

A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR)

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The target of rapamycin protein (TOR) is a highly conserved ataxia telangiectasia-related protein kinase essential for cell growth. Emerging evidence indicates that TOR signaling is highly complex and is involved in a variety of cellular processes. To understand its general functions, we took a chemical genomics approach to explore the genetic interaction between TOR and other yeast genes on a genomic scale. In this study, the rapamycin sensitivity of individual deletion mutants generated by the *Saccharomyces* Genome Deletion Project was systematically measured. Our results provide a global view of the rapamycin-sensitive functions of TOR. In contrast to conventional genetic analysis, this approach offers a simple and thorough analysis of genetic interaction on a genomic scale and measures genetic interaction at different possible levels. It can be used to study the functions of other drug targets and to identify novel protein components of a conserved core biological process such as DNA damage checkpoint/repair that is interfered with by a cell-permeable chemical compound.

Rapamycin is a macrocyclic immunosuppressive antibiotic. When complexed with its immunophilin receptor FKBP12, rapamycin inhibits the functions of two redundant related proteins, target of rapamycin protein (Tor)1p and Tor2p, in yeast and their homolog FRAP/RAFT/mTOR in mammalian cells (reviewed by refs. 1 and 2). In yeast, mutations at a conserved serine residue, Ser-1972 in Tor1p or Ser-1975 in Tor2p, confer dominant rapamycin resistance (3–6). These mutations occur in the FKBP12-rapamycin-binding domain and disrupt the binding of FKBP12-rapamycin (7–9). These results establish that Tor1p and Tor2p are the physiological targets for rapamycin and demonstrate that they share a redundant function(s) in rapamycin-sensitive growth. The best characterized function of TOR is translational control, which is conserved in both mammalian cells and yeast (reviewed by refs. 1 and 2). However, evidence suggests that TOR signaling is highly complex and is potentially involved in many cellular processes. In yeast *Saccharomyces cerevisiae*, rapamycin treatment results in starvation responses, including G₁ cell cycle arrest, glycogen accumulation (3, 10), autophagocytosis (11), reduced protein synthesis (10, 12), and sporulation (13). Recent studies show that TOR regulates transcription of genes involved in ribosomal biogenesis (14, 15) and nutrient responses in yeast (16–19). Understanding the general roles of TOR is important for dissecting the intracellular signaling network as well as further evaluating rapamycin as a therapeutic medicine.

Genetic analysis of *S. cerevisiae* has contributed much to our knowledge of eukaryotic cell regulation. By using phenotypic readouts such as cell viability and morphological alterations, the genetic interaction between two genes can be examined by mutations in both genes, which has been a powerful tool in yeast to discover and characterize biological pathways. Conditional loss of functions of proteins by chemical inhibitors has greatly facilitated such genetic analyses (reviewed by refs. 20 and 21). The relative sensitivity of yeast mutants to drugs has been widely used in genetically identifying and characterizing a variety of important cellular processes. For example, study of the relative sensitivity of yeast mutants to benomyl led to the identification of microtubule

network components and discovery of the spindle checkpoint (22, 23). In such studies, loss-of-function mutations in a protein acting as an enhancer of the function of the drug target protein causes drug hypersensitivity. Conversely, mutations in an inhibitor of the target lead to relative resistance. Identification of genes whose mutations confer drug-sensitivity phenotypes has led to detailed construction of many biological pathways or processes.

The completion of the *S. cerevisiae* genome sequence has revolutionized research with yeast as a model organism and has led to new tools for analyzing gene expression, protein–protein interaction, and enzymatic activities on a genome-wide basis (24–27). The *Saccharomyces* Genome Deletion Project is an ongoing consortium devoted to generating a complete set of yeast mutant strains with each individual ORF being completely deleted (28). Here we describe a genome-wide analysis of genetic interactions of TOR based on the systematically generated *Saccharomyces* deletion mutants. We explored the interactions of TOR with other yeast genes by measuring the relative sensitivity of the yeast deletion mutants to rapamycin and constructed a genome-wide genetic linkage map for TOR. Our results provide a global view of the possible global functions of TOR.

Materials and Methods

Sources and Handling of Yeast Deletion Mutants. Haploid *MATa* nonessential yeast deletion strains were purchased from Research Genetics (Huntsville, AL). The collection of *MATa* and *MATa/α* heterozygous deletion strains of chromosome VIII was a gift from Mark Johnston (Washington University, St. Louis). The deletion strains were generated by replacing the target gene with a kanamycin resistance cassette, KanMX4 (28). The *MATa* and *MATa/α* deletion strains were generated from parent strains BY4741 and BY4743, respectively (28). Throughout the primary screen, all deletion strains were identified by their internal code provided by Research Genetics and Mark Johnston. The identity of the corresponding genes was retrieved only after the screening process was complete. All strains were tested for resistance to 200 mg/liter Geneticin (G418 sulfate) purchased from Life Technologies (Rockville, MD) at 30°C before the rapamycin-sensitivity assay.

Scores of Previously Known Rapamycin-Hypersensitive (RH) and -Resistant (RR) Mutants. Overnight yeast extract/peptone/glucose (YPD) cultures containing wild-type strain BY4741, RR strain *TOR1^{RR}* expressing Tor1p(S1972I), and deletion strains *gln3Δ*,

Abbreviations: YPD, yeast extract/peptone/glucose; RR, rapamycin resistant; RH, rapamycin hypersensitive.

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rrd2Δ, *bmh2Δ*, *ure2Δ*, and *tor1Δ* were serially diluted at 10-fold increments and spotted onto YPD +/- 25 nM rapamycin. All deletion strains exhibited comparable growth rate with wild type on YPD. After the deletion strains were grown at 30°C for 4 days on YPD with rapamycin, scores were assigned to them, with “-” representing hypersensitivity and “+” representing resistance when compared with wild type (which has a score of 0). Each “-” or “+” represents approximately a decrease or an increase in rapamycin resistance by an order of magnitude. *gln3Δ* has a score of “+++.” Therefore, according to this scale, deletion of *GLN3* confers rapamycin resistance by ≈1,000-fold when compared with wild type.

Primary Screens. At the initial phase of screening, all deletion strains were streaked onto YPD +/- 25 nM rapamycin with the wild-type BY4741 or BY4743 (where appropriate) as the control. Plates were incubated at 30°C for 4 days. A strain was marked as RH or RR only if it showed marked difference in growth when compared with wild type on YPD plates with rapamycin but not on YPD plates alone. The second phase involves assigning scores to putative RH and RR strains. Overnight cultures were serially diluted at 10-fold increments and spotted onto YPD +/- 25 nM rapamycin. These plates were incubated at 30°C for 4 days. In this study, we focused on strains with a score of “++” or “--” or greater. Therefore, only those more hypersensitive to rapamycin compared with *bmh2Δ* (which has a score of “-”) and strains that are equally or more resistant to rapamycin compared with *rrd2Δ* (which has a score of “+++”) were retained. A small number of deletion strains show slow growth on YPD plates. Scores for these mutants are calculated by subtracting the differences between wild type and the slow-growing mutant on YPD +/- rapamycin to reflect real rapamycin sensitivity.

Confirmation of TOR Dependency and Specificity. All RH strains were transformed with plasmid pYDF80 expressing RR Tor1p(S1972I) or control plasmid pYDF81 harboring wild-type *TOR1*. Transformants were first selected on synthetic defined Leu- agar plates and then replica plated onto YPD +/- 25 nM rapamycin. Plates were incubated at 30°C for 4 days. If the RH strains are truly hypersensitive to rapamycin because of inhibition of TOR, Tor1(S1972I) should revert its hypersensitive phenotype.

Rapamycin binds to its intracellular receptor FKBP12 and inhibits TOR. Because FKBP12 is involved in other biological processes, to eliminate the possibility that the RH or RR phenotype is merely a side effect of FKBP12 depletion, all RH and RR strains were streaked onto YPD +/- 25 nM FK506 and incubated at 30°C for 4 days. Strains showing hypersensitivity in the presence of FK506 were eliminated. To test whether the RH or RR phenotype is only a secondary effect because of (i) inhibition of protein synthesis by rapamycin, or (ii) activation of general multidrug resistance response by rapamycin treatment, strains were streaked onto YPD +/- 100 nM cycloheximide, incubated at 30°C for 4 days, and scored for sensitivity.

Identification of Mutants and Functional Group Classification. The identity of both RH and RR strains was uncovered at this stage. The reported or predicted functions of these RH and RR genes are retrieved from the Yeast Proteome Database (<http://www.proteome.com/databases/index.html>) (29). Genes reported or predicted to be functioning in the same biological process are classified into the same functional group. In the end, there are eight functional groups (groups I to VIII) together with group IX, which consists of genes that cannot be classified or genes with no known function.

Results

We have initiated a large-scale genome-wide genetic interaction study of TOR by measuring the relative sensitivity of individual

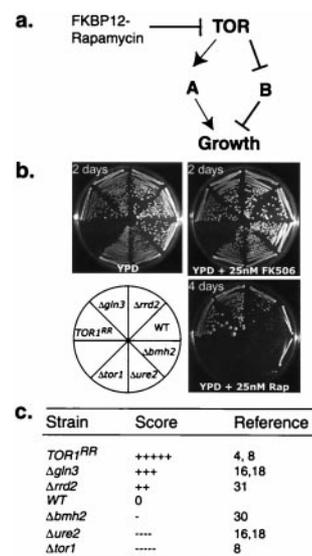


Fig. 1. Rapamycin-sensitivity assay for yeast deletion strains. (a) A simplified model for genetic interaction between TOR and other components in the rapamycin-sensitive pathways. (b) The rapamycin-sensitivity assay for yeast deletion mutants of genes known to play a role in rapamycin-sensitive signaling. Wild type yeast (WT), RR strain (*TOR1^{RR}*), expressing Tor1p(S1972I) or strains with deletion of *GLN3*, *RRD2*, *BMH2*, *URE2*, or *TOR1* were streaked onto YPD, YPD+25 nM rapamycin, or YPD+25 nM FK506 plates and incubated at 30°C. (c) Scores for rapamycin-sensitivity for strains in b.

yeast deletion mutants to rapamycin. Deletion of an inhibitor in TOR signaling or a gene in a cellular process negatively regulated by TOR is predicted to confer partial rapamycin resistance. In contrast, deletion of an enhancer in TOR signaling or a gene in a cellular process positively regulated by TOR renders rapamycin hypersensitivity (Fig. 1a). Such examples of rapamycin-sensitivity phenotypes have been described in several studies as *bmh1*, *bmh2*, *gln3*, and *ure2*, important players in TOR signaling (16, 18, 30). For simplicity, we call this genome-wide genetic analysis with chemical inhibitors a chemical genomics approach. Because cell growth is used here as a readout for our screen, nonessential genes can be tested with their haploid or homozygous diploid deletion strains, whereas essential genes can be examined with their heterozygous diploid deletion strains. The rapamycin sensitivity of each mutant was scored on the basis of its relative growth to wild-type, RR *TOR1(S1972I)* (*TOR1^{RR}*) and *tor1Δ* strains in the absence and presence of rapamycin. We chose “-” or “+” to describe the relative rapamycin sensitivity. The majority of our mutants did not exhibit growth defects in the absence of rapamycin. The scores of the few strains showing slow growth were normalized against the wild-type strain in the absence or presence of rapamycin. By a series of dilution experiments, we quantitatively estimated that each “-” or “+” represents approximately an increase/decrease in drug sensitivity by one order of magnitude. We chose to further analyze only the mutants scored with at least “--” or “+++” (100-fold change in rapamycin sensitivity). Furthermore, the majority of our positive mutants (>95%) do not exhibit slow growth under normal conditions. Thus, the heightened drug sensitivity or resistance of our positive mutants reflects their genetic interactions with TOR rather than an additive effect of the relevant mutation and rapamycin on cell growth.

We have thus far focused on the nonessential haploid mutants and screened 2,216 nonessential deletion mutants. These mutants represent more than half of the total nonessential genes in yeast (28). We have also carried out a small-scale screen with 50 heterozygous essential diploid deletion strains derived from genes on chromosome VIII. It is important to note that genes known to

Vacuolar Biogenesis, Functioning, and Protein Targeting. Functional group VI (vacuolar function) has 11 genes involved in protein sorting from the Golgi to the vacuole, vacuolar protein targeting, vacuolar biogenesis, and functioning. This is in contrast to the role of the vacuole in autophagocytosis, which is negatively regulated by TOR (11, 37). Autophagocytosis is a process that generates internal supplies of small molecule nutrients by degrading cytosolic organelles, proteins, and ribosomes during carbon and nitrogen starvation (38). Because all these vacuolar mutants were hypersensitive to rapamycin, their corresponding genes are positively involved in TOR-dependent processes (Table 3). Thus, TOR appears to be involved in both positive and negative aspects of vacuolar functions. The yeast vacuole is an important compartment for storage of nutrients and other small molecules and for maintaining homeostasis between biosynthesis and degradation (reviewed in ref. 39). Regulation of vacuolar functions by TOR may also play a role in its ability to regulate cellular responses to nutrient availability.

Ubiquitin-Dependent Proteolysis. Functional group VII (ubiquitin-dependent proteolysis) contains three genes involved in the ubiquitin-dependent proteolysis pathway: two proteasomal subunits (Pre9p and Rpn1p) (reviewed in refs. 40 and 41) and one subunit (Cdc23p) of the anaphase-promoting complex or cyclosome (reviewed in ref. 42). Mutations in this group resulted in hypersensitivity to rapamycin, suggesting that the ubiquitin-dependent degradation apparatus is required for a growth-promoting function of TOR. In T lymphocytes, the degradation of the cyclin-dependent kinase inhibitor p27Kip1 is mediated by ubiquitin-dependent proteolysis, which is inhibited by rapamycin (43). Therefore, ubiquitin-dependent proteolysis appears to be important to a conserved function of TOR.

Microtubule-Related Functions. Functional group VIII (spindle function/stability) has six genes required for mitotic spindle assembly and stability. Consistent with this finding, we recently showed that both Tor1p and Tor2p interact with Bik1p, a microtubule-associated protein (44). Rapamycin treatment rapidly causes microtubule instability and defects in microtubule-related functions, such as spindle elongation and orientation, chromosomal segregation, nuclear migration, and karyogamy (44). Rapamycin treatment also leads to chromosomal missegregation in both mammalian cells and yeast (44, 45). Chromosomal instability (CIN) is an important contributing factor to human cancer (reviewed in ref. 46). These observations reveal that CIN may be a severe side effect for rapamycin and may therefore limit its future clinical application as an immunosuppressive medicine. In that sense, this type of approach also offers valuable information regarding the potential side effects of therapeutic drugs.

Discussion

We have taken a chemical genomics approach to conduct a large-scale genetic interaction analysis by using systematically generated yeast deletion strains for rapamycin sensitivity. Thus far, we have identified 106 mutants displaying rapamycin-sensitivity phenotypes of more than 2,266 yeast genes or over one-third of total yeast genes. The majority of the RH and RR genes have known functions and cluster into eight functional groups: protein synthesis, carbon and nitrogen catabolite repression, metabolic biosyntheses, mitochondria biogenesis and functioning, general transcription, vacuolar biogenesis and functioning, ubiquitin-dependent proteolysis, and spindle stability and functioning (Table 3). These results provide a global view of the potential functions of TOR. In addition, RH and RR genes may provide important links to future mechanistic studies of the roles of TOR as well as these genes in various intracellular processes.

Table 1. Rapamycin sensitivity of the cyclin deletion mutants

ORF	Gene name	Score
G1/S-specific cyclins		
YMR199W	CLN1	0
YPL256C	CLN2	0
YAL040C	CLN3	0
G2/M-specific cyclins		
YGR108W	CLB1	0
YPR119W	CLB2	0
YLR210W	CLB4	0
YPR120C	CLB5	--
YGR109C	CLB6	0
G1/S-specific cyclins; Pho85p associated		
YNL289W	PCL1	0
YIL050W	PCL7	0
YPL219W	PCL8	0
Other Pho85p associated cyclins		
YGL215W	CLG1	0
YOL001W	PHO80	0

Many genes of important cellular functions are notably absent from the TOR functional groups. For instance, among 13 cyclin mutants screened, including six G1 cyclins and five *Clbs*, only the *clb5Δ* strain exhibited slight hypersensitivity to rapamycin (--) (Table 1). Because Clb5p is redundant with other Clbs in regulating the Cdc28p kinase during mitosis (reviewed in ref. 47), the rapamycin-sensitivity by *clb5Δ* must attribute to a unique function of Clb5p. One such plausible function is its role in mitotic spindle stability (48). Cln3p has been suggested to be an important mediator of TOR signaling (10). Surprisingly, however, none of the three *cln* mutants (*cln1Δ*, *cln2Δ*, and *cln3Δ*) exhibited any significantly heightened sensitivity to rapamycin. Within each individual rapamycin-sensitive functional group, only highly selective genes were scored in our screen. Only five ribosomal protein genes were isolated among mutants including 45 ribosomal proteins and six translation initiation and elongation factors (Table 2). Thus, this approach is specific and appears to reveal genes that may play important roles in the pathway/process involving TOR. Therefore, further study of the RH and RR mutants in each functional group should give mechanistic insights into the functions of TOR as well as their own unique roles. These results also indicate that this approach is specific and does not randomly identify any gene important for cell growth.

Like any methodology, this approach has its limitations. Tor1p and Tor2p share the redundant function(s) sensitive to rapamycin (6, 8). Tor2p also has a rapamycin-insensitive unique essential function necessary for a normal actin cytoskeleton (8, 49). Because this approach is based on rapamycin sensitivity, it will not uncover the rapamycin-insensitive functions of Tor1p and/or Tor2p. In agreement with previous findings, deletion of *ROM1* and *ROM2*, two important effectors in the rapamycin-insensitive unique function of Tor2p (49) did not affect rapamycin sensitivity (data not shown). In addition, mutations in certain genes functioning in mitochondria and vacuoles are known to have pleiotropic effects on drug sensitivity. Although TOR is linked to the functions of these organelles (11, 17), caution must be taken in interpreting the phenotypes of mutations associated with these organelles.

Traditional genetic screens contribute much to our current knowledge of the molecular control of life. However, many limitations exist in present genetic approaches. In a traditional yeast genetic screen, a collection of mutants needs to be generated by mutagenesis, which tends to be biased and incomplete. Many genes may be missed as a result of their weak phenotypes

Table 2. Rapamycin sensitivity of the deletion of protein synthesis genes

ORF	Gene name	Score	ORF	Gene name	Score
Translation initiation factors					
YJL138C	TIF2	0	YMR012W	CLU1	0
Translation elongation factors					
YDR385W	EFT2	0	YLR289W	GUF1	0
YKL081W	TEF4	0	YOR133W	EFT1	0
Ribosomal proteins					
YPL220W	RPL1A	0	YGR214W	RPS0A	0
YPL198W	RPL7B	0	YLR048W	RPS0B	0
YLL045C	RPL8B	0	YML063W	RPS1B	0
YGR085C	RPL11B	0	YBR181C	RPS6B	0
YEL054C	RPL12A	0	YOR293W	RPS10A	0
YDR418W	RPL12B	---	YMR230W	RPS10B	0
YKL006W	RPL14A	0	YOR369C	RPS12	0
YNL301C	RPL18B	0	YJL191W	RPS14B	0
YMR242C	RPL20A	0	YMR143W	RPS16A	0
YOR312C	RPL20B	0	YML024W	RPS17A	0
YLR061W	RPL22A	0	YML026C	RPS18B	0
YGR148C	RPL24B	0	YNL302C	RPS19B	0
YGR034W	RPL26B	+	YHL015W	RPS20	---
YER056C-A	RPL34A	0	YJL190C	RPS22A	0
YMR194W	RPL36A	0	YGR118W	RPS23A	0
YLR185W	RPL37A	0	YPR132W	RPS23B	0
YJL189W	RPL39	0	YER074W	RPS24A	0
YOL039W	RPP2A	0	YGR027C	RPS25A	0
YDR382W	RPP2B	--	YKL156W	RPS27A	0
YDR115W		0	YHR021C	RPS27B	---
YDR116C		0	YOR167C	RPS28A	0
			YLR264W	RPS28B	0
			YLR287C-A	RPS30A	0
			YOR182C	RPS30B	0

or partial inactivation. Furthermore, activating or gain-of-function mutations often complicate the screens. Once the mutants are isolated, complicated crosses have to be performed to eliminate dominant mutations and mutations on the same loci. Finally, a plasmid library must be transformed into each mutant strain for complementation to identify the relevant gene. Even screening a portion of the yeast genome often takes considerable effort. A recent new technology uses transposon insertion mutagenesis (50) to improve the efficiency of identifying mutated genes. Our approach takes advantage of the available genomics resources and offers a simple, thorough, and relatively unbiased approach at a genomic scale. Each deletion mutant contains a complete deletion of the entire ORF and is well defined. Because each deletion strain is bar coded, the procedure can be readily automated for high-throughput screens (28, 51, 52). This approach measures genetic interactions at different possible levels,

weak or strong. It tests the interaction of all genes in *S. cerevisiae*. With emerging new drug design and screening technologies, many important conserved proteins have or will have their specific chemical inhibitors. This approach should find increasing application in the understanding of important regulatory proteins and the cellular regulatory network. In addition, it can be used to evaluate the global effects of a therapeutic drug on cell growth and functioning and to provide valuable information regarding the potential side effects and additional benefits of the drug.

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