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Growing Yeast for Fun and Profit: Use of *Saccharomyces cerevisiae* as a Model System in Drug Discovery

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Yeast has great utility as a surrogate system to study aspects of mammalian biology. This utility extends to the drug discovery process, where yeast has been used to reveal the mechanism of action of compounds, to discover and characterize components of signaling pathways and to dissect protein function. These applications of yeast are illustrated by examples of research published by major pharmaceutical companies.

2.1 Introduction

This chapter is intended to illustrate the use of yeast (*Saccharomyces cerevisiae*) as a model organism in drug discovery research. Yeast has had a long utility as the workhorse of pharmaceutical discovery research, whether as a representative of its pathogenic cousins or as a living eukaryotic vessel for bringing together reagents such as the two-hybrid system components or carrying reporter constructs for screening. However, I will confine this review to applications where yeast has been used as a true 'model' for vertebrate biology in the area of disease. To demonstrate the value of yeast in applied

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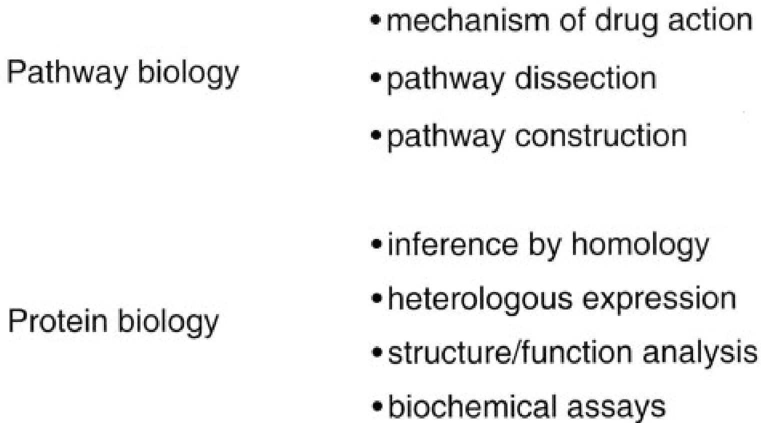

Figure 2.1 Outline of the areas in which yeast has been used as a model system for the biology of higher eukaryotes. Pharmaceutical research in these areas is described in the text

pharmaceutical research, my examples and citations are almost completely culled from publications by research scientists at major pharmaceutical companies (i.e. roughly the top 20 companies based on the market share). This approach results in the omission of many fine pieces of academic work that may have had publication priority, but the aim of this chapter is to demonstrate the type of yeast research that drug discovery organizations historically have regarded as worthwhile, informative and likely to affect their bottom line. Unfortunately this approach also unwittingly leads to the omission of much excellent biomedical research using the fission yeast *Schizosaccharomyces pombe*, because few examples of its application have been published by pharmaceutical companies. The uses of yeast described in this chapter are laid out in Figure 2.1; they include sections on the use of yeast in elucidating pathways and their components, including pathways that are not native to yeast and pathways involved in the mechanism of action of compounds. I will also describe more targeted experiments to characterize the functions of specific proteins. Finally, I will review the 'post-genome' tools, technologies and information resource advances that now enable yeast research.

2.2 *Saccharomyces cerevisiae* and its genome: a brief primer

Commonly known as baker's, brewer's or budding yeast, *S. cerevisiae* has been a standard laboratory microorganism since the 1950s. It has many endearing attributes, including the ability to fill a laboratory with a pleasant 'warm-bread' odor, yet also to survive years of abandonment in a fridge or

freezer or even on a desiccated piece of agar in a forgotten petri dish. (Almost every yeast biologist has had the need to test this last assertion.) It is cheap to feed, non-pathogenic and divides every 2 h. It can grow either aerobically or anaerobically, depending on the nutrients provided, and in solid or liquid media. It can exist stably as a haploid or a diploid, and haploids can be mated and put through meiosis to recover haploid progeny in a matter of days. Although a unicellular organism, it can on occasion display such group characteristics as pseudohyphal growth, intercellular signaling and programmed cell death. Finally, a highly versatile transformation (transfection) system has been available for several decades. You can choose a vector that is linear, circular or integrating, high or low copy number, with a positive or negative selection system, and you can express your favorite gene from several types of regulated promoters. In addition, homologous recombination occurs with high efficiency, allowing the integration of transformed DNA into chromosomes at precise locations, replacing and deleting host DNA as desired.

The *S. cerevisiae* genome sequence was completed and almost entirely annotated for genes in 1996 (Goffeau *et al.*, 1996) but it has not remained static (Kumar *et al.*, 2002c). By comparison to most eukaryotes, coding regions are enviably simple to identify in yeast: about 70% of the genome encodes protein, and only about 4% of yeast genes contain introns, usually as a small insertion very near the 5′ end of the coding region. For expedience, the primary annotaters of the genome set the ability to encode a 100-amino-acid protein as the cutoff for a gene (unless other evidence existed). Each resulting open reading frame (ORF) was given a unique and informative seven-character identifier, e.g. YOR107w. This name immediately tells a yeast biologist that the gene lies on the Watson strand of the right arm of chromosome XV, 107 genes distal from the centromere. Unlike the Dewey decimal system, this left no room for additions; fortunately there have been relatively few subsequent modifications of genes because these have had to be dealt with by inelegant suffixes (A, -B, etc.; inconsistencies in their syntax are a common source of error in data handling). This systematic name complements and conforms to the yeast genetic nomenclature adopted by consensus in the 1960s, in which the upper case notation informs us that a wild-type gene is being discussed, a lower case notation would indicate a mutant and Yor107w is the name of the encoded protein. All yeast genes thus have a systematic ORF name; about half of them also have one or more traditional three-letter gene names that are intended to reflect some property of interest, e.g. *RAD1* to identify the first gene identified from a mutant screen for radiation sensitivity. Yeast biologists have concluded that clarity in the literature is more important than their egos and nowadays they commonly agree on a single, rational primary gene name maintained in a central registry. These names are certainly duller than those for *Drosophila* – yeast never had *ether-a-gogo* but for over a

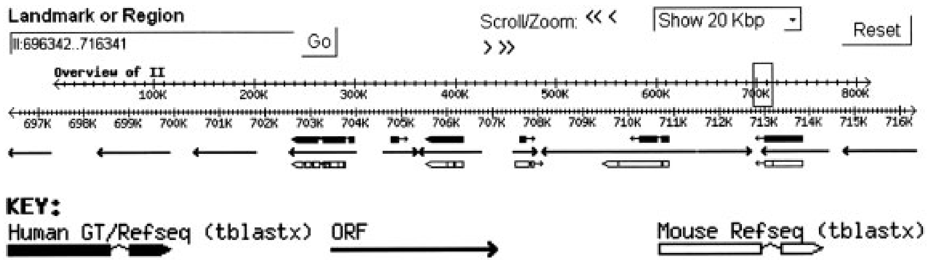

Figure 2.2 A graphical view of a 20-kilobase region of yeast chromosome II, showing 11 open reading frames (ORFs) encoding proteins. The NCBI Reference Sequence project (RefSeq) clones from human and mouse that show significant homology at the protein level are overlaid on their yeast homologs. The view was generated using the browser created by the Generic Model Organism Database Project (www.gmod.org) following customization by Dr N. Siemers

decade it did have *WHI1* (whiskey1, named in a pub in Scotland) until the title's overturn by the more prosaic name *CLN3* (cyclin3).

The number of recognized genes in yeast hovers just above 6000, remaining in flux due to continued research on which of these are spurious and what additions should be made (see Kumar *et al.*, 2002c). Figure 2.2 provides a visual snapshot of a region of the yeast genome and illustrates the significant homology between some of the proteins coded therein and proteins from the mouse and human genomes. Unfortunately, no quantitative cross-comparison between yeast and human genomes has been published since 'completion' of the human genome sequence. An analysis performed in 1997 found that about one-third of yeast proteins had significant homology to a mammalian GenBank sequence (Botstein *et al.*, 1997); by 1997 the results from Bassett *et al.* (1996) had been updated to suggest the existence of yeast homologs for 34% of the 84 disease-related human genes that were positionally cloned at the time. In 1998 a very stringent comparison between yeast and the newly finished *Caenorhabditis elegans* genome (Chervitz *et al.*, 1998) predicted that about 40% of yeast proteins were orthologous to about 20% of those encoded in worm. Many of the remaining 80% of worm proteins contained domains also present in yeast, but their arrangement within proteins was not identical. Because 80% of *C. elegans* proteins apparently lack a close relative in yeast, it might seem that there is a low probability of a given gene from a multicellular organism having a yeast homolog that can be studied productively. However, these numbers are skewed by the 'bulking out' of the *C. elegans* proteome by gene duplication events that lead to huge multigene families such as that for the nuclear hormone receptors. Within core metabolic and structural functions there is virtually complete conservation across eukaryotes. A recent comparison between the predicted proteins of the *S. pombe* and *S. cerevisiae*

genomes and 289 human disease proteins found 182 *S. cerevisiae* proteins with significant similarity with about 50 probable orthologs (Wood *et al*., 2002). The shared proteins covered a range of human disease areas from neurological to metabolic, the largest group being those implicated in cancer. Also, in many situations where a more intensive analysis has been brought to bear, proteins previously cited as absent from yeast have been found. A recent example is the identification of a caspase-type protein in yeast (Uren *et al*., 2000) and demonstration of its orthology to metazoan caspases (Madeo *et al*., 2002).

2.3 Yeast in pathway and mechanism elucidation

Selection of appropriate targets remains a major hurdle in drug discovery. When a biological pathway is of interest for therapeutic intervention, a broad understanding of its components is essential to allow the design of assays that can address both desired and undesired effects of that intervention. Knowledge of pathway biology is at its most advanced in yeast, owing to the ground cleared by decades of academic yeast research. Observations of cell cycle mutants of *S. pombe* and *S. cerevisiae* in the 1970s led directly to identification of the same pathway in humans and to the first generation of cyclin-dependent kinase (CDK) inhibitors currently in the clinic (Senderowicz, 2000). Yet examples of the use of yeast by pharmaceutical companies in further dissection of this pathway are rare, although Novartis has reported a yeast system to screen for Cdk4-specific antagonists (Moorthamer *et al*., 1998). It seems that translation of observations in yeast to the relevance in mammalian systems and into pharmaceutical application continues to be underutilized. Mammalian biologists often feel that yeast is too simple to be of relevance to the process they study, or they point to incongruities in data to insist that yeast 'does it differently'.

Such reservations are partly justified: there are many examples of mammalian target proteins or drug effector mechanisms that are simply not present in yeast. For example, components of the cholesterol biosynthesis pathway, including the target for basic biochemical inhibitory action of the statin drugs, are largely conserved from yeast to humans. Yeast was used extensively by companies such as Bristol-Myers Squibb (Robinson *et al*., 1993) and Zeneca (Summers *et al*., 1993) in the identification and characterization of targets within this pathway. However, statins exert the majority of their cholesterol-lowering effect in humans by a feedback mechanism that leads to upregulation of the hepatic low-density lipoprotein (LDL) receptor, and this protein is not conserved in yeast (although feedback mechanisms responding to lowered sterol level do exist). Yeast also has no nuclear hormone receptors and thus lacks a form of regulation that overlays many conserved metabolic pathways in higher eukaryotes. Conversely,

examples also exist of cases where yeast has proved to contain the target for a drug, even though that drug has its therapeutic effect in a process such as immunity, which has no apparent parallel in yeast. There are also cases where a very clear conservation exists and yet the published work is almost exclusively academic, e.g. the use of yeast in the determination of the mechanism of action of the topoisomerase inhibitors (reviewed by Bjornsti *et al*., 1994). A search of the literature on camptothecin produces only one example of the use of yeast by industry: Takeda laboratories used *S. pombe* to demonstrate that the mechanism of a novel topoisomerase I inhibitor differs from that of camptothecin (Horiguchi and Tanida, 1995).

2.4 An example of mechanism elucidation: immunosuppressive agents

Three sterling examples of how yeast can contribute to the identification of a drug target and characterization of the responding pathway are provided by the immunosuppressive agents cyclosporin A, FK506 and rapamycin. The story of this research is also the story of what would have been an overwhelmingly difficult mechanism of action study without yeast, because it is a case where compounds interact with structurally unrelated binding partners to affect the same target and, conversely, compounds interact with the same binding partner to affect different targets (see Figure 2.3). The mechanism runs contrary to established wisdom on the feasibility of modulating protein–protein interactions. Finally, the binding partners are not the therapeutic target but, to throw in a couple of red herrings, they do have a common enzymatic activity that is inhibited by the compound! Without academic and industry groups striving neck and neck for the answer, and without yeast to identify additional components and provide genetic dissection and stringent hypothesis-testing, determination of their mechanisms within a decade of research is extremely unlikely to have occurred. Ironically, the ultimate targets are a kinase and a phosphatase, and today no right-thinking pharmaceutical company would put any money into a compound that took such a convoluted path to reach these targets. But these compounds were clinical successes before their mechanisms were established, and their efficacy has yet to be matched by small molecules from a rational development process. Cyclosporin A was identified in the 1970s at Sandoz (now Novartis) and approved for use as a transplant rejection therapeutic in 1983. As an interesting footnote, Novartis's own web page states that the initial observations on the natural product indicated a very weak compound that was regarded as being of little practical value. Fortunately an intellectual curiosity prevailed and allowed work to continue until Dr Jean Francois

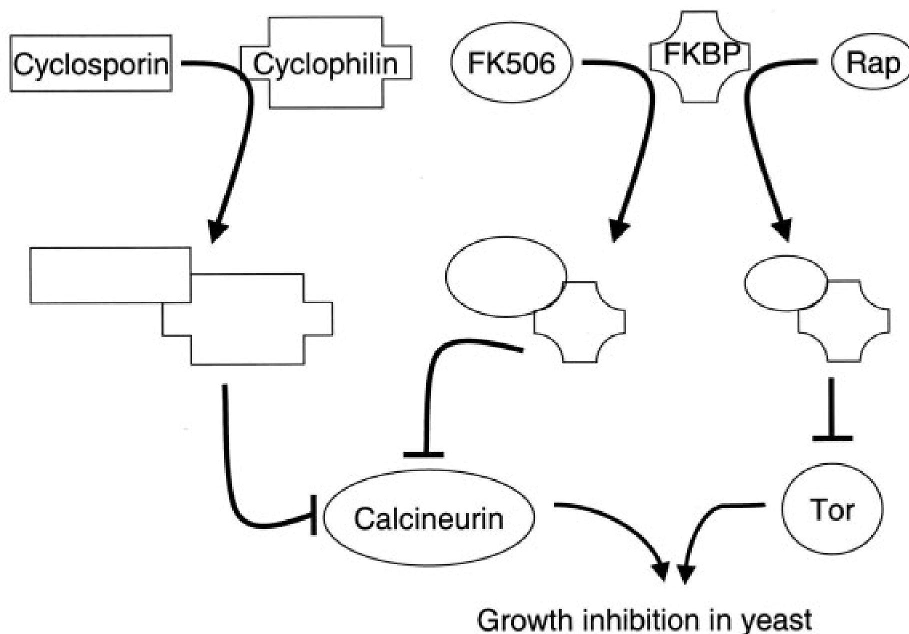

Figure 2.3 Binding partners and mechanism of action of cyclosporin A, FK506 and rapamycin (Rap). Cyclosporin A binds the cyclophilins, which are members of a family of proteins with peptidyl–prolyl isomerase activity. Both FK506 and rapamycin bind the same targets – a family of FK506-binding proteins (FKBPs). The FKBPs are members of a class of peptidyl–prolyl isomerases that are structurally unrelated to the cyclophilins. The cyclosporin A–cyclophilin and FK506–FKBP complexes both inhibit the protein phosphatase calcineurin. The rapamycin–FKBP complex inhibits the Tor kinases

Borel's team discovered the selective T-cell effects and purified the compound that became Sandimmune and Neoral, both long-running blockbusters for Novartis. Tacrolimus (FK506), marketed by Fujisawa as Prograf, was discovered in 1984 and gained FDA approval in 1994, whereas the related macrolide rapamycin (sirolimus), marketed by Wyeth as Rapamune, was discovered in 1975 and approved in 1999.

Far flung in origin, produced by fungi in the soil of Norway or bacteria from the shores of Easter Island or the Tsukuba region of Japan, all these immunosuppressive agents selectively block T-cell activation, with FK506 and cyclosporin A acting to block the transcription of early activation genes, and rapamycin blocking downstream events. However, they had begun their pharmaceutical careers as antibiotics, and scientists in academia (most notably the groups of Michael Hall and Joseph Heitman) and in industry applied yeast to understanding their mechanism and a search for the molecular target. The contributions of many scientists to this work are covered in a comprehensive review by Cardenas *et al.* (1994).

By 1990, cyclosporin A had been determined biochemically to bind and inhibit a target protein named cyclophilin that had been purified also as a peptidyl–prolyl *cis–trans* isomerase (PPIase). Academic work had shown that cyclophilin existed in yeast, and that CsA resistance in yeast correlated with the loss of cyclophylin interaction (Tropschug *et al.*, 1989). Yeast contributed to extensive structure/activity investigations of cyclosporin A at Sandoz (Baumann *et al.*, 1992). In 1990, Merck scientists reported that FK506 also bound and inhibited a protein that had PPIase activity. This protein (FKBP12) was from a novel class of PPIases. It was not lymphoid-specific and it was conserved from yeast to humans (Siekierka *et al.*, 1990). Because FK506 and CsA each inhibited the PPI activity of their binding partner, these 'immunophilins' were obvious candidates for the biological effector in mammals and for the lethality observed in yeast. Yet cloning and disruption of the yeast gene for the protein FKBP, *FKB1* (now *FPR1*), revealed that it was non-essential (Wiederrecht *et al.*, 1991). Scientists from SmithKline Beecham identified a second yeast cyclosporin-A-binding protein, Cyp2 (Koser *et al.*, 1990), and then a third (McLaughlin *et al.*, 1992), suggesting that a protein family was also targeted in humans. Although both Cyp1 and Cyp2 had PPI activity that could be inhibited by cyclosporin A, a triple deletion (*cyp1 cyp2 fpr1*) was viable. Although the existence of further PPI proteins giving functional redundancy was possible, these strains had very little PPIase activity, thus separating PPIase inhibition from lethality. Intriguingly, a genomic disruption of the *CYP1* gene gave cyclosporin A resistance in yeast, providing some of the first unequivocal evidence that the drug–immunophilin complex was a toxic agent (Koser *et al.*, 1991).

Following research from Stuart Schreiber's laboratory suggesting that the target of that toxicity was the protein phosphatase calcineurin (Liu *et al.*, 1992), Merck scientists showed that, like human FKBP12, yeast Fpr1 complexed with FK506 had the ability to inhibit this enzyme (Rotonda *et al.*, 1993). They also observed that the compound L-685,818, which acted as an FK506 antagonist in an immunosuppression assay and failed to inhibit calcineurin when complexed with human FKBP12, nonetheless proved to be an active inhibitor in complex with yeast Fpr1. Despite such differences in the behavior of drug–protein complexes, the crystal structure of yeast Fpr1 with FK506 was very similar to that of human FKBP12 with FK506, and pointed to structural modifications that could be made to improve potency (Rotonda *et al.*, 1993). Structure/function relationships between FKBP and its ligands were also explored by a group at SmithKline Beecham, who correlated the effects of an amino acid alteration with catalytic and ligand-binding properties and with protein function in yeast (Bossard *et al.*, 1994).

Work from Merck had been among the first to suggest that FK506 and rapamycin had different biological effects, indicating different targets (Dumont *et al.*, 1990), and yet the compounds acted as reciprocal antagonists

and appeared to compete for binding to FKBP12. Scientists at SmithKline Beecham attempted to resolve this paradox by identifying rapamycin target proteins *in vivo* using yeast. The gene that they cloned by virtue of the rapamycin resistance of a mutant, *RBP1*, proved identical to that for the FK506 binding protein Fpr1. They showed that both rapamycin and FK506 inhibited the PPIase activity of Fpr1, and that heterologous expression of human FKBP12 restored rapamycin sensitivity to the rapamycin-resistant *fpr1* mutant, indicating a true functional equivalence (Koltin *et al*., 1991). They identified mutations in two further genes, *DRR1* and *DRR2*, that showed a dominant phenotype of rapamycin resistance. Both *DRR1* and *DRR2* were proved to encode proteins of the phosphatidylinositol 3-kinase family (Cafferkey *et al*., 1993), and are now called Tor1 and Tor2. Further characterization revealed that for both proteins it was a point mutation of a conserved serine residue that had been responsible for the resistance to the FK506–Fpr1 complex (Cafferkey *et al*., 1994). The Tor proteins are now known to be part of a conserved signaling pathway that activates eIF-4E-dependent protein synthesis (reviewed by Schmelzle and Hall, 2000).

Although publications from industry have waned, academic research using yeast continues to illuminate the processes affected by these immunosuppressants and to indicate new targets in the pathway. Some of this work illustrates the application of genomic tools that will be described in the second part of this chapter, e.g. genome deletion collections (Chan *et al*., 2000) and microarrays (Shamji *et al*., 2000).

2.5 Application in pathway elucidation: G-protein-coupled receptor/mitogen-activated protein kinase signaling

The area of G-protein signaling pathways is one where the relevance and utility of yeast biology was not appreciated for many years. G-protein-coupled receptors (GPCRs) represent the most fertile area of therapeutic intervention, with GPCR agonists and antagonists accounting for over 50% of marketed drugs (cited in Gutkind, 2000). Targeting the receptor itself usually provides the requisite specificity and yet an understanding of the biology around the coupled heterotrimeric G protein and downstream signal transduction events is essential to address issues such as desensitization. Yeast possesses two GPCR-coupled pathways, and the biology of the mitogen-activated protein kinase (MAPK) cascade coupled to the mating receptor via $G\beta/G\gamma$ is unparalleled in the degree to which it has been dissected into molecular components (Dohlman, 2002). However, for many years mammalian GPCR effects were considered to be mediated solely via the $G\alpha$ subunit, and yeast was regarded as an oddity for signaling via the $G\beta/G\gamma$ subunits. It was not until

the mid 1990s that mammalian $G\beta/G\gamma$/MAPK interactions were characterized and the direct analogy between yeast and metazoan pathways became obvious (reviewed by Gutkind, 1998). Components of GPCRs such as regulator of G-protein signaling (RGS) proteins, and MAPK pathway components such as scaffold proteins, were first identified in yeast but continue to find metazoan counterparts (see review by Gutkind, 2000). The genetic tractability of yeast allows for intelligent investigation of their function: see the use of scaffold/pathway fusion proteins to dissect control and specificity in MAPK signaling (Harris *et al.*, 2001).

As an adjunct to their extensive use of the yeast mating signal transduction pathway as a reporter system for GPCR ligand screening, two companies have published further characterizations of its components. Scientists at Glaxo Wellcome have characterized interactions between the $G\alpha$ subunit and the pathway scaffold protein Ste5 (Dowell *et al.*, 1998) that may have relevance to the recent identification of scaffold proteins in mammalian pathways (reviewed by Gutkind, 2000). Wyeth-Ayerst researchers collaborated in a study of the interplay between $G\alpha$ and the RGS protein Sst2, succeeding in uncoupling the regulation (DiBello *et al.*, 1998). Such observations raise the possibility that small molecules could modulate RGS function and thus GPCR signaling (Zhong and Neubig, 2001).

2.6 Applications in pathway deconstruction/reconstruction

An alternative use of yeast in the study of pathway biology has been to select a pathway where yeast lacks (or appears to lack) components, and to add these back. For example, a group at Glaxo used *S. pombe* as a host to reconstitute signaling through platelet-derived growth factor β to phospholipase $C\gamma2$ (Arkinstall *et al.*, 1995) and to investigate the structure/function behavior of the SHP-2 phosphatase (Arkinstall *et al.*, 1998). A more widely applied example is the use of yeast to study apoptosis. Until recently components of programmed cell death had seemed lacking in yeast, and observations suggesting that apoptosis did exist (reviewed by Frohlich and Madeo, 2000) were largely ignored. Thus, yeast seemed an ideal vessel in which to investigate determinants of the process. Researchers from Novartis were among those to observe that the apoptosis effector Bax can induce cell death in yeast, and that this effect was overcome by mammalian apoptosis inhibitors such as Bcl-2 and Bcl-x(L) (Greenhalf *et al.*, 1996). Novartis used the yeast system to identify two novel inhibitors of apoptosis – BASS1 and BASS2 (Greenhalf *et al.*, 1999) – and to characterize the structure/function behavior of Bax (Clow *et al.*, 1998) and Bfl-1 (Zhang *et al.*, 2000). Glaxo Wellcome used a Bak-mediated lethality screen in *S. pombe* to characterize host proteins involved in mediating that lethality, identifying calnexin 1 as a necessary component (see Torgler

et al., 2000). Researchers at Merck recently used homology to a yeast protein to clone sphingosine-1-phosphate phosphatase (SPP1), a human enzyme with a key role in the interconversion of metabolites that regulate apoptosis. Human SPP1 partially complements the loss of the yeast gene function, and overexpression induces apoptosis in mammalian cell culture (Mandala *et al.*, 2000). It remains to be seen whether interpretation of such data will be modified by the recent demonstration of a yeast caspase-related protease that regulates a genuine apoptotic effect (Madeo *et al.*, 2002), and the identification of molecules that induce the process (Narasimhan *et al.*, 2001).

2.7 Applications to the study of protein function

The conservation of protein structure and function among eukaryotes, and the ease of genetic and molecular manipulation make yeast a natural choice for studies of protein function. These range from inferring a human protein's function based on that of its yeast homolog, to detailed dissection of structural dependencies.

Inference of function

It is perhaps a measure of the acceptance of conserved roles that nowadays researchers seeking a role for a mammalian gene may cite the involvement of the yeast in a particular process as a powerful reason for examining that same role in mammalian biology. For example, scientists studying the REDK kinase at SmithKline Beecham note that the homologous yeast protein is a negative regulator of cell division. The function of the yeast homolog is presented as evidence in support of their hypothesis that REDK acts as a brake upon erythropoiesis (Lord *et al.*, 2000). Where the existing academic literature on a homolog is not sufficient to the needs of industry, researchers have performed studies to validate the function of a yeast protein. Thus, functional studies by Glaxo on the yeast Duk1 (Tok1) protein, which proved to be the founder member of a new structural class of potassium channels (Reid *et al.*, 1996), were only narrowly preceded by the same work from an academic group (Ketchum *et al.*, 1995).

Heterologous expression

The ability of proteins from multicellular eukaryotes to substitute for the yeast function has long been recognized. Back in 1996, the XREFdb project (Ploger *et al.*, 2000) had already reported the existence of 71 examples of human/yeast

complementation. Use of a cloned mammalian gene to substitute functionally for a yeast protein has been widely used in industry, both as a means to isolate proteins and to prove their equivalence. Several examples were presented above in the research on mechanism of action of immunosuppressives, and in the characterization of cell cycle control components and apoptosis-regulating proteins. An additional example is the demonstration by researchers from Roche that three human RNA polymerase subunits could correctly assemble into multiprotein complexes and functionally substitute for the essential role of their yeast homologs (McKune *et al.*, 1995).

In some cases, human genes have been isolated deliberately based on their homology to a yeast protein. Examples include mSPP1, discussed in the section on apoptosis (Mandala *et al.*, 2000), or Chk2, the mammalian homolog of the *S. cerevisiae* Rad53 and the *S. pombe* Cds1 kinases. The latter was cloned by scientists at SmithKline Beecham and subsequently shown to complement partially the Cds1 function and to act as a downstream effector in the DNA damage checkpoint pathway (Chaturvedi *et al.*, 1999). Alternatively, novel proteins identified from a mammalian screen may be analyzed subsequently in yeast. For example, research at Eli Lilly identified a novel kinase, pancreatic eukaryotic kinase (PEK), from rat pancreatic islet cells and noted primary and structural homology to elongation initiation factor 2 kinases (eIF-2α kinases) but also a substantial and distinctive amino-terminal region. Despite this difference, they were able subsequently to demonstrate functional substitution by PEK for the yeast eIF-2α kinase GCN2, including use of the correct phosphorylation target site on eIF-2α (Shi *et al.*, 1998). These examples of functional complementation underscore the remarkable conservation of cellular machinery in eukaryotes.

Structure/function and structure/activity

Going one step beyond functional complementation are examples where heterologously expressed proteins are altered, or mutant forms of medical significance are used, in an attempt to correlate their structure with their properties. One example of an attempt to correlate the effects of a mutation with the role of a protein in disease is the use of yeast as a model to study the conductance regulator that is mutated in cystic fibrosis. Although academic research is still active in this area, pharmaceutical industry interest in this approach (as measured by publication) seems to have waned after research at Glaxo found that an early yeast model did not correctly mimic the mammalian disease biology (Paddon *et al.*, 1996). However, the general concept of using yeast for such analyses is undoubtedly of merit. There are several recent examples from academia where yeast has proved successful, e.g. in providing a model for the cellular defect (Pearce *et al.*, 1999b) and even

suggesting a therapeutic route (Pearce *et al.*, 1999a) in Batten Disease, a progressive neurodegenerative disorder of a class that affects one in 12 500 births.

There is a more successful example of simple structure/function analysis from the pharmaceutical industry: after identifying SAG as a novel human protein involved in apoptosis that had a yeast homolog (Duan *et al.*, 1999), scientists from Warner Lambert (now Pfizer) demonstrated complementation of the yeast *hrt1* mutant function with SAG and showed a requirement for the RING protein (Swaroop *et al.*, 2000); SAG proved to be a novel homolog of the ROC1/Rbx1/Hrt1 protein, which interacts with the Skp–cullin–F-box protein complex to generate an active E3 ubiquitin ligase. This ligase promotes degradation of CDK inhibitory proteins, and mutants that had lost E3 ligase activity were unable to complement the yeast *hrt1* mutant. Upon withdrawal of SAG expression, an *hrt1* mutant arrests with a very heterogeneous DNA content, and transcription profiling identified responsive genes from both the G1/S and G2/M checkpoints (Swaroop *et al.*, 2000). This group also used complementation of *hrt1* in yeast to test whether a SAG splicing variant encoded a functional protein (Swaroop *et al.*, 2001).

Another example of the use of yeast in structure/function analysis is provided by the human phosphoacetylglucosamine mutase genes HsAGM1 and HsAGX1, which were cloned using yeast by scientists at the Nippon Roche Research Center. Gene HsAGX1 encodes a UDP-*N*-acetylglucosamine pyrophosphorylase that may be involved in antibody-mediated male infertility. After cloning based on homology, it was shown to substitute functionally for the loss of yeast Qri1 (Uap1), and key catalytic residues were investigated by site-directed mutagenesis (Mio *et al.*, 1998). Gene HsAGM1 was cloned by functional complementation in yeast and, after sequence comparisons with other family members identified as likely key residues, site-specific mutagenesis was successfully combined with *in vitro* and *in vivo* yeast assays to identify residues essential for catalytic activity (Mio *et al.*, 2000). In both cases, identification of likely catalytic residues to target for mutagenesis was facilitated by extensive characterization of the hexose phosphate mutase family in yeast (Boles *et al.*, 1994).

Mitogen-activated protein kinases and their associated pathways are currently a hot area of pharmaceutical research. The p38α kinase is an active target of several major anti-inflammatory programs (Drosos, 2002). As always with kinases, issues of specificity are at the forefront (Scapin, 2002). The potential utility of yeast in this field is shown by research at SmithKline Beecham directed at dissecting functional differences between p38/CSBP1 and an uncharacterized splice variant that they called CSBP2. They were able to demonstrate complementation of yeast *hog1* mutants by human CSBP1 and by mutants of CSBP2, but not native CSBP2, and to obtain structure/function information for kinase activity and the salt-responsiveness of the enzymes

(Kumar *et al.*, 1995). Hog1 is a yeast MAPK that responds to osmotic stress; the mutant phenotype also can be rescued partially by stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) 1. Kinase p38α and SAPK/JNK activation by hyperosmolarity also seems to be conserved in some mammalian cell lines (reviewed by Kultz and Burg, 1998).

Several cases where the yeast protein structure could be correlated with compound activity (structure/activity relationship studies) in drug discovery research have been cited above in the work on cyclosporin, FK506 and rapamycin. Another published example is the inclusion of yeast farnesyl–protein transferase and geranyl–geranyl–protein transferase in structure/activity evaluations of several chaetomellic acid chemotypes under study at Merck for inhibition of prenyl transferase activity (Singh *et al.*, 2000). Finally, yeast may also act as an *in silico* surrogate for mammalian proteins in structure/activity work: scientists at Novartis have described the use of the yeast crystal structure for the 20S proteasome to guide analog design for mammalian proteasome inhibitors that have therapeutic potential as antitumor agents (Furet *et al.*, 2001).

Biochemical assays

Many studies published by pharmaceutical companies have used yeast as a source of biochemical data; however, the majority most likely represent enzymes that are targets for antifungal drug discovery rather than those that model a vertebrate protein. Respiratory uncoupling proteins, which are implicated in the regulation of energy expenditure and the development of obesity, represent an area where *in vivo* biochemical studies in yeast have been used to characterize function. For example, when Merck scientists identified a novel member of the uncoupling protein family, they used expression in yeast to show that it caused a loss of mitochondrial membrane potential (Liu *et al.*, 1998). Novartis has also described heterologous expression of human uncoupling protein 1 (UCP1) and UCP3, measurement of their effects on mitochondrial polarization and modulation of their effects with purine nucleotides (Hinz *et al.*, 1999). The type of detailed kinetic data that can be obtained on a yeast enzyme *in vitro* is illustrated by an analysis of the steady-state mechanism of decarboxylation by orotidine-5′-phosphate decarboxylase, published by scientists from Glaxo Wellcome (Porter and Short, 2000).

2.8 Reagents and resources available in yeast

Ironically, from this fairly complete survey of publications from major pharmaceutical companies, it seems that utilization of yeast as a model system

in drug discovery has fallen even as the ease of working with it has increased. Perhaps this is because mammalian systems have also become tractable enough to tip the balance, or perhaps exploratory biologists working in industry are publishing less of their basic research. Financial considerations have driven yeast researchers in academia to seize upon the information and tools generated by the complete genome, and direct their research in ever-more disease relevant and commercially applicable directions. However, the gap between an interesting observation and a drug in humans is still huge, and even the best academic research cannot substitute for the applied use of model systems within 'big pharma'.

Yeast is often cited for its ease and rapidity of use and for the range and sophistication of techniques available for genetic manipulation. Common techniques for manipulating yeast are covered in numerous texts; an excellent basic laboratory manual is provided by Guthrie and Fink's *Guide to Yeast Genetics and Molecular Biology* (Guthrie and Fink, 1991). Below I shall describe the range of 'genomic tools' now broadly available and the information resources they have generated, wherever possible giving examples of their use in disease-related research. The topics to be covered are laid out in Figure 2.4.

Expression profiles
- specific questions
- compendium approach

Genome deletion mutant collection
- complementation analysis
- phenotype screens
- synthetic lethality
- haploinsufficiency
- mutant mapping

Regulated expression
- drug resistance
- phenotypic analysis
- pathway analysis

Proteomics
- genome-wide two-hybrid set
- analysis of complexes by mass spectrometry
- genome-wide biochemical assays
- large-scale cellular localization

Figure 2.4 Reagents and techniques available in yeast, and their applications as discussed in the text

2.9 Gene expression profiling using microarrays

From the seminal publication in 1996 (Shalon *et al*., 1996), yeast served as the test bed for academic and commercial development of microarrayed DNA probes and was the first organism for which whole genome arrays were available. Incyte's Yeast Proteome Database (YPD; Costanzo *et al*., 2001) currently contains data culled from nearly 50 yeast genome microarray publications, including some covering as many as 119 experimental conditions (Roberts *et al*., 2000). There is currently a variety of sources for yeast genome arrays, including primer sets for polymerase chain reaction (PCR) amplification of each ORF (Invitrogen), sets of 50-mer (MGW Biotech) or 70-mer (Invitrogen) oligonucleotides that each probe one ORF, ready-arrayed 50-mer oligonucleotides (MGW Biotech) and Affymetrix gene chips that use sets of 25-mer oligonucleotides to provide a readout for each gene. These reagents are listed in ascending order of price; they are also listed in descending order of effort to implement. The relative merits in this tradeoff can be harder to determine for yeast than other systems. In the use of mammalian-based assays for a transcriptional profiling experiment, the cost of generating samples is often much higher than that of the chip. Yeast, however, allows many hundreds of assays to be run in relatively short times with low reagent cost. This allows the creation of a database of profiles to which new entries can be compared in a manner analogous to BLAST searches on GenBank. The creation of an agglomerated public database of yeast transcriptional profiles still lies in the future, but the usefulness of such a resource was demonstrated by work at Acacia Biosciences. After creating a large dataset of response profiles generated by mutations and compounds of known mechanism, they were able to use clustering algorithms to categorize new compounds or compounds presented 'blind' into functional groups. Complexities of blocking a pathway such as isoprene synthesis at different steps could be revealed (Dimster-Denk *et al*., 1999). This approach was extended by scientists at Rosetta Inpharmatics who compared transcription profiles of cyclosporin-A- and FK506-treated cells with those from null mutants for the immunophilins CPH1 and FPR1 and for the ultimate target protein calcineurin. They demonstrated that the ability of a compound to inhibit pathways other than its intended target can be quantified by such experiments, providing a means to group and rationally select desirable chemotypes (Marton *et al*., 1998). By 2000 Rosetta had constructed a reference database of transcription profiles for 300 diverse mutations and chemical treatments in *S. cerevisiae*. 'Homology' of profiles within this database suggested functions for eight uncharacterized ORFs; such functions were then confirmed by more detailed individual analysis. As an example of the utility of such a database in compound classification, the observation that the profile for yeast treated with dyclonine resembled those of yeast mutants with blocks in sterol synthesis, and

specifically that of an *erg2* mutant, suggests that this compound might mediate effects in humans by binding the sigma receptor, the closest homolog of Erg2 (Hughes *et al.*, 2000). The sigma receptor binds a number of neuroactive drugs, including the antipsychotic haloperidol (Haldol; Ortho-McNeil), which also likely inhibits yeast Erg2 (Acacia Biosciences, unpublished data). Dyclonine is a widely used topical anesthetic (Dyclone; Astra) that is reportedly longer lasting than benzocaine; if you want to test the effect on your own sigma receptors, there are a couple of milligrams of dyclonine in every Sucret throat lozenge (except original mint flavor)!

2.10 Deletion collections: reinventing traditional screens

Saccharomyces cerevisiae was early to benefit from reagents that allowed analysis of gene function on a genome-wide scale (Ross-Macdonald *et al.*, 1999). It now has a resource that will remain unparalleled in any system: a set of strains that comprise a start-to-stop-codon deletion for nearly every annotated ORF in the genome (Winzeler *et al.*, 1999; Giaever *et al.*, 2002). One immediate and obvious utility of the Yeast Genome Deletion Collection for the use of yeast as a model organism is that complementation of a particular gene defect by a putative human ortholog can be tested very rapidly because the necessary mutant strain already exists. Where the function of the ORF proved essential for viability under normal growth conditions, a heterozygous diploid containing one wild-type copy was created; for all other genes, both haploid and diploid homozygous disruptants were made in addition to the heterozygous diploid (Winzeler *et al.*, 1999). There are thus four collections: the heterozygous diploids (ca. 6000 strains); the haploid disruptants of each mating type; and the homozygous diploids (ca. 4800 each). These strains can be obtained from either ResGen (Invitrogen, Carlsbad, CA) or the American Type Culture Collection (Manassas, VA) as individual tubes, in microtiter format, or as pools.

An additional feature of the collection is that for each ORF the deletion construct was individually designed and constructed, allowing the insertion of two unique 20-base elements into the genome at the site of the deletion (Figure 2.5). The existence of these 'tags' allows the identity of a particular strain to be confirmed rapidly by sequencing of a PCR product. It also allows the presence or absence of a particular strain among a group of strains to be measured by various PCR strategies. One strategy is to generate a labeled PCR product containing the tag sequence as shown in Figure 2.5 and then hybridize to microarrays of oligonucleotides that are complementary to the tags (Shoemaker *et al.*, 1996). Figure 2.6 shows an example from my laboratory of such a pool of PCR products hybridized to the 'Tag3' array, custom produced by Affymetrix. Use of these 'tag arrays' represents a very new area

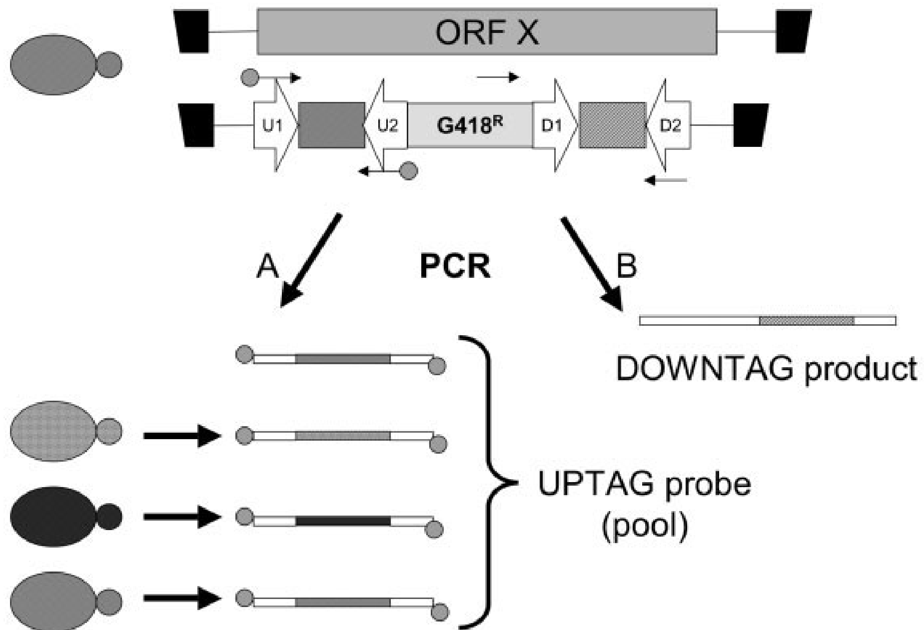

Figure 2.5 Utility of features incorporated into the Yeast Genome Deletion Collection. For each ORF in the *S. cerevisiae* genome (ORF X in the example), a specific deletion cassette was constructed. This cassette contained flanking sequences that targeted it to replace the ORF from the start codon to the stop codon. The DNA substituted for the ORF contained a gene conferring resistance to the antibiotic G418 (G418^R). At each end, it also contained unique 20-base-pair sequences not found in the yeast genome. Called the 'uptag' and the 'downtag', these 20-mer tags are flanked by short sequences that are common to each construct, indicated as U1, U2, D1 and D2. These common sequences can be used as priming sites for polymerase chain reaction (PCR), allowing every tag present in a pool to be amplified in a single reaction. In this example, (A) shows an amplification using primers U1 and U2, where U1 carries a molecular probe such as biotin or a fluorophore. The resulting pool of PCR products could be hybridized to an oligonucleotide array to determine its composition. Alternatively, PCR (B) uses a primer complementary to the G418^R marker region in combination with primer D2 to generate a longer PCR product suitable for sequencing. Because the downtag is unique to the strain carrying the deletion of ORF X, this sequencing reaction immediately reveals the identity of the strain

with few publications, and the availability of arrays is a limiting factor. However, it is anticipated that the ability to pool hundreds or indeed all 6000 strains, perform a selection and then identify all the changes that have occurred in the population in a rapid, multiplex fashion will enable new types of screens that were too onerous to perform by traditional methods. Although they have been available for less than 2 years, the collections are already finding wide use as detailed below.

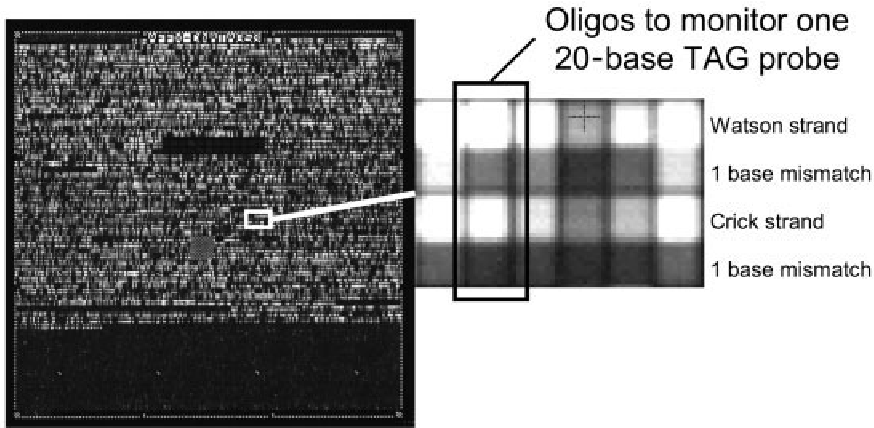

Figure 2.6 Use of 'Tag array' chips to assay the Yeast Genome Deletion Collection. Using genomic DNA from the collection of ca. 6000 heterozygous deletion mutant strains as a template, a pool of biotinylated polymerase chain reaction (PCR) products was generated as shown in example (A) of Figure 2.5. Both the 'uptag' and the 'downtag' regions were amplified in separate reactions. The pool of ca. 12 000 PCR products were hybridized to a 'Tag3' custom oligonucleotide array provided by Affymetrix (Santa Clara, CA). This array allows probing with 16 000 distinct sequences and because only ca. 12 000 of these sequences were used in generating the Yeast Genome Deletion Collection the bottom quarter of the chip does not show hybridization. Each 'tag' that was used in the Yeast Genome Deletion Collection is represented by four features on this array: two features that represent a perfect match to either strand of the tag region and two features that contain a single base mismatch substitution in the center of the perfect match sequence. As shown to the right, the PCR product containing a particular tag sequence should hybridize strongly to the two 'perfect match' features and with reduced efficiency to the 'mismatch' features

Phenotypic screens

The most immediate application of the deletion collection is to direct phenotypic screening. Publications on screens for genes involved in rapamycin sensitivity (Chan *et al.*, 2000), autophagy (Barth and Thumm, 2001), glycogen storage (Wilson *et al.*, 2002), mitochondrial function (Dimmer *et al.*, 2002), DNA repair (Ooi *et al.*, 2001), proteasome inhibition (Fleming *et al.*, 2002) and intracellular transport (Muren *et al.*, 2001) have already appeared. These screens have used analysis of individual strains. Only one published example to date has employed a chip-based assay on the pooled collection: Ooi *et al.* reported the identification of known and novel yeast gene deletion mutants that are incapable of circularizing a plasmid introduced in linear form. Instead of performing over 4800 individual assays, one pool was used (Ooi *et al.*, 2001).

Synthetic lethality

Synthetic lethality is another established genetic technique to receive a new twist from the deletion collections. In this approach, mutations that are individually of little or no effect are revealed to synergize and cause cell death. This approach is particularly useful for characterizing pathways because it can identify redundant functions or synergistic effects. Historically, this was a laborious technique that involved random mutagenesis followed by close visual inspection of thousands of colonies, followed by a cloning attempt that might prove unsuccessful. Now, Tong *et al*. (2001) have described a system for the use of the haploid deletion collection to perform systematically a version of synthetic lethality analysis that is scalable and amenable to automation. A haploid containing the mutant of interest is mated to the ca. 4800 viable haploid deletion strains. Following meiosis, the viable haploid progeny are examined for co-segregation of the mutation of interest and the deletion mutation. This sounds complex, but in fact is achieved by a series of simple steps using selective media. Lack of growth occurs when there is an inability to carry the two mutations in the same haploid cell, indicating that a synthetic lethality is occurring.

Haploinsufficiency analysis

Another utility for the deletion collection that has published proof-of-principle is 'haploinsufficiency' screening for drug targets (Giaever *et al*., 1999). Conceptually, this approach relies on the increased drug sensitivity of a strain that has reduced gene dosage for the target protein. Such hypersensitive strains can be identified by individual assays on each strain from the heterozygous mutant collection; alternatively, the inclusion of the individual 'tags' in each deletion construct allows analysis of pooled strains with a chip-based readout. The ability to use pooled strains is a major advantage for drug discovery applications, where limited amounts of compound are available. Haploinsufficiency is a sound theory, but in practice many mutant strains will be hypersensitive to a given compound, and robust statistical analysis of a large body of compound and control data is essential to identify strains that respond specifically to a test compound (D. Shoemaker, personal communication; Rachel Kindt, personal communication).

Mutant gene mapping

Use of a complete collection of gene deletion mutants eliminates much of the uncertainty involved in phenotypic screens: issues of mutagenesis of multiple

genes, screen saturation and gene recovery are eliminated. What is lost is the ability to generate specific changes such as conditional alleles of essential genes; such point mutants historically have been the richest source of information. To illustrate this assertion: although complete deletion of the gene for yeast immunophilin Fpr1 results in rapamycin resistance, isolation of point mutations in the essential Tor proteins by Cafferkey *et al.* (1994) was required for a complete understanding of the mechanism of action. However, another way to look at the deletion collection is as a comprehensive set of mapped markers. Thus, a point mutation of interest can be mapped by mating a haploid mutant to the ca. 4800 viable haploid deletion strains and then examining the haploid meiotic progeny of each diploid for linkage between the mutation and the G418 resistance marker. The same system applied by Tong *et al.* to high-throughput synthetic lethal screening can be applied here to render this process rapid and automated (Tong *et al.*, 2001).

2.11 Overexpression analysis: enough is enough

As yet, no genome-wide reagent for systematic overexpression of yeast genes exists, although several are in construction. Such a collection will have broad utility. Historically, several drug targets have been identified in yeast by virtue of the resistance caused by introduction of a genomic fragment containing the gene on a high-copy plasmid (Rine *et al.*, 1983) and 'high-copy suppressors' of mutant phenotypes are a standard tool in analysis of gene function. More recently, overexpression analysis has been used to examine effects on MAPK signaling, identifying new kinases that can modulate a well-characterized pathway (Burchett *et al.*, 2001). Overexpression was also used by Stevenson *et al.* to identify new proteins implicated in cell cycle control (Stevenson *et al.*, 2001). Kroll *et al.* (1996) described synthetic lethality when a protein of interest is overexpressed in the background of an otherwise benign mutation as a method of detecting specific genetic interactions. This technique was applied in a screen for genes whose overexpression is lethal in a proteasome-impaired mutant, and revealed six novel genes capable of inducing apoptotic death in yeast (Ligr *et al.*, 2001). A standardized regulated genome-wide collection of expression constructs is arguably the next great yeast genomic reagent.

2.12 Proteomics: would you like chips with that?

Although drug discovery is in a period where protein targets are screened in splendid isolation, that is not how they exist in the cell and ultimately some information about interactions and modifications is likely to prove necessary. Our ability to study such characteristics of a protein has greatly increased, and

yeast has served as both the test bed for many techniques and as a surrogate for mammalian target proteins. Several complete-genome reagents and their use have been described.

Two-hybrid analysis of interactions

Two-hybrid analysis originated in yeast, and the ease of high-throughput assays in this system has made it the host of choice for most commercial and academic analysis of mammalian protein interactions (Uetz, 2002), although this is likely to change as mammalian systems become more tractable. The assay requires two fusion constructs to be expressed in the same cell: if an interaction occurs between the proteins under test, it reconstitutes their attached domains into a protein that can generate a measurable output (e.g. a transcription factor). Performing a comprehensive analysis involves mating of a strain with one fusion construct (the 'bait') to an array of strains carrying possible interactors (the 'prey'). Although a genome-wide analysis of every protein in yeast is theoretically possible, it would require over 38 million matings. However, if you wish to perform your own screen on a protein of interest, the Fields' laboratory makes the complete set of yeast fusion 'prey' constructs (Uetz *et al.*, 2000) available to all interested researchers.

Several large-scale two-hybrid studies have been reported to date: each tested only a subset of the genome and/or used pooling strategies (Fromont-Racine *et al.*, 1997; Flores *et al.*, 1999; Ito *et al.*, 2000; Uetz *et al.*, 2000). Such data can be synthesized to provide an interaction map for a eukaryote proteome and to suggest a function for uncharacterized proteins (Schwikowski *et al.*, 2000). Integration of the data into yeast information resources such as YPD and MIPS mean that results for orthologs of human proteins are readily accessible. An example of yeast as a model for a target of therapeutic relevance is a recent dissection of interactions within the 26S proteasome. Thirty-one proteasome components were screened against the entire proteome, and novel interacting components could be validated further by mutant analysis and reporter assays (Cagney *et al.*, 2001).

Analysis of complexes by mass spectrometry

This relatively recent addition to the set of techniques available is fast proving valuable. For various reasons discussed in the publications below, it usually produces quite different answers than two-hybrid analysis, and the datasets that are obtained complement each other. To achieve sufficient specificity, mass spectrometry must be applied to protein complexes that can be purified physically. Usually this means epitope tagging of the protein of interest and then passing through multiple rounds of affinity purification (TAP) followed by gel

purification, although one report on the 40s ribosomal subunit directly analyzed complexes physically separated by other means (Link *et al.*, 1999). Honey *et al.* reported the use of TAP and mass spectrometry to characterize components of the active yeast CDK complex tagged on its cyclin subunit (Honey *et al.*, 2001). More recently two commercial entities reported far larger scale projects: Cellzome's work included 1143 yeast orthologs of relevance to human biology (Gavin *et al.*, 2002), whereas MDS Proteomics tagged 725 proteins, including a large number implicated in DNA damage responses (Ho *et al.*, 2002). These commercial projects represent pilots for mammalian work, as well as providing a large body of data for many yeast proteins that have mammalian homologs.

Biochemical analysis

A surprising pursuit, in this day and age, is to click around the links in various metabolic pathway websites and to discover how many of the described biochemical activities do not have a yeast gene linked to them. To eliminate the onerous task of purifying such activities to identify the responsible protein, Martzen *et al.* (1999) created expression constructs for all yeast ORFs in which the yeast protein was fused to glutathione-*s*-transferase (GST). These GST-fusion proteins can be purified and screened for enhancement of a particular activity; they are also a useful resource for hypothesis testing with cross-linkable ligands. As a further refinement, Zhu *et al.* report attachment of such tagged yeast proteins to microarrays and their screening for kinase activity (Zhu *et al.*, 2000) and for affinity to calmodulin and phospholipids (Zhu *et al.*, 2001). It is easy to conceive of future use of such arrays to identify molecular targets for labeled compounds.

Localization data

In addition to the most complete protein interaction data resources, yeast has a large volume of information on subcellular protein localization. Greatly extending data provided by individual studies and by an earlier large-scale project (Ross-Macdonald *et al.*, 1999), Kumar *et al.* conducted a genome-wide epitope-tagging and immunocytochemistry project resulting in annotation of nearly half the proteins in yeast to one of six subcellular localization sites (Kumar *et al.*, 2002a,b).

2.13 Web-accessible databases: bringing it all back home

- Saccharomyces Genome Database: http://genome-www.stanford.edu/Saccharomyces/

- Comprehensive Yeast Genome Database: http://mips.gsf.de/proj/yeast/CYGD/db/index.html

The community of yeast researchers numbers in the tens of thousands and, coupled with the tools described above, the capacity to generate 'omic' scale information is almost overwhelming (Zhu and Snyder, 2002). In addition, the ability to measure and modulate so many parameters in yeast means that it is a natural test bed for systems biology (Ideker *et al*., 2001). Gene-centric information for yeast is compiled into several databases that have made commendable efforts to cross-reference each other. Principal among these are the Saccharomyces Genome Database (SGD) based at Stanford, USA (Dwight *et al*., 2002), the Comprehensive Yeast Genome Database (CYGD) at MIPS-GSF (Germany) (Mewes *et al*., 2000) and Incyte's YPD (Costanzo *et al*., 2001). The latter is a commercial subscription database historically provided free to academic researchers and has served as a template for Incyte's Human-PSD and GPCR-PSD databases. Many other databases exist to collate specialized information in greater detail; these are indexed off the sources listed above.

2.14 Conclusion

Analysis of the genomic sequences of both humans and yeast has led to a renewed appreciation of the shared biology of these long-separated eukaryotes. Although the understanding of this relationship is broader in the academic community, this review illustrates the wide range of uses that yeast has served in the pharmaceutical industry. As the technologies available become more powerful every year, it is to be hoped that we do not lose our appreciation of the insight that this small organism can continue to provide.

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