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High-throughput approaches to functional characterization of genetic variation in yeast

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Expansion of sequencing efforts to include thousands of genomes is providing a fundamental resource for determining the genetic diversity that exists in a population. Now, high-throughput approaches are necessary to begin to understand the role these genotypic changes play in affecting phenotypic variation. *Saccharomyces cerevisiae* maintains its position as an excellent model system to determine the function of unknown variants with its exceptional genetic diversity, phenotypic diversity, and reliable genetic manipulation tools. Here, we review strategies and techniques developed in yeast that scale classic approaches of assessing variant function. These approaches improve our ability to better map quantitative trait loci at a higher resolution, even for rare variants, and are already providing greater insight into the role that different types of mutations play in phenotypic variation and evolution not just in yeast but across taxa.

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Introduction

Genetic diversity present across the budding yeast *Saccharomyces cerevisiae* population has produced the extraordinary phenotypic diversity that we see in this species today [1,2]. With a variety of wild ecological origins and over 12 000 years of domestication across the globe, *S. cerevisiae* isolates in different clades have distinct polymorphisms that facilitate adaptation to specific environments [3–5]. The overarching question that still remains after decades of genetics and genomics work is which of the genotypic differences and changes in genetic architecture between individuals give rise to phenotypic variation. A change in a single locus alone can be the cause for phenotypic differences and is known as a Mendelian trait. However, Mendelian traits are rare as most traits are complex and thus governed by multiple loci and gene-by-environment interactions. Understanding the genetic basis of complex traits has many implications for advancing therapeutics for disease, industrial applications, agricultural output for our changing climate, and our knowledge of evolution.

Curation, deep sequencing, and genomic analysis of 1011 *S. cerevisiae* isolates collected from natural and domestic environments revealed the sheer number of variants present in the population [3]. Containing over 1.6 million single-nucleotide polymorphisms (SNPs), this collection highlights that foundational approaches such as quantitative trait locus (QTL) mapping for understanding the effects of these SNPs would be prohibitively labor-intensive, time-consuming, and oftentimes impossible, given the current limitations of these strategies [6]. Copy number variants (CNVs) further confound our understanding of the genetic architecture of traits; almost all open-reading frames in the *S. cerevisiae* genome have a CNV in at least one of the 1011 strains [3]. The huge diversity in populations compared with the little that is known about the effects of genotypic changes, with the added layer of intricacy that environmental factors play, necessitates high-throughput experiments that can confidently determine the impacts of variants of all types.

Even the huge number of variants as yet observed is dwarfed by the number of variants that could possibly exist. Obviously, this general problem is not limited to the yeast system: modern genetics is going to require high-throughput approaches to understanding variation across taxa. Technological solutions developed in yeast can immediately be applied to other genomes either as a testbed for methods development to port to other systems, or by heterologously expressing genes in yeast.

While these challenges are daunting, the throughput of systems-level approaches for determining and measuring variant function has increased considerably, particularly in *S. cerevisiae*, pointing to a path forward. The expansive genetic diversity of yeast, coupled with the extensive toolkit for dissecting genetic traits and engineering

variants, provides an excellent foundation for understanding the impact of polymorphisms, learning fundamental principles underlying these effects, and ultimately predicting the effects of potential mutations. Here, we describe the latest technologies and strategies developed for addressing genotypic changes on a massive scale and what questions applications of these approaches can answer.

Approaches

Genome-wide association study (GWAS) and QTL mapping are the major approaches used to understand the effects of natural variants on complex traits [3,6,7]. Even though over one million samples have been incorporated in human GWAS analyses, the missing heritability problem, where mapped variants explain a small proportion of the total heritability [8], is still not resolved. Previously, in *S. cerevisiae*, due to the high degree of mosaicism and presence of population structure, GWAS required additional statistical corrections to avoid false positives [9–13]. However, increases in sample size and diversity have improved the power of using GWAS for understanding the genetic basis of complex traits in yeast [3,7,11–14].

QTL mapping, on the other hand, controls environmental factors and leverages the segregation of parental genotypes to pinpoint causal genetic variants, leading to measurable phenotypes of interest [22] (Figure 1, left panel). Phenotyping approaches for QTL mapping in yeast vary widely and include those that detect changes in molecular phenotypes such as gene expression or protein abundance [23•–29], colony or cell morphology [20,30,31], flocculation patterns [20], growth rate [3,15••,16,20], enzymatic function [32], compound production [33], translation-termination efficiency [34], and many more [1,2].

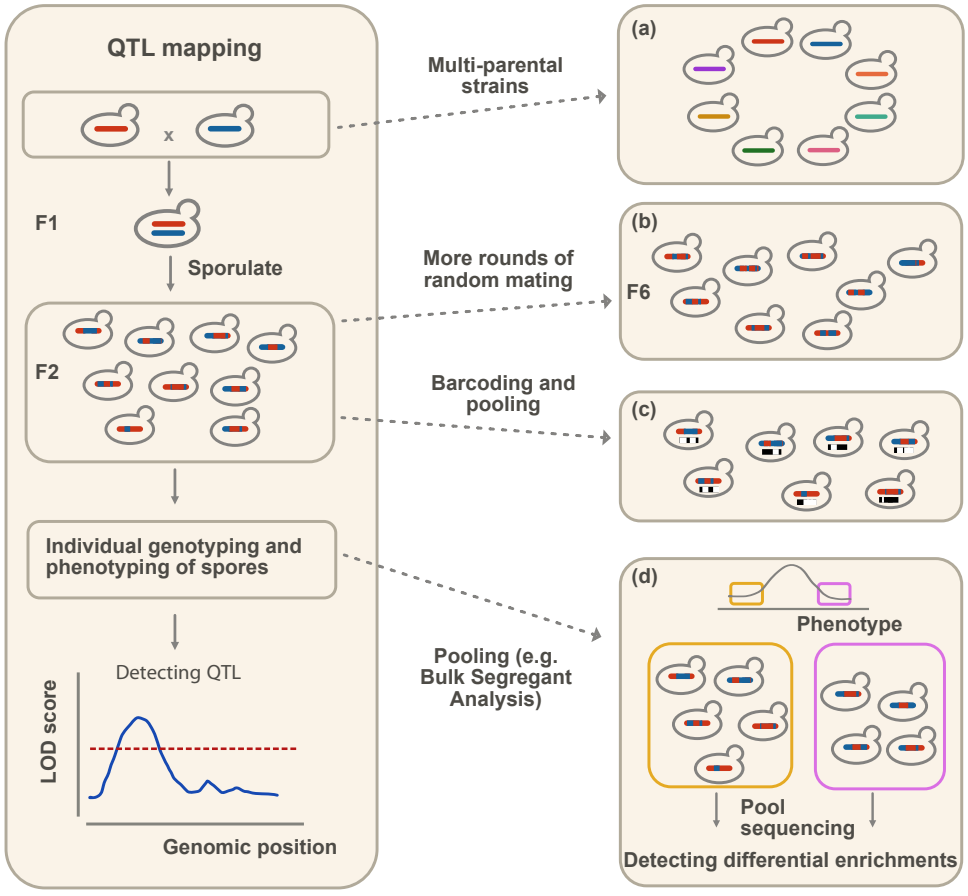
High-throughput genome-wide identification of genes contributing to trait differences

Pooled approaches — such as bulk segregant analysis, which sequences pooled individuals with extreme phenotypes [21], and barcoding multiple parental strains [18••] or individual segregants [19•] in order to trace lineages of pooled segregants throughout a screening — improve the throughput of QTL mapping (Figure 1, right panel). Although these approaches can be enriched for false positives due to beneficial mutations, genomic instability, and diploidization events that may arise during the growth phase [20], advancements in automated workflows now allow phenotyping of a remarkable number of individual segregants. The largest QTL mapping study in yeast to date phenotyped an astounding 100 000 F2-barcoded segregants [18••]. Collectively, QTL mapping studies have measured over 100 complex traits [15••–18••,20,21]. Furthermore, deliberate selection of parent strains can survey most of the natural variation in the *S. cerevisiae* population, with 82% of biallelic SNPs captured by just 16 parental natural isolates [15••]. Progeny of these 16 isolates, as well as hybrids from a diallele cross with 55 natural isolates, are also enriched for rare variants [15••,35••]. Screening of these crosses confirmed that variants of large effects are usually rare in the total population, consistent with negative selection on these alleles [15••,18••,35••].

Ascertainment biases in QTL mapping still exist, resulting in variants of large effects being overrepresented in functional studies and variants of small effects being overshadowed. Identification of polymorphic differences between two strains and individually introducing the variants from one strain to another exposes the functional effects of those variants in an unbiased manner. Several advances made in CRISPR/Cas9 precise editing techniques have been used to introduce SNPs identified between two strains in a high-throughput manner (Figure 2b) [36–39]. Such studies leverage the use of retrons, base editing, and protein fusions to increase the efficiency of homology-directed repair over non-homologous end joining in yeast [36–38]. These pooled variant studies have been able to interrogate the impacts of over 16 000 SNPs (in some cases over 30 000 SNPs) in one experiment, although variants of small effect tend to have low reproducibility and high false-discovery rates [36,37,39]. Once generated, libraries can be screened in various conditions to measure the interaction between each variant and the environment. Overcoming other limitations such as decreased editing accuracy with increased distance to protospacer-adjacent motifs (or the cut-site motif recognized by Cas9) sites or high oligonucleotide error rates will improve the power and accuracy of these assays.

Precise editing approaches have been useful for identifying expression QTLs (eQTLs), or *cis*-regulatory variants that alter expression and affect phenotype, making it possible to pinpoint causal *cis*- and *trans*-acting mutations in *S. cerevisiae* [23•,27•,40]. Similarly, genome-wide perturbations that upregulate or downregulate nearly all genes in one assay can identify eQTLs as well, although not always at nucleotide resolution [41•–44]. Scaled eQTL studies can now simultaneously measure expression and protein abundance with greater statistical power, providing a better understanding of how

Figure 1



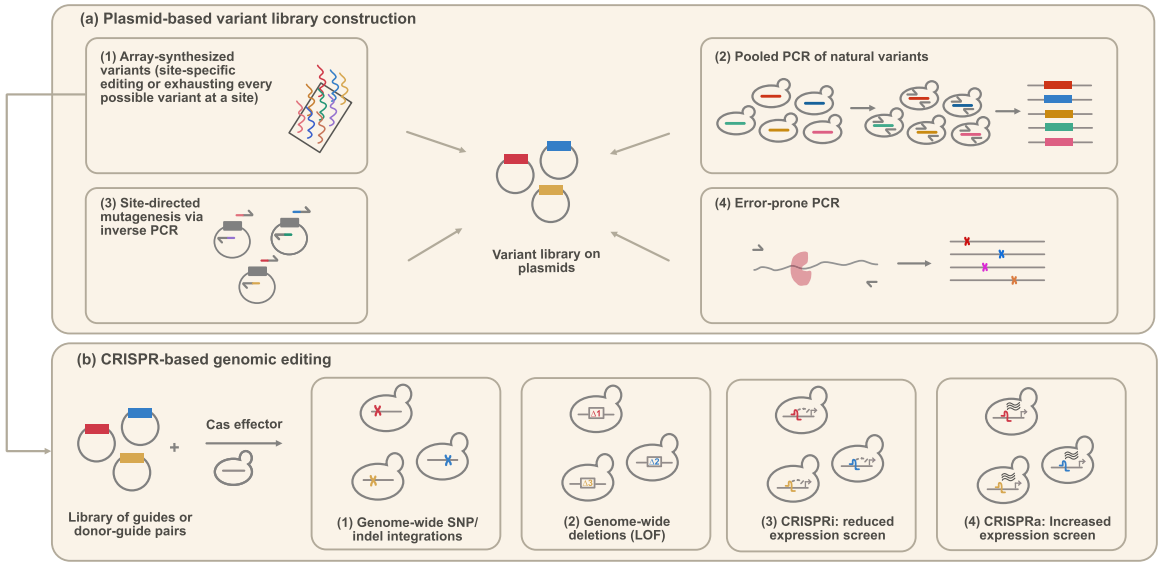
Traditional QTL mapping procedures (left panel) and recent advancements (right panel). The LOD (logarithm of odds) score indicates the probability that a QTL is present at that genomic position. QTL are identified by regions with LOD scores above a significant threshold. Recent advancements increase mapping precision by **(a)** using multiple parental strains [15••], **(b)** more rounds of random mating [16,17], **(c)** high-throughput barcoding techniques [18••,19•], or **(d)** pooling assays [20,21]. In **(d)**, the yellow and purple boxes represent yeast with extreme phenotypes, which are pooled and sequenced separately to detect alleles that are differentially enriched.

promoters and post-transcriptional processes may affect complex traits [23•,27•,40]. In addition to changes in expression and protein-abundance patterns, complete loss of function (LOF) by full gene deletions, frameshift mutations, temperature-sensitive mutations, or introduction of premature stop codons can be investigated in a high-throughput manner to determine the impact of different types of LOF mutations [38,45].

High-throughput interrogation of variant effects at a causal locus

When genes of interest are identified by any of these mapping methods, the question arises of how natural genetic variation or potential new mutations in these genes impact function. Multiplexed assays of variant effects, or MAVEs, can interrogate the effects of substitutions and *cis*-regulatory mutations in one locus by creating large libraries of variants and measuring *en masse* how they affect fitness, protein interactions, expression, splicing, or enzymatic function [49]. MAVEs encompass a variety of methods such as Massively Parallel Reporter Assays, Deep Mutational Scanning, and Saturation Genome Editing. Variants for MAVEs can be generated through a variety of methods (Figure 2a): Error-prone polymerase chain reaction (PCR) is a cost-effective approach that introduces random mutations into a region of interest and can explore both single and combinatorial effects of mutations, but due to its random nature, specific mutations and variants of interest are not guaranteed to be generated [50,51]. Inverse PCR allows for site-directed mutagenesis and has now been scaled for MAVEs, but it remains quite labor-intensive

Figure 2



Summary of yeast library construction and genomic editing methods. **(a)** Plasmid-based variant library construction. The effects of these variants can be determined by transforming yeast cells with this plasmid library. Commonly used library construction methods include (1) array-synthesized variants [37,46,47,54,55], (2) pooled PCR of natural variants [48], (3) site-directed mutagenesis [46,52,53], and (4) error-prone PCR [50,51]. **(b)** CRISPR-based variant construction. Yeast cells are transformed with a plasmid library of guide or donor-guide pairs, so that genetic changes are incorporated into the genome. These methods include (1) Genome-wide SNP/indel editing [36–39], (2) Genome-wide deletions [41•], (3) CRISPRi to decrease target expression [41•–43], and (4) CRISPRa to increase target expression [41•].

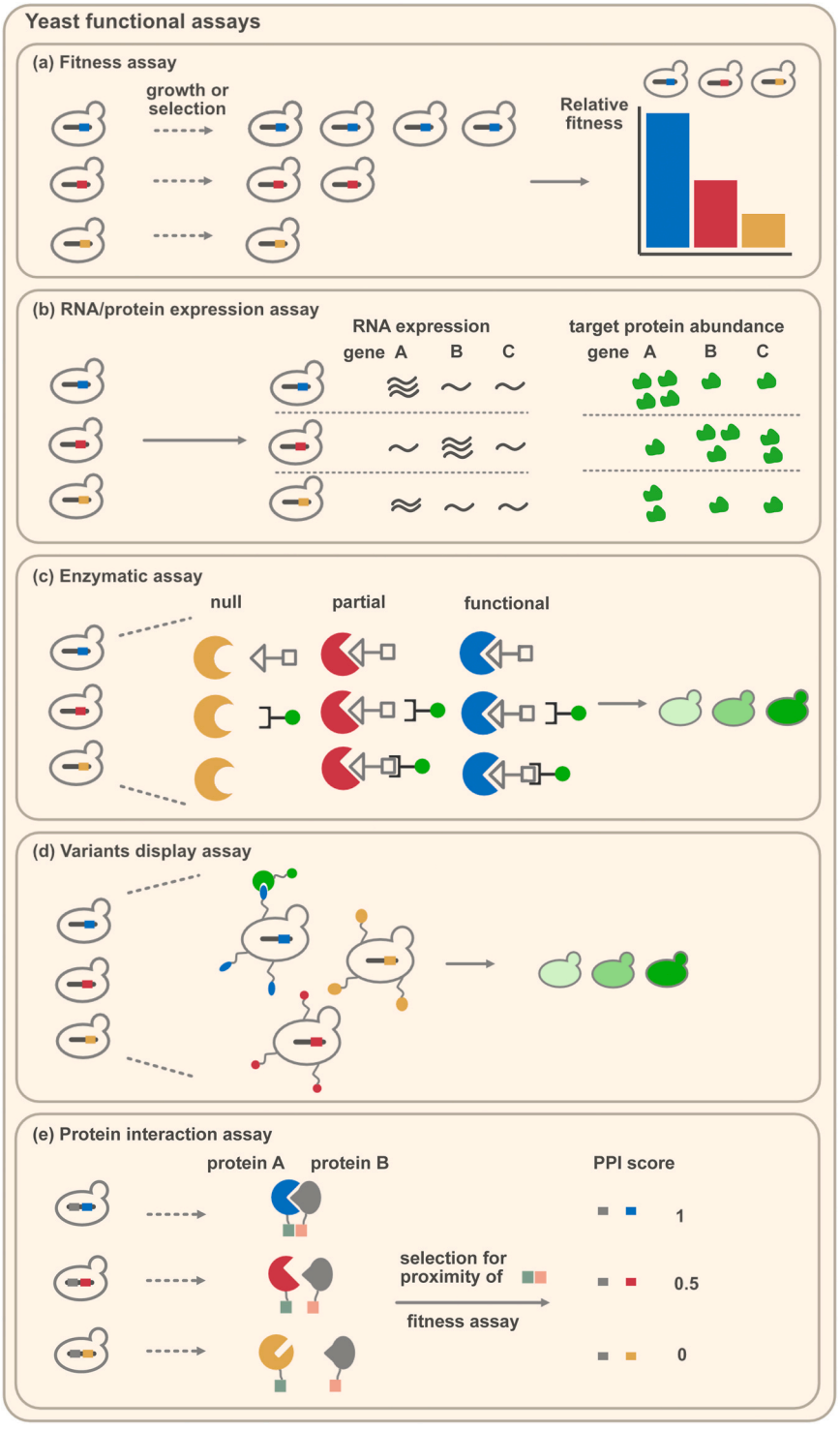
[46,52,53]. Oligonucleotide synthesis also allows for deep interrogation into single substitutions and can include more complex variants, or variants with more than one mutation, but has a higher cost and error rate [47,54,55]. Saturation mutagenesis can now be performed endogenously with CRISPR/Cas9 editing, namely by designing synonymous mutations in the donor DNA strand or introducing a heterologous intermediate sequence to prevent unintentional recognition and cleavage due to strand complementarity to the guide RNA [37,56] (Figure 2b). Similarly, improvements in the use of single-stranded oligodeoxynucleotides in yeast permit the scaling of precise, multisite editing without double-stranded breaks [57]. Sequencing full variants or short DNA tags that act as barcodes for each variant allows the tracking of these variants throughout an assay, which can be used to infer variant function (Figure 3).

MAVEs in yeast have been useful for predicting how new mutations alter phenotype, not just for yeast genes, but for genes from other systems as well (Figure 3). Remarkably, many human genes complement their orthologous *S. cerevisiae* gene knockouts, sequence homology can be taken advantage of for those that do not, and even genes without homologs can be functional when heterologously expressed in yeast [46,47,58–64•].

This style of high-throughput, pooled phenotype testing can also be applied to more complex allele libraries [48,65,67,68]. For example, we have generated and phenotyped a library of all natural alleles of one gene, including multiple SNPs and insertion/deletions in one haplotype [48]. This MAVE approach for understanding gene function on a species-wide scale reveals not only the natural fitness distribution of variants in this gene, but informs its evolutionary history as well. Decreases in cost along with increases in throughput and accuracy of long-read sequencing enable the genotyping of large libraries containing complex variants that are longer than what can be fully captured from high-throughput sequencing [68].

Finally, variant libraries need not be based on natural sequences at all. Generation of randomized sequences has been useful for understanding how translation and transcription factor-binding sites are utilized in promoter regions, with the effects of up to 100 million sequences

Figure 3



Summary of high-throughput functional assays in yeast. The starting library can be developed using plasmid, integrative, or CRISPR-based methods from Figure 2. These functional assays include but are not limited to **(a)** Fitness assay [48], **(b)** RNA or protein-expression assay [23•,27•–29], **(c)** Enzymatic assay [46], **(d)** Variants display assay [64•], and **(e)** Protein-interaction assay [65–67]. Note that for simplicity, we only represent one variant library in the protein-interaction assay panel **(e)**. In reality, two sets of protein variant libraries can be used to explore protein–protein interactions [65,67].

determined in one experiment [69–71]. The increasing number of studies focusing on exploring this space enhances our understanding of variant effects and is fundamental to computational programs more accurately predicting function [29,72–76].

Conclusions

Advancements in approaches for identifying QTLs as well as the underlying causal genes and nucleotides have revealed an extraordinary amount of information about complex traits. High-throughput methods such as those described here facilitate moving beyond individual example cases and allow for general patterns to surface that begin to reveal the categories of complex traits and what is required for a comprehensive understanding of their genetic basis. Complex traits vary in patterns themselves and can involve multiple common variants, multiple rare variants, or a combination of both [15••,18••], justifying the need for independent interrogations of variants under multiple environments. For some traits, variants clustered in one locus can have effects in the same direction [36,77]; for others, however, variants can have canceling effects that result in neither variant being detected in a QTL [23•]. These variants can be coding or noncoding, and the effect sizes of these mutations are relatively similar [16,36]. Effects of variants can be additive, although greater sample sizes of segregants revealed that the resulting phenotypes are more indicative of epistatic interactions [15••,18••,19•,35••]. Identifying epistatic interactions is still challenging, and most high-throughput approaches for doing so have been through whole-gene deletions or engineered mutant alleles [67,78–81] or by investigating genetic network changes as a result of single-gene perturbations [29,82]. Thus, the effects of most pairwise and complex SNP interactions at the genomic scale remain to be determined. Impacts of higher-order interactions are still rather elusive, although a recent study shows that this can be investigated using a hierarchical gene-drive system [83].

The end goal of these studies is to understand the principles behind how genotypic changes alter phenotype. Saturation-level analysis can be achieved by taking a gene-centric approach to understand how variation in a locus affects phenotype. Good candidates for such analysis are the causal genes identified by QTL studies. Certain genes (such as *MKT1*, *HAP1*, and *IRA2*) continually resurface in QTL maps, indicating a high degree of pleiotropy [16,18••,20,84–89]. Yet, how regulatory and substitution changes alter their function under these various environments and across many more alleles and genetic backgrounds is still largely unknown. Measuring the effects of genes using standing variation can reveal patterns of evolution and signatures of selection [48]. Insertion/deletion mutations are commonly seen in natural variants as well; although indel effects are poorly understood, they can now be investigated using MAVE approaches [90,91]. Moreover, coupling growth phenotypes with molecular phenotypes such as gene expression or protein abundance can lead to mechanistic understanding. They also illustrate a high-throughput way for studying distribution of fitness effects of genes, which is an important and long-standing question in understanding evolution.

Even with the high-throughput approaches developed to date, many challenges and prospects in identifying causal variants persist. In order to measure variant effects on complex traits, the traits must have phenotypes that can be measured accurately in high throughput. Additionally, knowing only one of the multiple traits affected by a pleiotropic gene may confound interpretations of how variation affects fitness. The impact of intergenic regions remains comparatively understudied as well. Increases in whole-genome sequencing have also revealed that causal CNVs explain a larger fraction of phenotypic variance when compared with SNPs [3]. Thus, future studies will need to move beyond nucleotide-level variants; increasing the throughput for studying the effects of mutations such as copy number variation, translocation, and aneuploidies will provide a more exhaustive view of how genotype affects phenotype. Finally, the success of heterologously expressing human genes in yeast to understand variant function is evidence that this versatile model organism can test gene function across other organisms as well. Applications of these high-throughput methods across taxa will inform the evolutionary history of selection, adaptation, and drift in spanning diverse populations.

Conflict of interest statement

None.

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