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A Genome-Wide Overexpression Screen in Yeast for Small-Molecule Target Identification

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Summary

We describe a multicopy gene suppression screen of drug sensitivity in *Saccharomyces cerevisiae* that facilitates the identification of cellular targets of small molecules. An array of yeast transformants harboring a multicopy yeast genomic library was screened for resistance to growth inhibitors. Comparison of array growth patterns for several such inhibitors allowed the differentiation of general and molecule-specific genetic suppressors. Specific resistance to phenylaminopyrimidine (1), an inhibitor identified from a kinase-directed library, was associated with the overexpression of Pkc1 and a subset of downstream kinases. Components of two other pathways (pheromone response/filamentous growth and Pho85 kinase) that genetically interact with the PKC1 MAPK signaling cascade were also identified. Consistent with the suppression screen, inhibitor 1 bound to Pkc1 in yeast cell lysate and inhibited its activity in vitro. These results demonstrate the utility of this approach for the rapid deconvolution of small-molecule targets.

Introduction

Phenotypic or pathway-specific high-throughput screens of small-molecule libraries are often used for the discovery of small-molecule effectors or inhibitors of cellular processes. However, the subsequent identification of the small-molecule targets can be quite challenging. The budding yeast *Saccharomyces cerevisiae* has been broadly employed for drug target identification and mechanism-of-action studies. It is a model organism representative of simple eukaryotes, but has only slightly greater genetic complexity than bacteria (~6,200 open reading frames; ORFs) and its genome can be easily manipulated [1, 2]. Significantly, about 30% of known genes implicated in human disease have yeast orthologs [3]. Target identification and validation in yeast have been especially useful in the case of antiproliferative agents [4], where the potential small-molecule targets are often components of highly conserved cell division or DNA repair machinery.

In recent years, various yeast genomics- and proteomics-based target identification strategies have been developed [5, 6]. In particular, the availability of a comprehensive collection of gene deletion strains [7, 8] has expanded the utility of yeast in small-molecule target deconvolution. Screens employing induced haploinsufficiency (the diploid strain heterozygous at the drug target locus being more sensitive to the small molecule than the wild-type strain) have been carried out with gene deletion strains in order to identify or confirm the targets of tunicamycin [9] and other drugs [10, 11]. Another approach involves comparison of gene expression profiles of drug-treated cells with a compendium of expression profiles derived from a large set of gene knockout strains. This method was used to identify a novel target of the commonly used drug dyclonine [12]. And yet another approach utilized the global pattern of genetic interactions obtained from synthetic genetic array (SGA) analysis [13] or synthetic lethality analysis by microarray (SLAM) [14]. SGA and SLAM allow for screening of mutations that exhibit synthetic lethal interactions either by crossing a query mutation with arrayed deletion mutants (SGA) or by introducing a query mutation via integrative transformation into a haploid deletion pool (SLAM). Based on the hypothesis that the drug hypersensitivity profile and the genetic interaction profile should match if the query mutation is in the drug target encoding gene, the mechanism of action of a small molecule can be inferred by comparing the growth defect pattern of haploid deletion strains after drug exposure with the "fingerprint" genetic interaction profile [15].

A complementary approach to the screens described above, which involve gene deletion strains, is a multicopy suppression screen for genes that confer resistance to an inhibitor upon overexpression [1, 2]. This screen is based on the principle that cells which express increased levels of a small-molecule target should tolerate higher drug levels. Such a screen is carried out by the introduction of a yeast chromosomal library harbored on a multicopy vector into the wild-type yeast strain with subsequent examination of individual transformants for resistance to the inhibitor. The molecular targets for various known antifungals, such as tunicamycin [16], ketoconazole [17], and soraphen [18], have been identified in this fashion.

The main limitation of the above approaches to drug target identification is that only a subset of potentially interesting compounds has a growth-inhibitory phenotype in yeast. This is caused partly by a lower permeability of yeast cells to small molecules as compared to mammalian cells, partly by the nonessential nature of equivalent yeast targets, and partly by the absence of such mammalian targets in yeast. In spite of these limitations, approaches utilizing budding yeast represent a powerful tool for drug target identification.

Here we report a variation of a gene dosage resistance screen that is amenable to a high-throughput format by using arrays of transformed *S. cerevisiae* strains. Such an approach allows target identification for small molecules that are active in mammalian cells

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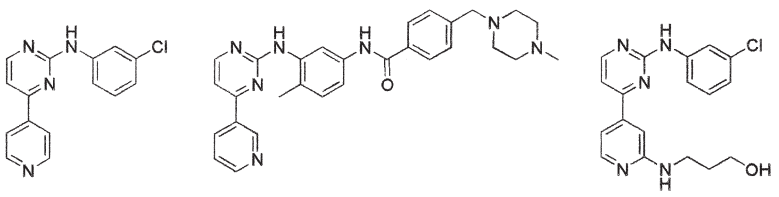


1 2 3

Figure 1. Structures of PAP Compounds **1** (This Study), **2** (STI-571), and **3** (CGP60474)

and simultaneously inhibit yeast growth. We initially applied this method to the identification of targets of a small molecule derived from cellular screens of kinase-directed combinatorial libraries [19]. Protein kinases represent an important class of drug targets because they are involved in the regulation of various cellular functions (e.g., proliferation activation or apoptosis) [20] and aberrant kinase activity has been linked to cancer and other diseases [21].

Results and Discussion

Small-Molecule Screen for Growth Inhibitors

A small library of ~1000 kinase-directed heterocyclic small molecules [19] was screened for inhibitors of growth using standard yeast strain YPH500. The screen was carried out in a 384-well format on soft agar and plates were visually inspected for growth inhibition. A number of active compounds were identified that inhibited yeast growth with IC_{50} values less than 20 μM. The structure of one of these molecules, *N*-(3-chlorophenyl)-4-(4-pyridinyl)-2-pyrimidinamine (**1**) (IC_{50} ~5 μM; Figure 1) [22], shares the phenylaminopyrimidine (PAP) scaffold with the anticancer kinase inhibitors STI-571 (Gleevec, **2**, Bcr-Abl inhibitor) [23] and CGP60474 (**3**, CDK1/CDK2 inhibitor) [24], and was selected for testing the target identification strategy by dosage suppression of drug sensitivity.

Generation of Overexpression Library

The YPH500 strain was transformed with the yeast genomic DNA library constructed in the multicopy YEp13 plasmid (average insert size, 5 kb) [25]. In total, 7296 randomly picked transformants were arrayed in 384-well plates (19 plates). To determine the frequency with which a typical gene is present in the array, the transformants were plated on minimal media lacking either histidine or tryptophan. Because YPH500 strain harbors inactivated *trp1* and *his3* genes, it cannot grow on either medium. However, the genomic DNA of the yeast strain from which the library was constructed has the functional *TRP1* and *HIS3* genes. Thus, the presence of a plasmid in the library that contains one of these two genes in YPH500 strain will restore growth to a transformant on medium lacking the respective amino acid. In total, the assembled array of transformants contained three Trp^+ and four His^+ independent clones, indicating that a typical library gene is represented in the array multiple times.

Screen for Suppressors of Growth Inhibition

To screen for suppressors of growth inhibition by pyrimidine **1**, a minimal inhibitor concentration was used that prevented the formation of control colonies (harboring YEp13 vector without an insert) in the course of 3-day cultivation. The arrayed transformants were first grown in 384-well plates and then stamped with a pintool onto –Leu agar in the absence of inhibitor to assess viability of the transformed strains (Figure 2A), and onto agar containing compound **1** at 15 μM (four 384-well plates were replicated onto one agar plate). The resistant colonies (Figure 2B) were first selected and then analyzed by determining the sequence of the vec-

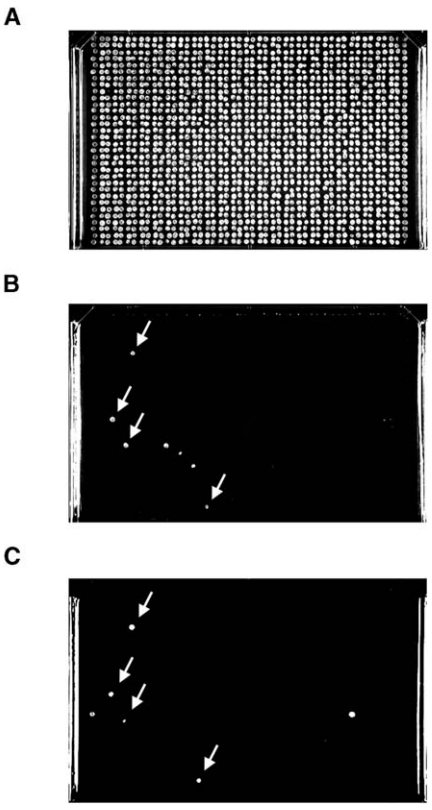


Figure 2. Photographs of a Representative Plate from the Array

A total of 1536 transformed yeast strains were replicated onto each –Leu agar plate and cultivated for 3 days (A) in the absence of inhibitor, (B) in the presence of inhibitor **1** (15 μM), and (C) in the presence of a structurally distinct inhibitor (see Supplemental Data). Nonspecific suppressors are indicated with white arrows.

Table 1. Genes from Overexpression Screen Conferring Resistance to Compound 1

Specificity	Category	Hits[b]
Specific	PKC1 MAPK pathway	*PKC1* (4), *BCK1* (2), *MKK2* (3)
	Cell cycle	*CLG1* (5)
	Filamentous growth[a]	*KSS1* (4), *STE5* (3), *STE7* (3)
Nonspecific	Drug pump	*PDR3* (4)
	Biosynthetic genes	*PAB1* (7), *PRS4* (7), *INO1* (3)

[a] Components are also involved in the pheromone response pathway.
[b] Numbers in parentheses indicate the number of independent resistant colonies harboring a given gene on plasmid insert.

tor insert they harbored through end sequencing and database comparison. To eliminate false positives due to plasmid-independent genetic variations among the individual transformants, only the genes recovered from at least two colonies in a particular small-molecule screen were considered. Furthermore, because many plasmids harbor several full-length ORFs, the exact localization of the resistance-conferring gene requires deletion analysis of inserts and reexamination of the resistant phenotype for the deletion constructs. Therefore, to avoid such laborious analysis, we considered only those genes that were recovered in a particular compound screen multiple times and in such sequence contexts that allowed unequivocal assignment of resistance to a single gene.

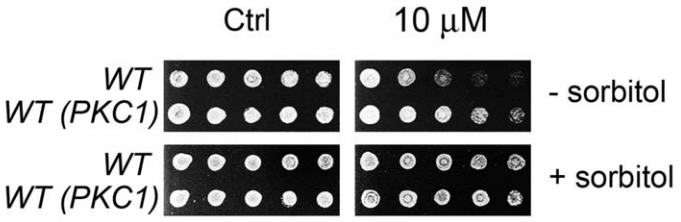
Genes, which conferred resistance to not only compound **1** but also to several other inhibitors identified in the small-molecule screens (see Supplemental Figure S1), were classified as nonspecific suppressors (compare Figures 2B and 2C). These included drug pump gene *PDR3*, ribose-phosphate pyrophosphokinase gene *PRS4* (catalyzing the rate-limiting step in the synthesis of the precursor molecule for the biosynthesis of nucleotides, tryptophan, and histidine), L-myo-inositol-1-phosphate synthase gene *INO1* (catalyzing the rate-limiting step in the biosynthesis of inositol), and poly(A) binding protein gene *PAB1* (essential function in protein translation). Because these genes rescue growth inhibition by structurally distinct compounds, the mechanism of suppression by these genes is likely not related to the specific mechanism of action of compound **1**.

Suppressors that selectively rescued growth inhibition by compound **1** were categorized into three classes (Table 1). The first group comprises members of the PKC1-MPK1 MAP kinase signaling pathway, which plays an essential role in several processes including bud formation, polarized cell growth, and maintenance of cell wall integrity during environmental stress [26]. In addition to *PKC1*, two other kinase-encoding genes were recovered in the screen: *BCK1* (MAPKKK) and *MKK2* (MAPKK); both act downstream in this pathway. The second class of suppressors contains a single gene, *CLG1*. Clg1 is one of ten cyclins (generally classified into two subfamilies) associated with Pho85 CDK and belongs to the Pcl1/Pcl2 subfamily, which is believed to play a role in polarized cell growth [27]. Interestingly, a *pcl1Δ pcl2Δ* double deletion is synthetically lethal when combined with *bck1Δ* or *mpk1Δ* (MAPK, PKC1-MPK1 signaling cascade) mutations [28]. This indicates that both pathways (Pkc1 and Pho85) fulfill partially redundant functions. Pho85 is nonessential but also capable of serving as an alternative to Cdc28, the main (and essential) CDK for cell cycle progression in yeast [29, 30]. The last class of suppressors included a subset of genes involved in the pheromone response/filamentous growth MAP kinase signaling pathway [31]. In addition to the scaffolding protein Ste5, two additional kinases—Ste7 and Kss1—rescued growth inhibition by compound **1**. Kss1, one of the two downstream effectors of this signaling cascade, is known to regulate the transcription of dozens of genes that contribute to pheromone response/filamentous growth [32, 33]. A prominent feature of Kss1 activation is an altered cell cycle and the upregulation of Cln cyclins (the Cdc28 CDK cyclins) [33, 34]. Similar to the Pho85 pathway (Clg1), the Cln cyclins are known to genetically interact with the Pkc1 signaling cascade, and overexpression of Cln2 suppresses certain *pkc1* mutations [35]. Furthermore, *pcl1Δ pcl2Δ* double and *pho85Δ* single mutants are synthetically lethal not only with mutations in genes of the Pkc1 pathway (see above), but also when combined with *cln1Δ cln2Δ* mutations [28]. Thus, all three pathways identified in the screen exhibit extensive genetic interactions with each other, which further underscores their relevance for the resistance to compound **1**.

Deletion Strain Analysis of Growth Inhibition

In the simplest case, if growth inhibition results from the biochemical inactivation of a protein by compound treatment, the target-encoding gene must be essential for yeast growth. Because *PKC1* was the only compound-specific gene recovered in the screen that is also essential for viability (Table 1), and because the other identified genes function in two other pathways known to genetically interact with the Pkc1 signaling cascade, Pkc1 was considered as the primary target for compound **1**. To test this hypothesis, we first reconfirmed that transformation of wild-type yeast strain with multicopy plasmid (YEp13) harboring *PKC1* as the only nonvector ORF increases resistance of the transformed wild-type strain to compound **1** (Figure 3A). It was also reported that growth defect of a *pkc1Δ* yeast strain can be rescued by increasing the medium osmolarity through the addition of high concentrations (1.0 M) of sorbitol [36]. Consistent with this hypothesis, the inhibitory effect of compound **1** was completely eliminated under such growth conditions (Figure 3A). To further extend these observations, we examined the sensitivity to **1** of several viable strains containing gene deletions in the cell wall integrity signaling pathway downstream of Pkc1 (*pkc1Δ/PKC1*, *bck1Δ*, *mkk1Δ*,

A



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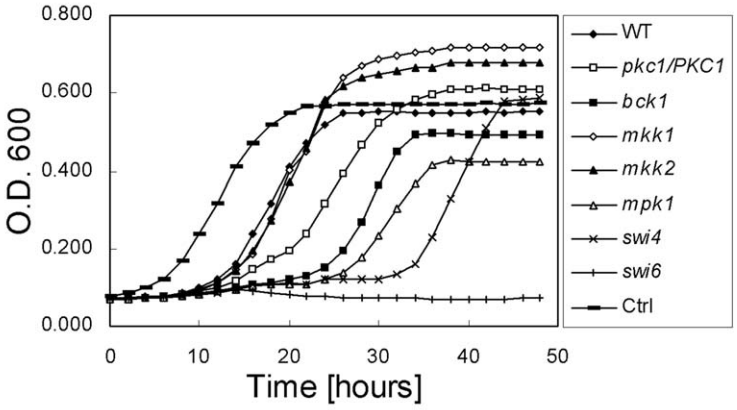

C

Strain	IC_{50} [μM] at 20 h
WT	13 μM
bck1Δ	6 μM
mkk1Δ	13 μM
mkk2Δ	13 μM
mpk1Δ	9 μM
swi4Δ	5 μM
swi6Δ	4 μM
pkc1Δ/PKC1	10 μM

Figure 3. Gene Dosage Effects

(A) Effect of overexpression of Pkc1 and the presence of 1 M sorbitol on yeast growth in the absence and presence of compound **1** (10 μM). Serially diluted stocks of the indicated yeast strains were replicated onto agar plates and incubated for 48 hr.

(B) Growth curves for homozygous (*bck1Δ*, *mkk1Δ*, *mkk2Δ*, *mpk1Δ*, *swi4Δ*, *swi6Δ*) and heterozygous (*pkc1Δ/PKC1*) diploid deletion strains in the presence of **1** (15 μM) and their comparison with treated and nontreated (ctrl) wild-type strain BY4743. All tested strains grew as well as the wild-type strain in the presence of equivalent DMSO concentration (data not shown).

(C) Compound **1** IC_{50} values for the tested yeast strains as determined in liquid medium.

mkk2Δ, *mpk1Δ*, *swi4Δ*, and *swi6Δ*). Unlike the initial screens, this experiment showed significant growth of wild-type strain in the presence of compound **1** already after 20 hr (Figure 3B). The enhanced growth was likely to be caused by several factors. First, the number of seeded cells was significantly higher in this experiment than it was during the screen. Second, yeast cells grew much faster in the liquid medium than on agar. And third, it is possible that compound **1** is a less potent inhibitor of yeast growth under the latter conditions. Nevertheless, there was significant inhibition of wild-type strain growth by **1** at 15 μM concentration (Figure 3B). When treated with compound **1**, strains lacking *SWI4* or *SWI6* (transcription factors downstream of the Pkc1 signaling cascade) [37] exhibited dramatic growth inhibition (Figures 3B and 3C). This observation is in agreement with the fact that *swi4Δ* and *swi6Δ* strains are synthetically lethal with mutations in the PKC1 MAP kinase pathway [38, 39] and strongly supports our proposal that compound **1** inhibits this pathway. Swi6 in combination with Swi4 form the heterodimeric SBF complex, while Swi6 and Mbp1 constitute the MBF complex [40]. Both complexes are involved in the transcriptional program that promotes entry into the cell cycle (START) by activating Cln1,2/Cdc28 (Figure 4). It is noteworthy that the *swi6Δ* strain was the most susceptible to **1** (Figures 3B and 3C), possibly because Swi6 is part of *both* transcription factor complexes. Mutant strains *bck1Δ*, *mpk1Δ*, and *pkc1Δ/PKC1* also displayed increased sensitivity to **1**. In contrast, *mkk1Δ* and *mkk2Δ* mutants were not susceptible to treatment with **1**, which might result from mutual compensation by Mkk1 and Mkk2 [41]. The synthetic interaction between the deletion mutations in the downstream components of PKC1 MAP kinase pathway and compound **1** indicates that the compound-induced phenotype involves additional pathway(s). It is indeed known that Pkc1 also plays a role outside the MAP kinase cascade

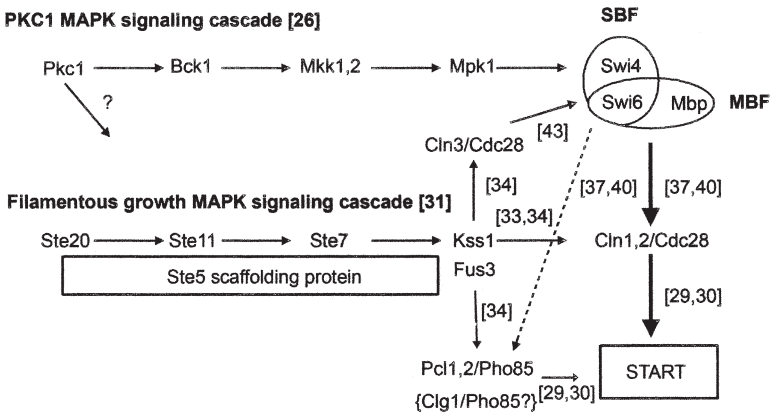

Figure 4. MAPK Signaling Cascades that Appear to Play a Role in Rescuing Growth Arrest Induced by Compound **1** (Simplified Representation)

A *pkc1Δ* mutant exhibits a more severe phenotype than the deletion mutants in the downstream genes and is thought to function in additional pathways (indicated by "?").

signaling, such as in the mobilization of chitin synthase III (Chs3) from the chitosomes to the plasma membrane [42]. Such extended functions of Pkc1 are also reflected in the phenotype of the *pkc1Δ* mutant, which is more severe than that of the downstream mutants of this MAP kinase pathway. Thus, the results of the gene deletion experiments are consistent with inhibition of Pkc1 by compound **1** and Pkc1 being a major cellular target responsible for the observed growth inhibition.

Biochemical Characterization of Mechanism of Action

In order to further characterize the molecular targets of **1**, affinity chromatography was carried out with yeast extracts. Resins derived from **1** (resin **4**) and an inactive analog of **1** (resin **5**; Figure 5A) were synthesized as outlined in Figure 5B. 2-Chloroisonicotinic acid (**6**) was converted to the vinylogous amide **8** in four steps. Compound **8** was then reacted with adduct **10** of 3-chloroaniline (**9**) and cyanamide to afford pyrimidine **11**, at which point the secondary aniline could be methylated to **12**. The linker was introduced by nucleophilic aromatic substitution on the pyridine moiety with ethylenediamine to give **13** and subsequently modified and extended by PEGylation. The affinity resins **4** and **5** were prepared by coupling compound **15** to AffiGel-15 (Bio-Rad, Hercules, CA) via amide bond formation under mildly basic conditions (see Supplemental Data for details). The 2-position of the pyridine ring was chosen as the site of linker attachment based on docking studies of the related pyrimidine CGP60474 (**3**) to the ATP binding hinge domain of the homologous mammalian kinase CDK2 [24]. The inactive resin **5** differs from **4** by the presence of an N-methyl group in the PAP skeleton (Figure 5A), which leads to a substantial loss of activity (IC_{50} > 100 μM for N-methylated **1**). This is consistent with the above docking study of CGP60474 (**3**), which predicts that the NH proton of the PAP-based kinase inhibitor forms a hydrogen bond with the ATP binding domain of the kinase. The affinity resins were incubated with cell extracts and then washed extensively. Proteins retained by the affinity resins were eluted by boiling in SDS, separated by SDS-PAGE, and visualized by silver staining. Several bands on the protein gel appeared to be specific for the nonmethylated resin **4** (Figure 5C). One specific band at 130 kDa was identified to be Pkc1 by immunoblotting with anti-Pkc1 antibody (Figure 5D). Moreover, addition of 100 μM compound **1** to the lysates during affinity chromatography blocked the binding of Pkc1. An in vitro kinase assay with GST-tagged Pkc1 overexpressed in yeast showed that compound **1** directly inhibits the enzyme (IC_{50} 0.8 μM). In this fluorescence quenching-based assay, the observed relative fluorescence intensity is inversely proportional to the extent of phosphorylation (Figure 5E; see Experimental Procedures for details). Furthermore, compound **1** did not inhibit Pkc1 in the presence of excess ATP, indicating that **1** binds competitively in the ATP binding site (data not shown). Consistent with these findings, molecules with a PAP scaffold such as **3** have been reported that bind the mammalian kinase PKC-α, a homolog of Pkc1 (Table 2) [22, 24].

Other specific protein bands from the affinity pull-down may represent additional intracellular targets of compound **1**, as the kinase-directed scaffolds are known to commonly bind multiple kinases [43]. Indeed, Western blot analysis with an anti-Cdc28 antibody suggested that compound **1** also interacts specifically with Cdc28 (Figure 5D). This is not surprising given the structural similarity of **1** to CGP60474 (**3**), which is an inhibitor of CDK1/2, a mammalian ortholog of yeast Cdc28. Inhibition of Cdc28 by compound **1** might also account for the cell cycle-related suppressors of growth defects in the dosage suppression screen because they are closely linked to Cdc28 (Figure 4), as well as for the high susceptibility to **1** of *swi4Δ* and *swi6Δ* deletion strains because Cln3/Cdc28 activates SBF and MBF [44]. Indeed, compound **1** was shown to inhibit a number of mammalian CDKs (Table 2). Even though *CDC28* was not identified as a suppressor in the screen, this might be due to the fact that Cdc28 functions in the complex with cyclins and to increase its in vivo activity would require simultaneous overexpression of both Cdc28 and a cyclin. To examine a possible role of Cdc28 in growth inhibition by compound **1**, we inspected the morphology of compound-treated yeast cells by light microscopy. Also, we quantified fractions of yeast cells in different cell cycle stages after compound treatment by cytometric analysis. Neither experiment revealed a cell cycle-specific arrest, leaving the physiological relevance of interaction be-

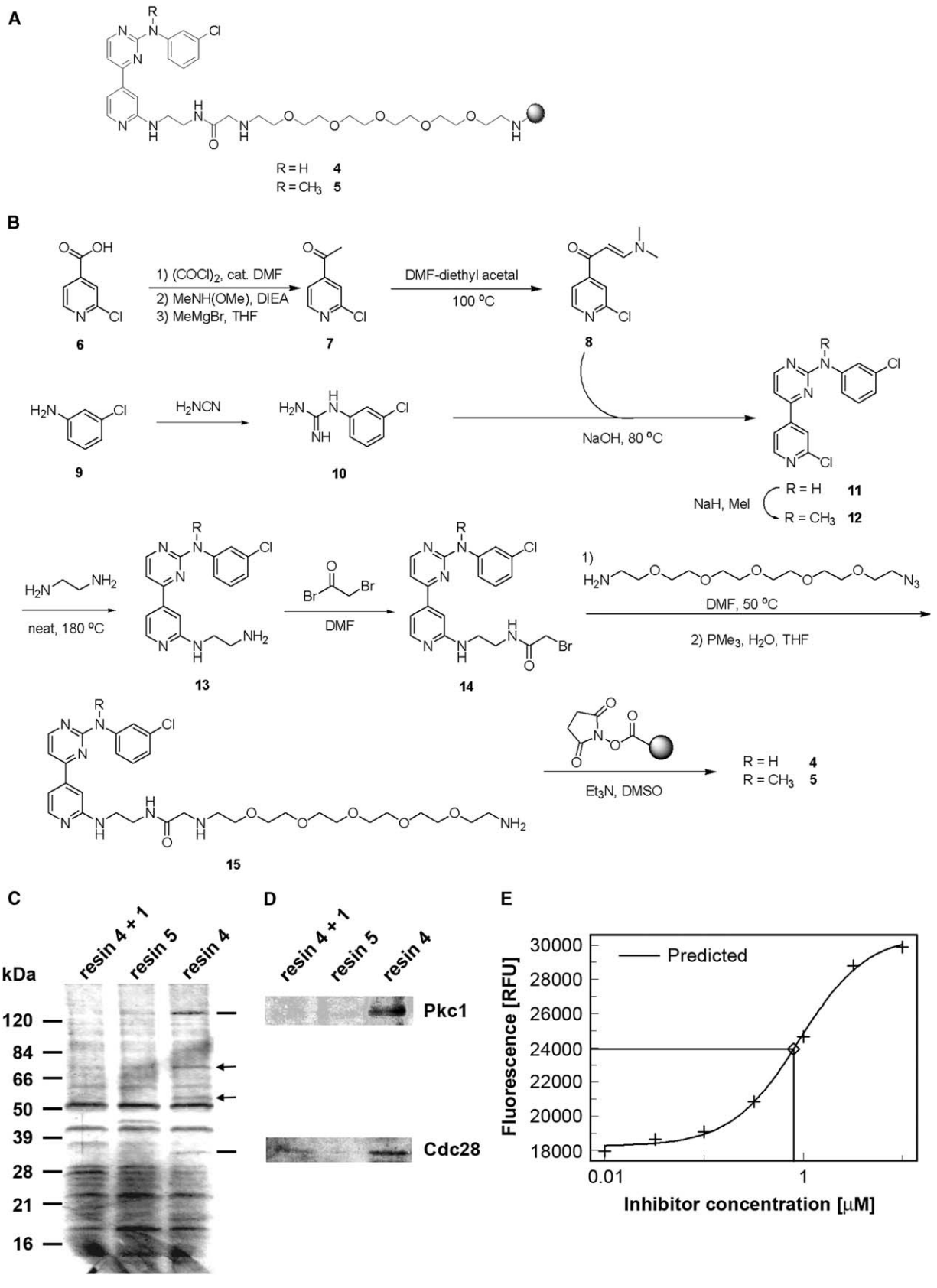

Figure 5. Biochemical Target Validation

(A) Structures of the immobilized ligands used for affinity chromatography.

(B) Synthetic scheme for the affinity resins.

Table 2. Inhibition of Mammalian Kinases In Vitro by Compounds **1** and **3** (IC_{50} Values)

Kinase	Compound **1**	CGP60474 (**3**)
PKC-α	2.2 μM	0.25 μM
CDK1/cycB	0.24 μM	17 nM
CDK2/cycA	n.d.[a]	80 nM
CDK5	0.12 μM	n.d.[a]

Assays were carried out by Novartis Pharma Inc., as described [24].
[a] n.d. = not determined.

tween Cdc28 and compound **1** unclear. Taken together, the results of the overexpression screen are consistent with the affinity chromatography experiment and suggest that the growth-inhibitory effects of compound **1** are due to inhibition of Pkc1 and possibly also Cdc28. In addition, biochemical experiments suggest that compound **1** binds other cellular targets, which may contribute to phenotypes observed in the dosage suppression screens.

Significance

In this study, a genome-wide yeast dosage suppression screen was used to identify the target of a PAP compound with yeast growth-inhibitory activity. The finding of Pkc1 as a target was confirmed by using affinity chromatography and biochemical assays. The high-throughput format of the screen is suitable for a rapid follow-up study of a small-molecule screen, particularly because imaging-based bioinformatics can readily be implemented to facilitate data analysis. Hits from small-molecule screens can putatively be classified according to growth patterns on the agar plates. As with any type of gene dosage-based experiment, the screen selects not only for the drug target but also for other members of the target pathway or for genes that can confer resistance to the cell through different mechanisms, including general stress-response genes, drug efflux pumps, or metabolic genes [45]. However, the array format represents an advantage over classical drug susceptibility screens that employ pooled overexpression strains. This format allows subtraction of nonspecific genes identified previously without the need for repeated sequencing. Another advantage of the screen, especially when compared to screens utilizing deletion strains, is that it can be easily repeated in different genetic backgrounds such as yeast strains sensitized to drugs through mutations in proteins affecting cell permeability, therefore extending its utility to drugs that are ineffective to wild-type yeast. Thus, the overexpression screen is a useful complement to screens exploiting homozygous or heterozygous deletion strains to aid in target identification. Genetic screens of this type may eventually be systematically transferred to mammalian systems to probe more complex pathways.

Experimental Procedures

Yeast Strains, Overexpression Library, and Media

The haploid *S. cerevisiae* strain YPH500 (*MAT***a**, *ura3-52*, *lys2-801*, *ade2-101*, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*) was used in minimal dropout media (–Leu) for both the small-molecule screen and generation of the overexpression library. The library was constructed in YEp13 shuttle vectors (2 μM *LEU2*) and was a gift from Dr. M. Meijer, University Hospital Rotterdam, The Netherlands. YPH500 was transformed with the genomic DNA fragment library using the standard lithium acetate procedure and individual colonies were arrayed into 384-well plates containing minimal medium lacking leucine. The homozygous and heterozygous deletion strains are based on a BY4743 diploid strain background and were obtained from Research Genetics (Invitrogen, Carlsbad, CA). Assays were performed with synthetic complete medium. A yeast clone (Yeast ExClone) containing a plasmid carrying the GST-Pkc1 encoding gene under the control of the Cu^{2+}-inducible CUP1 promoter was obtained from Research Genetics (Invitrogen).

Small-Molecule Screen

In the primary screen, YPH500 cells (1 μl of a saturated culture diluted 1:10) were spotted onto agar containing compounds at concentrations of 20 and 200 μM in a 384-well format. Plates were incubated for 3 days at 30°C and visually inspected for growth. Hits were tested in dilution series to determine the minimal growth-inhibitory concentration.

Overexpression Screen and Sequencing of Clones

Transformed yeast strains were grown on 384-well plates and replicated onto –Leu agar plates containing the inhibitor (1536 strains per plate) as described in the main text. Following 3-day incubation at 30°C, hit colonies were picked, streaked onto –Leu agar plates, and grown at 30°C. Subsequently, strains were grown overnight in 5 ml liquid cultures (–Leu) and yeast plasmid DNA was isolated as follows. Cells were harvested by centrifugation (1000 × g) for 5 min and the pellet was resuspended in 0.5 ml of 1 M sorbitol/0.1 M EDTA (pH 8). Zymolyase (5 μl of 0.5 mg/ml 100T; ICN, Costa Mesa, CA) was then added and the mixture was incubated at 37°C for 1 hr. Cells were centrifuged (1000 × g, 1 min) and plasmid DNA isolated using the Spin Mini Prep kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. *E. coli* KC8 cells were transformed with the isolated yeast DNA (by electroporation) and plated on ampicillin-containing LB plates. Colonies were grown in selective LB medium (ampicillin, 100 μg/ml), plasmid DNA was isolated as above, and inserts were sequenced using primers DL1 (5′-ACTACG CGATCATGGCGA-3′) and DL2 (5′-TGATGCCGGCCACGATGC-3′).

Deletion Strain Experiments

Growth curves of homozygous and heterozygous diploid deletion strains in the presence and absence of **1** (2% DMSO) were re-

(C) Silver-stained protein gel following affinity chromatography. Yeast protein extracts were loaded onto affinity matrices **4** and **5**. After **1** hr of incubation at 4°C under constant agitation and then three washes with bead buffer, bound proteins were extracted by boiling in SDS sample buffer, resolved by SDS-PAGE (4%–20% Tris-glycine), and visualized by silver staining. For the competition assay (left lane), 100 μM compound **1** was present during the incubation and wash steps. Bands corresponding to proteins that bind specifically to resin **4** and competed off by **1** are indicated.
(D) Immunoblot following affinity chromatography. Binding of Pkc1 and Cdc28 to resin **4** was assayed by Western blot analysis using goat anti-Pkc1 and goat anti-Cdc28 polyclonal antibodies, respectively, and a rabbit anti-goat peroxidase-conjugated secondary antibody.
(E) In vitro kinase assay using the IQ PKC Assay kit with the pseudosubstrate peptide substrate (Pierce) and GST-Pkc1 (overexpressed in yeast) in the presence of various concentrations of **1**. Fluorescence at 590 nm was measured (excitation at 560 nm) and indicated the extent of phosphorylation (inverse relationship).

corded using the Bioscreen C (Thermo Labsystems, Franklin, MA). IC_{50} values were determined based on cell growth after 20 hr by monitoring the optical density at 600 nm (OD_{600}). Values given in Figure 3C correspond to concentrations at which the OD_{600} reached half of the maximum.

Affinity Chromatography

Synthesis of the linker derivatives and affinity supports is described in the Supplemental Data. Yeast cells (YPH500) were grown in 1 L of YPDA medium (to OD_{600} 0.8), harvested by centrifugation (1000 × g, 5 min), and washed with 200 ml H_2O and then with 200 ml breakage buffer (30 mM sodium phosphate [pH 7.0], 60 mM β-glycerophosphate, 1 M KCl, 6 mM EDTA, 6 mM EGTA, 6 mM NaF, 10% glycerol). Cells were resuspended in a minimal amount of breakage buffer, protease inhibitors were added (1 mM PMSF, 10 μg/ml each of leupeptin, pepstatin, and chemostatin), and cells were frozen in liquid nitrogen. Frozen cells were ground with mortar and pestle while keeping the mortar cold. The cell powder was scraped into an Eppendorf tube, thawed, and pelleted in a microcentrifuge (14,000 × g, 15 min, 4°C). The supernatant was transferred into a new tube and pelleted again. The supernatant was aliquoted, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined using the BCA protein quantification kit (Pierce, Rockford, IL). The protein extract (400 μg) was diluted into cold bead buffer (50 mM Tris [pH 7.4], 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 100 μM benzamidine) to a total volume of 800 μl and added to 40 μl of affinity support (50% slurry in PBS) that had been prewashed twice with bead buffer. The mixture was incubated at 4°C for 1 hr under constant agitation, and thereafter centrifuged (3000 × g, 1 min, 4°C). The supernatant was discarded and the affinity support was washed three times with cold bead buffer (in the competition assay, the buffer contained 100 μM **1** during incubation and wash steps, final ethanol concentration 0.5%). After the final wash of the resin, 20 μl of SDS sample buffer was added and the mixture was heated at 95°C for 5 min. Samples were loaded onto a 4%–20% Tris-glycine SDS-polyacrylamide gel (Invitrogen). Detection of protein bands was achieved by silver staining using the Silver Stain Plus kit (Bio-Rad). For Western blot analysis, proteins were transferred onto nitrocellulose membrane by electrotransfer, and the membrane was blocked overnight at 4°C with 5% nonfat milk and then incubated with goat anti-Pkc1 (yC-20, 1:400) or goat anti-Cdc28 (yC-20, 1:200) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at room temperature. The membrane was washed three times with PBS/0.05% Tween 20 containing 5% nonfat milk, incubated with rabbit anti-goat peroxidase-conjugated secondary antibody (1:5000; Sigma, St. Louis, MO) for 1 hr at room temperature, washed with PBS/0.05% Tween 20, and developed using the ECL plus Western Blotting Detection system (Amersham Biosciences, Piscataway, NJ).

Protein Overexpression and Purification

The GST-Pkc1 fusion protein was purified from overexpressing *S. cerevisiae* strain grown in 250 ml of −Ura −Leu medium. Protein expression was induced by addition of $CuSO_4$ to a final concentration of 0.5 mM in logarithmic phase (OD_{600} 0.8) for 2 hr. Cells were harvested by centrifugation, resuspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 5 mM DTT, 10% glycerol, 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin and pepstatin), and homogenized by vortexing in the presence of glass beads. The lysis mixture was then pelleted, the supernatant was mixed with 2 ml of equilibrated 50% glutathione bead slurry (Clontech, Palo Alto, CA), and incubated at 4°C for 1 hr. The resin was washed with lysis buffer containing 1% Triton X-100 (3 × 4 ml) and the fusion protein was eluted with buffer containing 20 mM glutathione, 0.5 M NaCl, 50 mM Tris (pH 8), and dialyzed overnight in 20 mM Tris-HCl (pH 8), 0.5 mM EDTA, 1 mM DTT, 55 mM NaCl, 50% glycerol.

Recombinant Protein Assay

The kinase assay was carried out using the IQ PKC Assay kit with the pseudosubstrate peptide substrate (Pierce) in 96-well format as recommended by the manufacturer. The assay mixture contained ~0.5 μg GST-Pkc1, various concentrations of inhibitor **1** in DMSO, 20 mM HEPES [pH 7.4], 1 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mg/ml phosphatidylserine, 60 μM pseudosubstrate, 10 μM ATP. The reaction was stopped after 2 hr by addition of IQ working solution and fluorescence was measured at 590 nm with excitation at 560 nm. No enzyme inhibition was seen at 1 mM ATP.

Supplemental Data

General synthetic methods, selected spectroscopic data for intermediates of affinity resin preparations, and structures of other yeast growth inhibitors are available online at http://www.chembiol.com/cgi/content/full/12/1/55/DC1/.

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