Transposon insertional mutagenesis in *Saccharomyces uvarum* reveals trans-acting effects influencing species dependent essential genes

Running Title

Gene essentially is influenced by trans effects

Authors

Monica R. Sanchez[1,2], Celia Payen[1], Frances Cheong[1], Blake Hovde[1], Sarah Bissonnette[3], Adam Arkin[5], Jeffrey M. Skerker[5], Rachel Brem[6], Amy A. Caudy[4] and Maitreya J. Dunham[1]

Affiliations

[1]Department of Genome Sciences, University of Washington, Seattle, Washington, United States of America

[2]Molecular and Cellular Biology Program, University of Washington, Seattle, Washington, United States of America

[3]Department of Biological Sciences, California State University, Turlock, California, United States of America

[4]Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada

[5]Energy Biosciences Institute, University of California Berkeley, Berkeley, California United States of America

[6]Molecular and Cellular Biology, University of California Berkeley, Berkeley, California, United States of America

* Corresponding author

Email : maitreya@uw.edu (MD)

Abstract

To understand how complex genetic networks perform and regulate diverse cellular processes, the function of each individual component must be defined. Comprehensive phenotypic studies of mutant alleles have been successful in model organisms in determining what processes depend on the normal function of a gene. These results are often translated to the increasing number of newly sequenced genomes by using sequence homology. However, sequence similarity does not always mean identical function or phenotype, suggesting that new methods are required to functionally annotate newly sequenced species. We have implemented comparative functional analysis by high-throughput experimental testing of gene dispensability in *Saccharomyces uvarum*, a sister species of *S. cerevisiae.* We created haploid and heterozygous diploid Tn7 insertional mutagenesis libraries in *S. uvarum* to identify species dependent essential genes, with the goal of detecting genes with divergent function. Comprehensive gene dispensability comparisons with *S. cerevisiae* revealed that approximately 12% of conserved orthologs are predicted to display diverged dispensability, including 22 confirmed differentially essential genes. Surprisingly, despite their differences in essentiality, these genes are capable of cross-species complementation, demonstrating that other *trans*-acting factors that are background dependent contribute to differential gene essentiality. Furthermore, we identified an instance of swapped essentiality between two paralogs, *CDC25* and *SDC25* between these two species. This data set provides direct experimental evidence of gene function across species, which can inform comparative genomic analyses, improve gene annotation and be applied across a diverse set of microorganisms to further our understanding of gene function evolution.

Keywords

Functional genomics, gene function evolution, yeast, insertional mutagenesis, essential genes, orthologs

Introduction

The ability to accurately predict gene function based on DNA sequence similarity is a valuable tool, especially in the current stage of genomic research where numerous genomes are increasingly becoming sequenced. It has become crucially important to predict gene function based on sequence similarity due to the lack of experimentally determined functional information associated with each newly sequenced genome. Most functional predictive methods rely on similarities of DNA sequence homology, co-expression patterns as well as protein structure to help assign function to uncharacterized genes, using genes where known functions have been previously characterized (Eisen 1998; Usadel et al. 2009). However, these methods come with their own set of limitations and often produce a substantial number of predictive errors, highlighting the importance of implementing experimental methods to directly test gene function of previously uncharacterized genomes to improve current methods of gene function annotation.

The gold standard of gene function characterization relies on targeted deletions of predictive coding sequences to probe the contributions of each gene to specific biological processes. To get a global view of gene function within an organism, several genome-wide deletion collections have been created in model species, particularly in bacteria and yeast, (Baba et al. 2006; Berardinis et al. 2008; Porwollik et al. 2014; Winzeler et al. 1999) including highly diverged species (Kim et al. 2010; Schwarzmüller et al. 2014) as well as different strains within a species (Dowell et al. 2010). These systematic deletion collections are powerful tools for investigating molecular mechanisms of gene function, biological pathways, and genetic interactions, especially in the genetic workhorse *S. cerevisiae,* where gene function characterization and gene dispensability comparisons have been extensively performed amongst various deletion collections of yeast (Costanzo 2016; Dowell et al. 2010; Kim et al. 2010; Tong et al. 2001). These studies have identified approximately 17%

of essential genes to be differentially essential between highly diverged species (*S. cerevisiae* and *S. pombe*) and have discovered 6% of essential genes (57) that are differentially essential even between two strains of *S. cerevisiae.*

However, considerable effort and resources are required to create these targeted, systematic libraries and they are not a practical approach for validating gene function across a wide range of non-standard genetic backgrounds in a high-throughput manner. Alternative approaches to targeted gene deletion libraries are transposon based mutagenesis methods used to create random insertional mutant collections, eliminating requirements for *a priori* knowledge about defined coding regions and providing information about partial loss of function or gain of function mutations. Random insertional profiling has been widely applied across various species and has been instrumental in understanding virulence genes, stress tolerance mechanisms and even tumor suppressor genes in mice (DeNicola et al. 2015; van Opijnen and Camilli 2013; de la Rosa et al. 2017; Weerdenburg et al. 2015; Yung et al. 2015; Coradetti et al. 2017). Several transposon libraries have also been implemented across diverged yeast species, providing useful information about gene function, growth inhibiting compounds and essential functional protein domains (Gangadharan et al. 2010; Guo et al. 2013b; Michel et al. 2017; Oh et al. 2010; Ross-Macdonald et al. 1999; Zhao et al. 2017; Price et al. 2016) .

Here we utilize a random insertional method that has allowed us to assay gene dispensability of approximately 50,000 mutants in *Saccharomyces uvarum,* a species that diverged from *S. cerevisiae* approximately 20 million years ago and contains approximately 80% identity in coding sequences to *S. cerevisiae* (Dujon 2010; Kellis et al. 2003; Scannell et al. 2007). These species can inter-mate to create hybrids, allowing us to leverage the large genetic toolsets established in *S. cerevisiae* to more fully explore the genetic basis for possible differential gene dispensability among these species. Genes with different

dispensability patterns between these two species can be used as a preliminary indicator of divergent gene function, providing a model for investigating gene function evolution between two diverged species of yeast. While this Tn7 insertional density is modest in comparison to other insertional mutant libraries (50,000 compared to > 300,000 insertions) we successfully validated a subset of predicted differentially essential genes, proving this approach to be useful for prioritizing genes for testing viability (Michel et al. 2017; Guo et al. 2013a). Furthermore, this Tn7 transposon mutagenesis library provides a valuable resource for studying *S. uvarum* gene function and serves as a framework for comparative functional genomics studies across newly sequenced, previously uncharacterized species.

Results

Generating Tn7 insertional libraries in *S. uvarum* to predict essential and nonessential genes

One initial step to identifying genes with divergent gene function is to identify mutant phenotypes that are different between two diverged species. One of the most straightforward phenotypes to characterize is cell viability or gene dispensability. Therefore, we first sought to characterize gene essentiality in *S. uvarum*, with the aim of identifying genes that are differentially essential between *S. cerevisiae* and *S. uvarum*. Instead of creating a library of individual knock out strains, we applied a high-throughput approach of creating random insertional mutants and leveraged the power of sequencing to identify the insertion sites in a pooled collection. The Tn7 mutagenesis library approach described by Kumar *et al.,* (2004) was used to create a collection of *S. uvarum* mutant strains and has been previously described by Caudy *et al.,* (2013). Briefly, *in vitro*

transposition of the Tn7 transposon was performed in a plasmid library containing random *S. uvarum* genomic fragments. The Tn7 transposon was designed to carry a ClonNat resistance marker that carries stop codons in all reading frames near both termini. The interrupted genomic fragments were excised out of the plasmid and integrated at their corresponding genomic positions, likely producing truncations when inserted within coding regions (**Supplemental Fig. 1A**). The plasmid library contains ~50,000 unique genomic insertion sites that were integrated into a diploid and a haploid *MAT***a** strain at a 10X coverage (additional details can be found in **Supplemental Information**). Pools of mutants from each Tn7 library were grown up in liquid cultures as described in Materials and Methods. Insertion sites were determined using sequencing methods as described in detail in the **Supplemental Information** along with DNA sequencing library preparation protocols (**Supplemental Fig. 1C**).

The distribution of haploid, diploid and overlapping (present in both libraries) insertion sites are evenly distributed throughout the *S. uvarum* genome, as illustrated in **Supplemental Fig. 2.** (Detailed information about overall sequencing coverage is listed in **Supplementary File 4.**) Once the insertion sites were determined in both libraries, we counted the number of insertion sites in each annotated open reading frame using a custom Python script (Materials and Methods). **Supplemental Table 1** summarizes the number of insertion sites and the number of genes that contain insertion sites within each library, including the initial plasmid library. All annotated *S. uvarum* genes containing the number of insertion sites from each library are listed in **Supplementary File 5.** Of the 5,908 annotated genes, a total of 5,315 (90%) genes harbor insertion sites that were identified in at least one library. Comparisons between shared genes and unique genes harboring insertion sites are illustrated in **Supplemental Fig. 3.**

Since the essentiality of most genes is expected to be conserved between *S. cerevisiae* and *S. uvarum,* we used the known essential set in *S. cerevisiae* to test if essential genes in the haploid library contain fewer insertion sites. We identified a significant reduction in the number of inserts present in known *S. cerevisiae* essential genes in the haploid library (Wilcoxon test $p < 2.2\ e^{-16}$, essential average inserts/kb=0.88, SD=1.28 vs. non-essential average inserts/kb = 4, SD = 4.38) (**Supplemental Fig. 4**), indicating that essential genes are effectively targeted by this approach. However, due to the nature of the library, insertional events at different positions across a gene may result in a partial loss of function. Since essential genes may still tolerate some insertions, we instead relied on comparisons between the diploid and haploid libraries to make inferences about gene essentiality. Specifically, we calculated an insertion ratio using the number of inserts per gene in the haploid library divided by the number of inserts in the diploid library, which inherently normalizes for the length of the gene (Materials and Methods). Using the insertion ratio as a metric, we also identified a significant difference between *S. uvarum* genes whose orthologs are known to be essential (Wilcoxon rank tests Sc_E:Sc_NE $p < 2.2e^{-16}$) (**Supplemental Fig. 5B**). Using the insertion ratio, we categorized genes as essential or non-essential using a null distribution to rank genes above or below a cut-off metric of 0.25 (details described in **Supplemental Information**). Using this cut-off value, 1170 genes were categorized as essential genes. We applied an additional cut-off metric (more details in Material and Methods) to remove a class of low coverage genes, resulting in a total number of 718 (13%) predicted essential genes and 3,838 (65%) genes that are predicted non-essential, with 1299 genes (22%) undetermined (genes without inserts in the diploid library). We proceeded to characterize each gene set and validate the dispensability of each of the predicted gene categories.

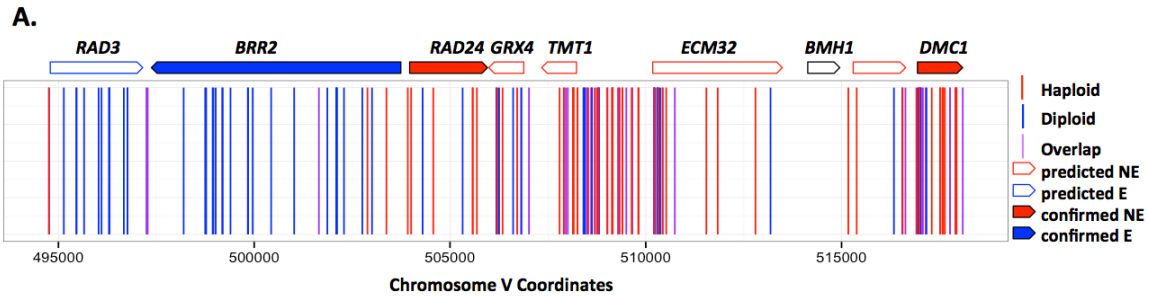
1

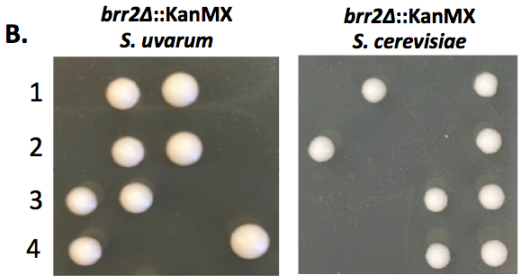
2 **Analysis of predicted gene dispensability**

3 The predicted gene list of *S. uvarum* essential genes was compared to known
4 essential genes lists from both *S. cerevisiae* and *S. pombe* to determine the amount of
5 conservation that exists between orthologs across diverged species. Of the predicted 718 *S.*
6 *uvarum* essential genes, 297 genes (42%) are shared amongst all three sets, with a total of
7 487 genes (68%) shared with at least one other set. Furthermore, 9 genes that are *S.*
8 *cerevisiae* strain specific, including 4 genes that are S288C specific and 5 genes that are
9 ∑1278b specific, overlap with *S. uvarum* predicted essential genes (**Supplemental Fig. 6)**.
10 Similar to what has been previously shown in *S. cerevisiae,* predicted essential genes in *S.*
11 *uvarum* were more likely to be unique, with 91% of essential genes (656/718) being
12 present in single copy compared to 76% of non-essential genes (2736/3604). Additionally,
13 comparisons between Gene Ontology (GO) molecular function terms of essential gene sets
14 from both species show significant enrichment (p-value < 0.01) for fundamental biological
15 functions. Processes such as DNA replication/binding, RNA and protein biosynthesis, as
16 well as structural constituents of the ribosome and cytoskeleton were enriched in both sets
17 of essential genes (**Supplemental File 6)**. In contrast, non-essential genes were
18 significantly (p-value < 0.01) enriched for regulatory functions (transcription factor
19 activity) and conditional responsive processes, such as transmembrane transporter activity
20 and cell signaling (kinase activity) (**Supplemental File 7)**.

21 Once we determined that many of the features of the predicted essential genes
22 were similar to confirmed essential genes in other species, we proceeded to create
23 heterozygous deletions to validate 13 conserved essential genes. Sporulating each
24 heterozygous deletion strain and performing tetrad analysis for cell viability confirmed
25 essentiality for 12 (92%) of the 13 strains (**Supplemental Table 2**). One example of a

confirmed essential gene can be found in **Figure 1A,** which illustrates the genomic positions of all insertion sites across a genomic locus of chromosome V that contains essential and non-essential genes. The color of the gene outline matches the predicted dispensability, which is determined by their insertion ratio. For example, the gene *BRR2* has an insertion ratio of 0.130 and is predicted to be a conserved essential gene (**Figure 1C**). The tetrad analysis of a *BRR2* heterozygous deletion strain displays a 2 viable:2 inviable segregation pattern in both species, validating this gene as a conserved essential gene (**Figure 1B**). Images of all other confirmed essential genes are located in **Supplemental Fig. 7.** We also tested three conserved non-essential genes and all three (100%) were confirmed as non-essential (**Supplemental Fig. 8**) (**Supplemental Table 2**). Additionally, we obtained an independent set of haploid deletion strains (see Materials and Methods), which was used as a validated non-essential gene set. Out of the total 356 gene deletions that were included in our library, 346 of those genes were predicted to be non-essential (97%) while the remaining 3% were predicted to be essential indicating that a significant proportion of our predicted non-essential genes were correctly predicted.





C.

Gene	Insert ratio (hap/dip)	Prediction
RAD3	0.0	E
BRR2	0.130	E
RAD24	2.0	NE
GRX4	1.0	NE
TMT1	3.33	NE
ECM32	1.25	NE
BMH1	N/A	N/A
DMC1	1.85	NE

Figure 1. Validation of conserved essential and non-essential genes. A) Mapped chromosomal insertion positions are plotted across chromosome V. Haploid inserts are indicated in red, diploid inserts are blue and overlapping inserts are indicated in purple. Genes indicated across the top are outlined according to predicted dispensability and filled in if confirmed. B) Tetrad analysis of a confirmed conserved essential gene *brr2Δ* in *S. cerevisiae* and *S. uvarum.* Segregants containing *brr2Δ* alleles are inviable in both species. C) Table indicating the insertion ratio (number of haploid inserts by the number of diploid inserts) per gene. The final column lists the predicted classification (NE, non-essential; E, essential; N/A, no data).

Gene dispensability comparisons of orthologous pairs between *S. cerevisiae* and *S. uvarum*

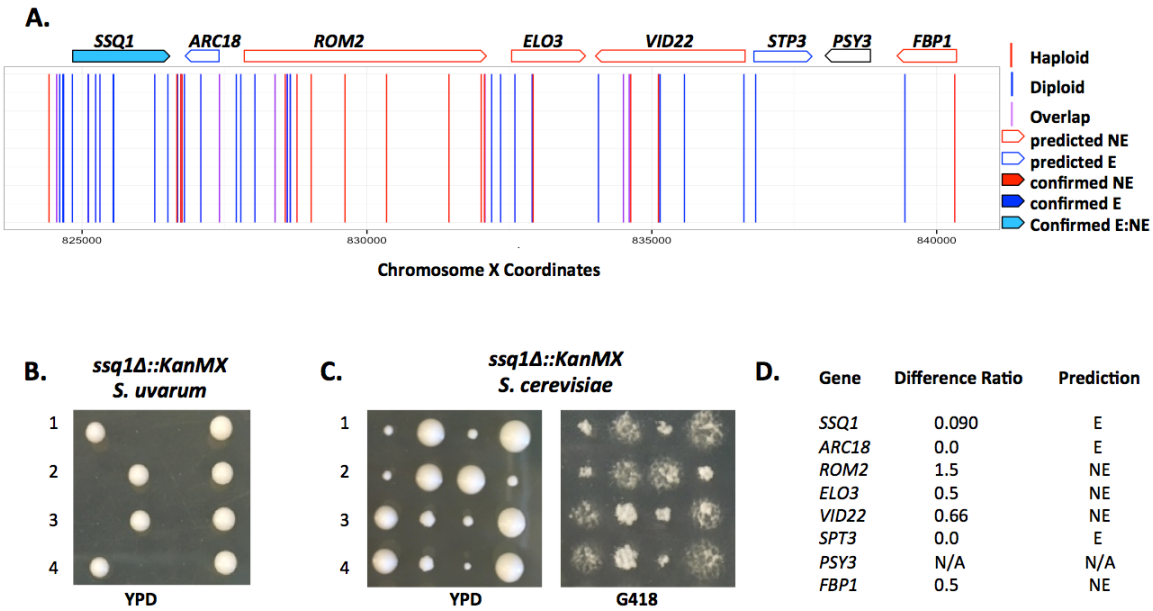
Our main goal of this project is to identify genes with differential essentiality to test for evidence of divergent function. While the previous section categorized all annotated *S. uvarum* genes, we narrowed our analysis to 4,543 orthologous genes for which we had data in the *S. uvarum* dataset to make direct comparisons of dispensability between *S. cerevisiae*

and *S. uvarum* (**Supplementary File 8**). Overall, 88% (4016/4543) of these genes display conserved dispensability between *S. cerevisiae* and *S. uvarum.* The remaining 12% of orthologs are predicted to differ in essentiality between the two species, with 304 (7%) of these genes only essential in *S. uvarum* and 221 (5%) genes only essential in *S. cerevisiae* (**Supplemental Figure 9**). Note the larger number of predicted essential genes in *S. uvarum* (304 in *S. uvarum* compared to 222 in *S. cerevisiae*). This difference may be attributed to the reliance on the absence of data (lack of insertion sites in a haploid gene) in a greater proportion of genes not previously characterized as an essential gene in *S. cerevisiae* (3687 non-essential genes vs. 765 essential genes), whereas, the latter category utilizes the presence of insertional data in a smaller proportion of genes that are known to be essential in *S. cerevisiae*. All predicted genes that differ in dispensability are listed in **Supplemental File 9.**

To analyze the two categories of genes that differ in essentiality further, we compiled a list of 222 genes from the *S. cerevisiae*-specific category and a more restrictive list (Materials and Methods) of 220 *S. uvarum*-specific genes to normalize the number of genes from each species. Using this list, we determined the proportion of *S. cerevisiae*-specific and *S. uvarum*-specific genes annotated for each function by performing Gene Ontology (GO) term finder using the molecular function ontology. The proportion of essential genes that differ between species for each functional category are represented in **Supplemental Figure 9B,** illustrating a subset of all significant functional categories. Interestingly, the most striking difference is in the functional category of the structural constituent of the ribosome. This category is enriched for genes that are predicted to be essential in *S. uvarum* (46/52). Additionally, differences exist between essential genes in the category of RNA polymerase activity, where 9/10 genes were identified from predicted

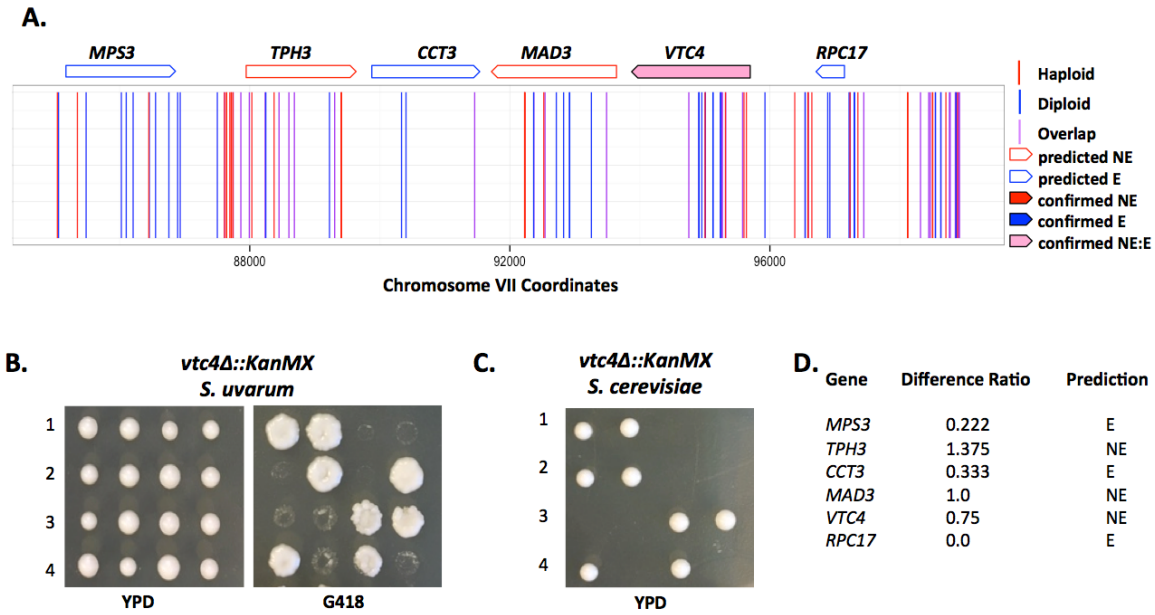
S. cerevisiae essential genes. Full lists of significant (p-value < 0.01) GO enrichment molecular function terms for each species individually are listed in **Supplemental File 10.**

To confirm a subset of these predicted essential genes within each genetic background, we applied the same method previously described to confirm conserved essential genes by sporulating heterozygous deletion strains to determine the viability pattern of the segregants. For example, gene *SSQ1* is an example of a confirmed *S. uvarum*-specific essential gene, illustrated in **Figure 2**. An example of one confirmed *S. cerevisiae*-specific gene is *VTC4,* illustrated in **Figure 3.** Overall, we confirmed a total of 22 predicted *S. uvarum*-specific and *S. cerevisiae*-specific essential genes (tetrad analysis can be found in **Supplemental Figs. 10 and 11** respectively). Interestingly, there are a variety of growth phenotypes associated with confirmed species-specific genes. All combined tetrad analysis results from the confirmation tests, also including false positives, are represented in **Supplemental Fig. 12**. **Supplemental Table 2** summarizes the total number of genes confirmed in each category. We note the higher false positive rate in the species-specific essential gene categories and attribute this to the low density of the overall library coverage.



Gene	Difference Ratio	Prediction
SSQ1	0.090	E
ARC18	0.0	E
ROM2	1.5	NE
ELO3	0.5	NE
VID22	0.66	NE
SPT3	0.0	E
PSY3	N/A	N/A
FBP1	0.5	NE

Figure 2. Validation of *S. uvarum*-specific essential gene *SSQ1*. A) Mapped chromosomal insertion positions are plotted across chromosome X. Haploid inserts are indicated in red, diploid inserts are blue and overlapping inserts are indicated in purple. Genes indicated across the top are outlined according to predicted dispensability and filled in, if confirmed. Light blue filling indicates a gene that is essential in *S. uvarum* and non-essential in *S. cerevisiae* (confirmed E_NE). B) Tetrad analysis of a heterozygous *ssq1Δ::KanMX* strain displaying inviable segregants containing the *ssq1Δ* allele in *S. uvarum.* C) Tetrad analysis of a heterozygous *ssq1Δ::KanMX* strain in *S. cerevisiae* containing viable segregants plated on YPD and G418. D) Table indicating the insertion ratio (number of haploid inserts by the number of diploid inserts) per gene. The final column lists the predicted classification (NE, non-essential; E, essential; N/A, no data).



Gene	Difference Ratio	Prediction
MPS3	0.222	E
TPH3	1.375	NE
CCT3	0.333	E
MAD3	1.0	NE
VTC4	0.75	NE
RPC17	0.0	E

Figure 3. Validation of *S. cerevisiae*-specific essential gene *VTC4*. A) Mapped chromosomal insertion positions are plotted across chromosome XII. Haploid inserts are indicated in red, diploid inserts are blue and overlapping inserts are indicated in purple. Genes indicated across the top are outlined according to predicted dispensability and filled in if confirmed. Light pink filling indicates a gene that is essential in *S. cerevisiae* and non-essential in *S. uvarum* (confirmed NE_E). B) Tetrad analysis of a heterozygous *vct4Δ* strain displaying viable segregants with the *vtc4Δ* allele in *S. uvarum* plated on YPD and G418. C) Tetrad analysis of a heterozygous *vtc4Δ* allele in *S. cerevisiae* resulting in inviable segregants. D) Table indicating the insertion ratio (number of haploid inserts by the number of diploid inserts) per gene. The final column lists the predicted classification (NE, non-essential; E, essential; N/A, no data).

In some cases, validation failures could be explained by errors in genome annotation, not errors in our insertional library results. One gene, *DRE2,* was predicted to be a non-essential gene but was confirmed as an essential gene through tetrad analysis**.** Although this gene was initially called as a false positive, closer analysis revealed that all the haploid insertions were clustered at the 5' end of the gene (**Supplemental Fig. 13**). Performing protein alignments of *DRE2* between *S. cerevisiae* and *S. uvarum* revealed an

annotated start codon in *S. uvarum* upstream of the annotated start codon in *S. cerevisiae.* It is likely that the gene was misannotated in *S. uvarum*, and instead shares the methionine start position further downstream. Using the reannotated gene coordinates, we would correctly classify *DRE2* as essential in *S. uvarum* since the haploid insertions were no longer included in the open reading frame. This one example highlights the utility of this library to improve gene annotation in addition to characterizing gene essentiality.

To determine if the differences in dispensability could be explained by gene expression, we compared a previously described metric used to quantify differences in gene expression between orthologous genes. We compared this metric in known genes that differ in essentiality and did not find evidence of genes enriched for expression differences, suggesting that gene expression alone cannot account for the differences in species dependent essentiality **(Supplemental Figure 14).**

Paralog divergence and duplicate gene loss explain some background effects on differential gene dispensability

Once we confirmed a list of genes that differed in dispensability between species and ruled out obvious expression differences, we set out to determine what genetic background effects could be contributing to the differences in dispensability between *S. cerevisiae* and *S. uvarum.* One explanation could be genetic redundancy due to gene duplications, such that a gene is nonessential in one species due to the presence of a paralog, whereas the other species contains only a single copy. To investigate this possibility, we began investigating genes that differed in dispensability and had paralogs. Of the 222 *S. cerevisiae*-specific essential genes, 11 were known to have paralogs. For example, *CDC25* is an *S. cerevisiae*-specific essential gene (nonessential in *S. uvarum)* and is a paralog to *SDC25*, which contains a premature stop codon in *S. cerevisiae.* We performed

complementation assays by cloning *S. uvarum* alleles of both paralogs into a CEN/ARS plasmid and testing whether the *S. uvarum* alleles could rescue the inviable phenotype of segregants from a heterozygous *cdc25Δ* deletion in *S. cerevisiae*. We found that *SDC25* from *S. uvarum* is functional and that both *SDC25* and *CDC25* alleles from *S. uvarum* can complement a *cdc25Δ* deletion in *S. cerevisiae* (**Table 1)**. Although we did not test for complementation in the *S. uvarum* background, the results from the complementation assays suggest that perhaps *CDC25* is required for growth in *S. cerevisiae* due to the lack of redundancy as a consequence of the non-functional copy of *SDC25.*

Following this same logic, we hypothesized that *CDC25* non-essentiality in *S. uvarum* could be attributed to the redundancy provided by the functional copy of *SDC25* in this species. To test this idea, we created an *S. uvarum* mutant heterozygous for both *cdc25Δ sdc25Δ* and performed segregation analysis on the dissected tetrads (**Table 1**). Unexpectedly, the segregation pattern of a double mutant displays a lethal phenotype for not only the double mutant but also the single *sdc25Δ* mutant. We confirmed this result by constructing an *sdc25Δ* heterozygous mutant in *S. uvarum* and found a 2:2 segregation pattern showing that *SDC25* is an essential gene in *S. uvarum.* Although there is no clear explanation for the requirement of *SDC25* in *S. uvarum,* this comparison displays one clear example of paralog divergence in essentiality between these two species.

Genotype	*S. cerevisiae*	*S. uvarum*
cdc25Δ	-	+
sdc25Δ	+	-
cdc25Δ + ScCDC25	+	N/D
cdc25Δ + SuCDC25	+	N/D
cdc25Δ + SuSDC25	+	N/D
cdc25Δ + ScSDC25	-	N/D
sdc25Δ + ScCDC25	N/D	-

Table 1. Viability summary of gene deletions and complementation assays. Signs in columns indicated by each species represent viability (-:inviable, +:viable, N/D:not done). Complementation assays are represented by the gene deletion with the addition of each gene expressed on a low copy plasmid.

In addition to redundancy differences that are attributed to divergence between pairs of paralogs, gene gains and losses may also contribute to genetic background effects that result in differential dispensability between *S. cerevisiae* and *S. uvarum.* Previous studies have investigated gene gains and losses across the *Saccharomyces* clade and identified genes that lost their duplicate in some species but not others. For example, *ALR1* is found as a singleton in *S. cerevisiae* but has retained the duplicate copy in *S. uvarum.* The *ALR1* gene is a confirmed *S. cerevisiae*-specific essential gene, which may be explained by the loss of the other copy of the duplicate pair. We created a heterozygous deletion of both *ALR1* copies in *S. uvarum* and tested the viability phenotypes of each mutant and of the double mutant separately. Surprisingly, each single mutant and the double mutant were viable. Furthermore, *ALR2* (the paralog of *ALR1)* is also nonessential, and even the triple mutants had no phenotype **(Supplemental Fig. 15).**

Divergent gene dispensability is largely due to trans effects

While genetic redundancy or gene loss is a possible explanation for a fraction of differentially essential genes, the remaining much larger portion of this class of genes remained unexplained. Because our main goal for this study was to find evidence of gene function divergence between these two species, we proceeded to further investigate the remaining differentially essential genes for functional differences. For a subset of these genes, we performed complementation assays in both species to test for divergent function. We cloned five *S. cerevisiae* alleles from the list of *S. uvarum*-specific essential genes (*SAC3, TUP1, CCM1, SSQ1,* and *AFT1)* and seven *S. uvarum* alleles from the list of *S. cerevisiae*-specific essential genes (*ALR1*, *SHR3*, *CDC25*, *INN1*, *LCD1*, *SEC24*, *VTC24*) into a CEN/ARS plasmid to perform complementation tests in *S. uvarum* and *S. cerevisiae* **(Supplemental Fig. 16)**. The results from these complementation tests revealed that all genes are able to complement the inviable phenotype in the other species, suggesting that the differences in essentiality are more likely to be due to *trans*-acting changes rather than functional differences of protein coding regions.

Discussion

In this study, we applied a comparative functional genomics approach to investigate how genetic background influences gene dispensability between two diverged species of yeast. Using insertional integration comparisons between haploid and diploid pools of mutants, we prioritized genes to validate as predicted essential, non-essential and differentially essential gene categories in *S. uvarum.* We predicted approximately 12% of orthologs to differ in dispensability between *S. uvarum* and *S. cerevisiae* and validated 22 genes in this category. Surprisingly, however, most genes that differ in dispensability have retained their function between these two species, suggesting that differences in gene

dispensability are likely due to *trans*-acting changes rather than the direct result of divergent coding sequence.

Specifically, our comparison of orthologous genes between *S. cerevisiae* and *S. uvarum* revealed that a majority of genes maintain conserved dispensability requirements (88%) while 12% of orthologs are predicted to be essential in one species but not the other. We confirmed 93% (15/16) of predicted conserved categories of essentiality and 49% (27/55) of genes predicted to be differentially essential. Although our rate of confirmed genes in this category was lower than the conserved category, we correctly identified a subset of genes that are differentially dispensable, despite the moderately dense insertional profile of the library and a less restrictive cut-off value applied to include more genes to be classified as this type. Further analysis of predicted species-specific essential genes revealed enriched GO ontology terms of molecular functions involved in structural constituent of the ribosome and DNA binding, although more precise analysis of functional enrichments may require more thorough validation to remove the influence of false positives. Finally, we utilized genetic tools in *S. uvarum* to test hypotheses about genetic background effects that contribute to differences in essentiality. We find that differences can be explained by paralog divergence and *trans*-acting factors.

Applying a random insertional approach has proved to be useful in functionally profiling *S. uvarum* and will be useful for studying other understudied species, with the goal of adding information to gene annotation methods. While this study was performed in standard laboratory conditions, it is easily amenable for testing stressful conditions, other nutrient sources as well as naturally relevant conditions. This library can be applied to probe previously un-annotated genes or even proto-genes for functional acquisition, since it is not restricted to *a priori* assumptions of genic boundaries. The identification of synthetic lethal interactions can also be determined by performing insertional profiling in the

background of a particular mutation of interest relatively quickly and economically. Additionally, pooled competition experiments *en masse* can be used to determine the frequency of particular insertional mutants, providing quantitative measurements of cellular fitness across conditions. Such a strategy could be efficiently employed using computational approaches to prioritize experimental conditions that are most likely to probe the most valuable phenotypic information for further functional characterization (Guan et al. 2010).

Gene regulation also plays a large role in evolution and is crucial for responding to environmental change (Carroll 2005). In previous studies, we aimed to functionally characterize differences in gene expression patterns between *S. cerevisiae* and *S. uvarum* and discovered species-specific responses to osmotic stress, peroxisome biogenesis and autophagy, suggesting that each species may been exposed to different selective pressures within their respective evolution histories (Caudy et al. 2013; Guan et al. 2013). Interestingly, we did not find that genes with different gene expression patterns between species were more likely to be differentially essential. Instead, *trans* genetic interactions dominate. Identifying the molecular basis of these *trans* effects can now be undertaken, potentially revealing principles of genetic interactions across species.

Materials and Methods

Strains, plasmids and primers

The strains, plasmids and primers used in this study are listed in **Supplementary Files 1**, **2** and **3** respectively. All *S. uvarum* strains are derivatives of the sequenced strain CBS 7001 (previously sometimes called *S. bayanus* or *S. bayanus* var *uvarum*), and all *S. cerevisiae* strains are of S288C background. Unless specified below, yeast strains were grown at 25°C for *S. uvarum* strains and 30°C for *S. cerevisiae* strains and standard media recipes were used.

Construction of the Tn7 mutagenesis library

The construction of the Tn7 plasmid library has been previously described in detail and was obtained from the Caudy lab (Caudy et al. 2013). Briefly, this mutagenesis approach uses a plasmid library of *S. uvarum* genomic DNA, containing random Tn7 transposon insertions. The construct has a selectable marker for transformation into yeast, allowing the selection of disruption alleles.

To make the plasmid library, genomic DNA was isolated and fragmented by sonication to an average length of 3 kb from a *rho0 S. uvarum* strain. The ends of the DNA were blunted and cloned into the pZero Blunt vector (Invitrogen). Approximately 50,000 colonies were recovered from the transformation into *E. coli* DH5α strain. The transformants were scraped from Kanamycin plates and pooled for plasmid purification. A version of the Tn7 transposon was constructed by amplifying the promoter from the Tet-on pCM224 (Bellí et al. 1998). The cassette of the Tet-on promoter and the ClonNAT resistance gene was amplified using PCR primers containing lox and BamHI sites and cloned into the BamHI site of the NEB vector pGPS3. This transposon construct was inserted into the *S.*

uvarum genomic DNA library *in vitro* using the transposon kit from NEB. Initial selection (50,000 colonies) was on ClonNAT/Zeo. HindIII and XbaI were used to digest the pZero backbone to release the linearized genomic DNA for efficient recombination. The library was then used to transform a haploid *S. uvarum* strain (ACY12) and a diploid strain (YMD1228) using a modified transformation protocol optimized for *S. uvarum* (Caudy et al. 2013). Transformant colonies were plated to YPD-ClonNat plates and allowed to grow for 5 days at 25°C. A total of ~ 500,000 colonies were scraped for each pool. Each final pool was well mixed at a 1:1 ratio with 50% glycerol and 2 ml aliquots were stored at -80°C.

Pooled growth of Tn7 *S. uvarum* libraries

To determine the initial complexity of the integrated pools, genomic DNA was extracted directly from the glycerol stocks of both haploid and diploid pools using the Hoffman and Winston method (Hoffman and Winston 1987). Additionally, we inoculated 500 µl of both libraries in separate YPD flasks for 24 hours to recover mutants after 24 hours of growth. Furthermore, to collect samples over time, we competed both pools under sulfate-limiting conditions in chemostats for approximately 30 generations at 25°C. A large-volume, ~300ml, sulfate-limited chemostat (Gresham et al. 2008) was inoculated with a single 2ml glycerol stock sample of each pool. After allowing the chemostat to grow at 25°C without dilution for ~24 hrs, fresh media was added to the chemostat at a rate of 0.17 h-1. This pooled growth assay was repeated twice, each including 5 time points with O.D. and dilution rate measurements as well as collected cell pellets for DNA extractions using the modified Hoffman-Winston prep referenced above.

Tn7 sequencing library preparation

Sequencing libraries were prepared by first extracting genomic DNA from pools of each library grown in YPD and sulfate limited conditions. Genomic DNA libraries were prepared for Illumina sequencing using a Tn7-seq protocol described previously (Wetmore et al. 2015). Briefly, the Covaris was used to randomly fragment DNA to approximately 200-800 bp in length. The fragments were blunt ended and A-tails were added to the fragments to ligate the Illumina adapter sequences. Custom index primers (listed in **Supplementary File 3**) targeting Tn7-specific sequence and Illumina adapter sequence were used to enrich for genomic DNA with Tn7 insertion sites. The barcoded libraries were quantified on an Invitrogen Qubit Fluorometer and submitted for 150 bp-paired end sequencing on an Illumina HiSeq 2000 by JGI. This method was also applied to make the plasmid library, from linearized plasmid DNA.

Sequencing analysis

Sequencing reads from the FASTQ files were trimmed to remove Tn7 specific sequences and adapter sequences, restricting the minimal length of reads to 36 bp using Trimmomatic (Bolger et al. 2014) and FASTX-Toolkit. Trimmed FASTQ files were aligned against the reference strain of *S. uvarum* (CBS 7001) using Burrows-Wheeler Aligner (BWA) with standard filters applied (Li and Durbin 2009). Specifically, non-uniquely mapping reads, reads in which the pair did not map, reads with a mapping quality less than 30 and PCR/optical duplicate reads were filtered out; the samtools C-50 filter was applied as recommended for reads mapped with BWA. To limit the insertional analysis to actively growing cells, SAM files were merged from the later time points in the growth assays of each pool using samtools (Li et al. 2009). The sequence coverage of the nuclear genome ranged from 70 to 300x (**Supplementary File 4**). Insertion sites were determined from SAM files using a custom Ruby script. Insertion sites that had 10 reads or more were

processed through a custom Python script that counted the number of insertion events in each coding region across the genome. This pipeline was applied to both libraries and further comparisons were made between the pools to determine essential genes. Read data have been deposited at the NCBI under the SRA accession number SRP115313.

Predicting gene dispensability between species

In order to determine a list of predicted essential genes, comparisons were made between the haploid and diploid libraries. We calculated an insertion ratio by dividing the number of insertions in the haploid pool by the number in the diploid pool. This direct comparison inherently accounts for the length of the gene, since the length is constant in both libraries. Therefore, a decrease in insertion sites in the haploid library indicates a reduction in the presence of mutants containing insertional sites that impact cellular viability. Ratios closer to zero represent insertional mutants that reduce the frequency of haploids harboring insertional sites in a coding region that is required for cellular growth.

To make an insertion ratio cut-off value to categorize essential and non-essential genes, we analyzed the distribution of insertion ratios within intergenic regions between 500 bp and 7 kb in length and positioned between Watson and Crick coding regions (so chosen because these are less likely to contain promoter sequences). The distribution of the insertion ratio calculated for these regions was similar to that of known non-essential genes in *S. cerevisiae.* Therefore, we used this distribution to rank the insertion ratios of all coding regions and set a cut-off value to 0.25 where 20% of the insertion ratio of coding regions fell below the intergenic distribution, which was similar to the kernel density estimates of known *S. cerevisiae* essential genes. The kernel density estimates were computed in R using X and visualized using ggplot2. To remove a class of low coverage genes in the essential gene category, we applied an additional cut-off value. Since the difference between 0 and 1

with a gene that is longer has a lower weighted difference than a shorter gene, we calculated the difference between the diploid pool and haploid pool and normalized this value to the length of the gene (normalized difference). Genes with less than a normalized difference of 2 were removed from the essential category.

Validating predicted essential and non-essential genes

We validated predicted essential genes by creating *S. uvarum* heterozygous diploid deletion mutants using primers listed in the **Supplemental File 3.** Primers containing 50 bp of homology upstream and downstream of each candidate open reading frame were used to amplify the KanMX cassette from the pRS400 plasmid. The PCR product was used to integrate into the *S. uvarum* genome using an *S. uvarum* specific transformation protocol. The proper integration of the construct was validated through clone purifying positive colonies and extracting genomic DNA to perform PCR using diagnostic primers listed in the **Supplemental File 3.** The diagnostic primers were designed to target ~150 bp upstream and ~150 bp downstream of the open reading frame to identify wild type and drug marker alleles. Positive clones were sporulated for 3-5 days at 25°C and 8 tetrads were screened for 2:2 viable segregation. Images were taken after 4 days of growth on YPD plates. Mutants conferring non-essential phenotypes were replicated on G418 plates and images were taken after 4 days of growth at 25°C (**Supplemental Fig. 9&10**). This method was also applied to making double mutants. A collection of 440 *MATα S. uvarum* strains was generated by standard methods in the Rine lab and used as confirmed non-essential genes.

Cross-species complementation assays

To determine if genes are diverging in gene function or in other trans-acting factors, we performed cross-species complementation assays with species-specific essential genes.

Essential genes that were *S. cerevisiae* specific were tested in a heterozygous diploid deletion strain from the magic marker collection. Alleles of each *S. cerevisiae* essential gene were amplified from *S. cerevisiae* and *S. uvarum* genomes and cloned into a CEN ARS plasmid. PCR using Phusion DNA polymerase was used to amplify 500 bp upstream and 5 bp downstream of the stop codon of each gene from *S. cerevisiae* and *S. uvarum.* Each gene was cloned into pIL37 by Gibson assembly using primers listed in **Supplemental File 3** using standard methods (Thomas et al. 2015)**.** All plasmids used in this study are listed in **Supplemental File 2.** The *S. cerevisiae* heterozygous diploid deletion strains were transformed with a plasmid containing a corresponding allele from each species and selected on C-URA plates. Similarly, *S. uvarum* specific essential genes were also tested by making each heterozygous diploid deletion strain *ura3Δ/ura3Δ,* and transformed with a plasmid containing a corresponding *S. cerevisiae* allele from the MoBY-ORF collection (Ho et al. 2009).

Transformed strains were sporulated for 5 days at 30°C and 25°C for *S. cerevisiae* and *S. uvarum* species, respectively and tetrad analysis was performed on YPD plates. After 3 days of growth, plates were replica plated on C-URA and YPD+G418 plates and imaged after 2 days of growth (**Supplemental Fig. 15)**.

Data Access

The transposon insertion data in this study have been submitted to the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra/) under SRA accession number SRP115313.

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Competing financial interests

The authors declare no competing financial interests.

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