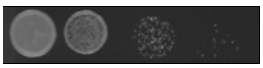
PUS4/YNL292W		
QDR2/YIL121W		
RAD18/YCR066W		
RAS1/YOR101W		
RNH1/YMR234W		
RNR3/YIL066C		
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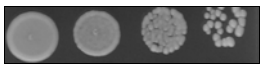
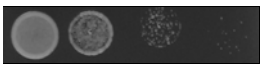
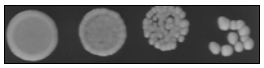
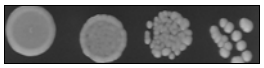
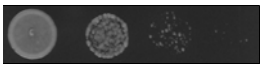
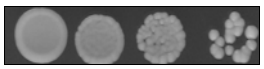
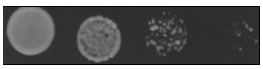
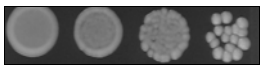
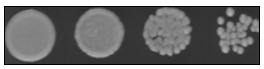
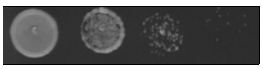
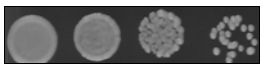
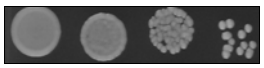
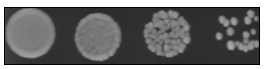
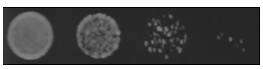
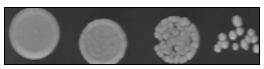
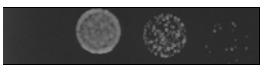
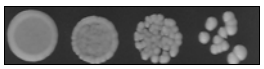
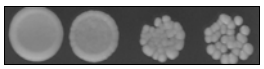
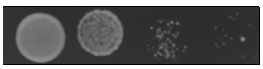
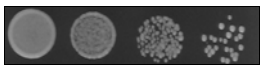
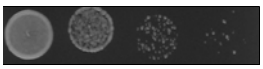
RPS10B/YMR230W		
RPS12/YOR369C		
RSE1/YML049C		
RSM25/YIL093C		
SHM1/YBR263W		
SLX1/YBR228W		
SMY2/YBR172C		
SPO1/YNL012W		
SPO20/YMR017W		
SYG1/YIL047C		
TIR3/YIL011W		
UIP3/YAR027W		
UMP1/YBR173C		
UTR1/YJR049C		
VPS53/YJL029C		
WSC2/YNL283C		
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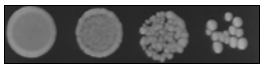
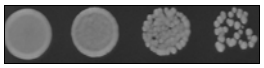
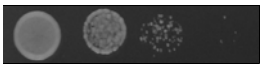
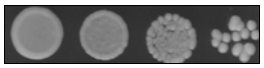
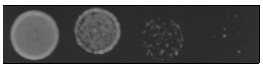
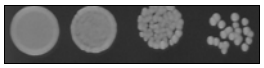
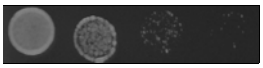
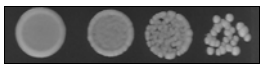
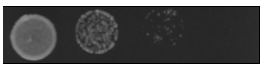
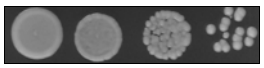
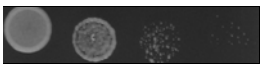
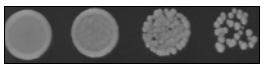
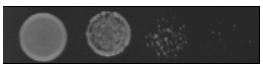
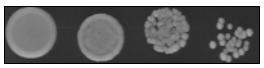
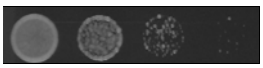
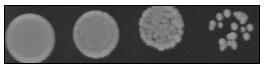
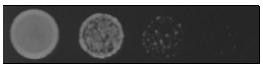
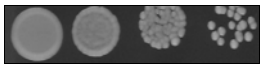
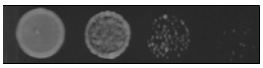
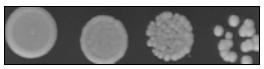
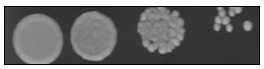
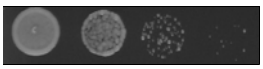
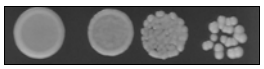
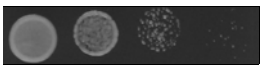
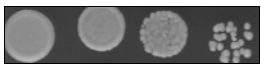
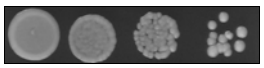
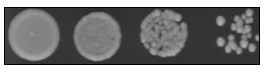
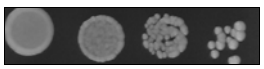
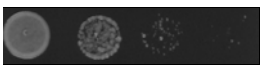
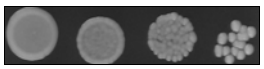
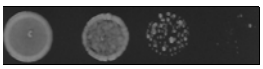
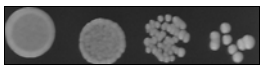
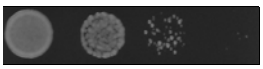
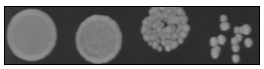
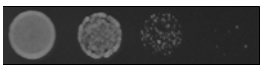
YOS9/YDR057W		

Gene/ORF	YEPD	YEPD + 0.5 mM TPZ
YAL065C		
YAR023C		
YBL083C		
YBL096C		
YBR203W		
YCL023C		
YCL039W		
YDL156W		
YDR330W		
YDR387C		
YDR514C		
YER049W		
YGR290W		
YHR131C		
YIL025C		
YIL096C		
YIL141W		
YIL161W		
YIL165C		
YJL028W		
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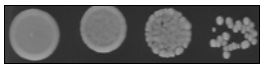
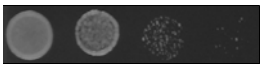
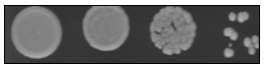
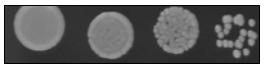
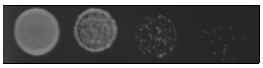
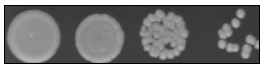
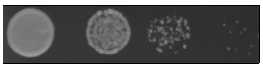
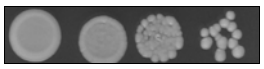
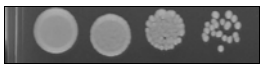
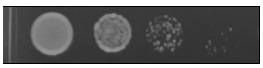
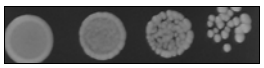
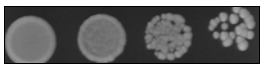
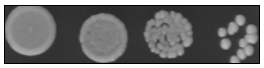
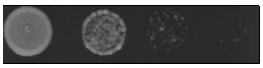
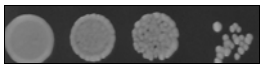
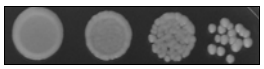
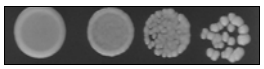
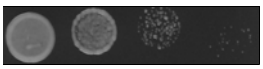
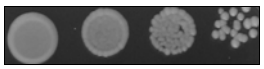
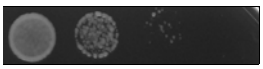
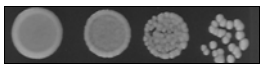
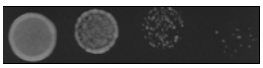
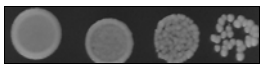
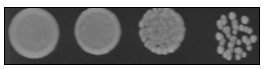
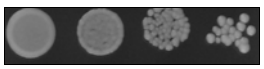
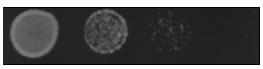
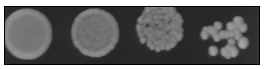
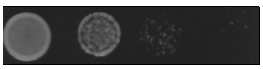
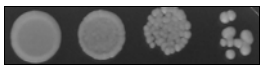
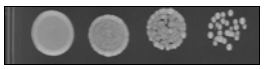
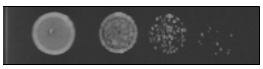
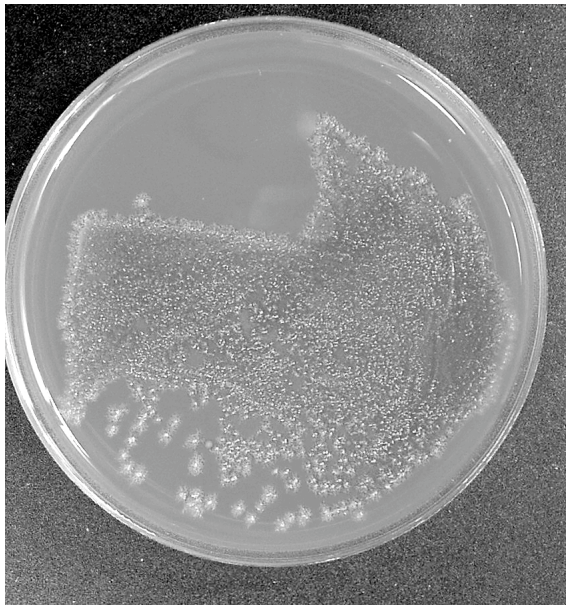
YJR054W		

Gene/ORF	YEPD	YEPD + 0.5 mM TPZ
YJR056C		
YJR087W		
YJR149W		
YKL161C		
YKL202W		
YKR096W		
YLR112W		
YLR187W		
YML050W		
YMR253C		
YNL046W		
YNL144C		
YNL156C		
YNR024W		
YOL163W		
YOR044W		
YOR302W		
YPL067C		
YPL206C		
YPR022C		

Supplementary Figure S3

- O_2 (2 days)



+ O_2 (5 days)