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Comparing the utility of *in vivo* transposon mutagenesis approaches in yeast species to infer gene essentiality

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ABSTRACT

Background:

In vivo transposon mutagenesis coupled with deep sequencing enables large-scale genome-wide mutant screens for genes essential in different growth conditions. Six large scale studies have now been performed with three yeast species (*S. cerevisiae, S. pombe* and *C. albicans*), each mutagenized with two of three different heterologous transposons (*AcDs*, *Hermes,* and *PiggyBac*).

Results: We analyzed predictions of gene essentiality for each of the six studies and evaluated the ability of the data to predict gene essentiality using a machine-learning approach. Important data features included a sufficient number of independent insertions and the degree of random insertion distribution. All transposons showed some bias in insertion site preference, both because of jackpot events, specific insertion sequence preferences and preferences for short-range vs long range insertions. For *PiggyBac*, a stringent target sequence limited the ability to predict essentiality in genes with few or no target sequences. Furthermore, the machine learning approach is robust for predicting gene function in less well-studied species by leveraging cross-species orthologs. Finally, comparisons of isogenic diploid vs haploid *S. cerevisiae* isolates identified several genes that are haplo-insufficient, while most essential genes, as expected, were recessive.

Conclusions: We provide recommendations for the choice of transposons and the inference of gene essentiality in genome-wide studies of eukaryotic microbes such as yeasts, including species thathat have been less amenable to classical genetic studies. These include maximizing the

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number of unique insertions, avoiding transposons with stringent target sequences and a method for cross-species transfer learning.

INTRODUCTION

Work with model yeasts such as *Saccharomyces cerevisiae* and *S. pombe* has pioneered the combination of genetype/phenotype comparisons at a genomic scale. For these yeasts, with genome sequences available for over 20 years[1, 2] and facile gene replacement protocols, have relied heavily on comprehensive collections of deletion mutants[3, 4] for high throughput dissection of specific genotypes as well as for genetic interactions with drugs (reviewed in Lehár et al.[5]) and for gene-gene interactions through systematic analysis of double and triple mutant analysis (e.g., Reguly et al.[6], Kuzmin et al.[7]). In animals and plants that are less amenable to such directed molecular manipulations, the use of heterologous transposons *in vivo* has facilitated genetic analysis, within the limitations imposed by the transposon excision/insertion process[8, 9]. With the advent of deep sequencing, such studies have also become more facile and have been performed in the two model yeasts as well[10, 11].

In vivo transposon mutagenesis generally involves the introduction of a heterologous DNA transposon, along with the genes (e.g., the relevant transposase) required to induce its active transposition into a clonal isolate of a species of interest. Upon induction, the transposase excises the transposon from its original location (excision site) and inserts it into a single new position in the genome. Each cell harbors, at most, a single transposition mutation because the frequency of transposon excision and reinsertion is quite low. The transposon is usually engineered for facile

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selection of excision and/or reinsertion events, allowing detection and enrichment of these rare events.

In vivo transposon insertion provides several advantages, as it rapidly yields large numbers of mutants in a single step and easily can be performed in parallel strains with different mutations or genetic backgrounds. Because it does not require much prior knowledge, it can also be performed in non-model species, where each experiment is likely to be highly informative. The only transformation steps required are those used to engineer the starting strain. This bypassing the problem of low transformation efficiency in many species. It also avoids the unintended genome alterations (e.g., aneuploidies) that often accompany DNA transformation[12]. The sites of transposon insertion throughout the genome can be identified *en masse* using very large collections of independent insertion event clones, coupled with deep sequencing of the DNA immediately adjacent to the new transposon locus. For example, a high throughput genotype/phenotype analysis of 30 bacterial species grown under >170 different nutrient and stress conditions recently assigned functions to thousands of genes including ~300-600 genes per bacterium that are essential for viability[13].

Three different transposon systems have been used for *in vivo* mutagenesis in yeasts: *AcDs from Zea mays, Hermes from Musca domestica and PiggyBac, from Trichoplusia ni*. *AcDs* has been used primarily in plant species but was also engineered for increased efficiency in the model yeast *S. cerevisiae*[14] and, later, in *C. albicans*[15]. *AcDs* does not display any insertion sequence preference, although it has a higher frequency of insertions into intergenic regions than coding regions and has a bias for reinsertion near the initial site of excision. *Hermes* has been used for mutagenesis in *S. pombe* and *S. cerevisiae*[17, 18]; it prefers to insert at genomic positions with

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the target sequence TnnnnA. *PiggyBac* (PB) has been used in mammalian systems such as rat, mouse[19] and also in *S. pombe*[17]. *PB* has a strong preference for insertion at TTAA sequences, which are generally more frequent in A-T-rich intergenic regions than within coding sequences.

Transposon insertion within an ORF is generally assumed to cause a loss-of-function mutation. Identifying the phenotypes associated with loss-of-function mutations in specific genes allows the prediction of genetic functions. Cells in which the transposon inserted into a gene essential for viability will fail to grow and thus be lost from the population. By contrast, cells with mutations in non-essential genes are expected to be well-represented in the cell population. Insertion of a transposon carrying a strong promoter into an ORF could activate expression inappropriately; can be useful for the study of gain-of-function mutations.

In vivo transposon mutagenesis studies of yeasts include analysis of *S. cerevisiae* with *Hermes* (this study) or with the mini-Ds derivative of the *AcDs* system[10], in *S. pombe* with *Hermes*[11] and *PB*[20] and in *C. albicans* with *AcDs*[21] and with *PB*[22] (Fig. 1A). In earlier work, we applied a machine learning (ML) approach to infer gene essentiality from the *C. albicans AcDs* data. Here, we modified the ML approach to predict the likelihood of essentiality for the complete set of predicted open reading frames these sixth *in vivo* transposon datasets. We compared the strengths and challenges of the different transposons in each species, with the goal of reaching insights concerning the number of insertion events required for accurate predictions, the distribution of mutations, and the degree to which different transposons, with different sequence dependencies, provided similar or different conclusions. The goal was to provide metrics that assist in determining the advantages and disadvantages of different transposon systems so as to

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optimize the data produced in a given *in vivo* transposon system and to suggest approaches for generating whole genome data in understudied yeast species.

RESULTS AND DISCUSSION

A comparative analysis of the transposon mutagenesis studies

We compared six *in vivo* transposon insertion mutagenesis experiments, produced using three different heterologous transposons (*AcDs, Hermes* and *PiggyBac*) in three different species (*S. cerevisiae, S. pombe and C. albicans*). Details of the datasets are provided in the methods section and relevant parameters are highlighted in Table 1. The number of transposition events detected in the different studies varied considerably, from over 500,000 unique insertion sites (hits) and 84 M total reads for the *C. albicans AcDs* (*CaAcDs),* to as few as 37,500 unique hits and 6.1 M reads in the *S. pombe PiggyBac* (*SpPB*) data set (Table 1). The number of reads per hit also varied considerably, from 41 to 170.

Overview of ML approach for gene essentiality prediction

We first mapped the hit and read frequency of the transposons in each of the three reference genomes (Fig. 1C). Sites of transposon insertion were identified based upon targeted sequencing of regions adjacent to the inserted transposon. Slightly different sequencing protocols were used in the different studies, but all six essentially amplified Tn-adjacent sequences and mapped them. Theoretically, essential genes have no hits and non-essential genes have many hits; however, distinguishing essential and non-essential genes from the data is not entirely straight-forward

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(e.g., Fig. 1C, Gene X_n). To address this ambiguity, we extended a previous approach for gene essentiality prediction[21]. We first chose input features from transposon data that were likely to be informative in the essential/nonessential decision: the number of unique insertion sites (hits) per ORF, the degree to which those insertion sites were enriched in the population (reads), as well as normalization factors that consider the insertion frequency as a function of chromosome position.

Training sets were built using information from the two model species with gene essentiality data available from classical genetic approaches (e.g., comprehensive ORF deletion analysis) (reviewed in Giaever and Nislow[23] and Spirek et al.[24]) (Table S1 (training sets)). The specificity and sensitivity of the approach was analyzed using the AUC (area under the receiver operating characteristic curve (Fig. 1D). For *C. albicans,* which did not have extensive prior knowledge of gene essentiality, we constructed a training set from a core set of genes whose orthologs were known to be essential in both model yeasts. This approach is likely to be useful for other species that lack sufficient prior knowledge of gene essentiality to construct a within-species training set.

We assessed the performance of each classifier by producing training sets using genes known or inferred to be essential and non-essential, as described in the Methods. The classifiers were trained and their performance, assessed using the AUC measure, was high across most examined studies (>0.94). The one exception was the *SpPB* study, which had far fewer unique insertion sites (Table 1) and had an AUC of 0.785. The highest AUC levels were seen with the *AcDs* in both *C. albicans* and *S. cerevisiae*. Of note, these two studies also had the largest number of total hits and reads. All the considered ML features for each ORF in every study and the predicted verdicts of

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essentiality are provided in Tables S2-S7. Below, we describe the main insights gained from this comparison.

Insight #1: Optimize the number of independent insertion sites (hits) for highest quality predictions of gene essentiality

The total number of unique insertion sites (hits) and the performance (AUC values) were high highly correlated, and this correlation was statistically significant (Fig. 2a; Pearson's r = 0.892; p-value = 0.0169). By contrast, the total number of sequencing reads showed a weaker correlation with the AUC that was not statistically significant (Fig, 2b; Pearson's r = 0.636; p-value = 0.1741). If we disregard the worst performing *SpPB*, the correlation of the AUCs with the total number of hits rises dramatically to Pearson's r = 0.995; p-value = 0.0003, and the correlation with the total number of sequencing reads remains similarly weak: Pearson's r = 0.652; p-value = 0.2327. Thus, it a library with many independent hits will improve performance and simply increasing the number of sequencing reads is not likely to be sufficient to obtain optimal results. Increasing the number of independent hits requires collection of sufficient numbers of independent colonies soon after transposase induction and the resulting transposon excision and reinsertion. An advantage of *Hermes* is that most insertions occur during stationary phase, so transposase-inducing conditions can be tolerated throughout the growth period. Experimental designs that optimize isolation of independent events are critical.

Insight #2: Avoid libraries with high levels of jackpot events

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Jackpot events are the appearance of extraordinarily high numbers of reads in a very small number of insertion sites. When the number of reads greatly exceeds the theoretical number of cell divisions in the experiment, this is likely due to a transposition event happened prior to the induction of transposon excision in the experiment. Jackpot events are a major pitfall in that much sequencing capacity is wasted on detection of a single insertion site. Jackpot events with >1M sequencing reads were present in 4 of the 6 data sets; *SpPB* and *ScHermes* had no major jackpot events (no hits with ≥1000-fold more reads than the average read/hit) (Table 1). Both of these libraries also had far fewer total sequencing reads than the other studies (6M and 18M vs 24-84M for the other libraries).

Of note, within a data set, some individual experiments had jackpot events that were far more than others (Table 1), which would be expected if jackpot events occur stochastically. Importantly, jackpot events were not clearly associated with one of the three species or with the transposon type. This suggests that jackpots arise from technical, rather than biological issues.

Avoiding jackpot events is important because they reduce data quality considerably: the higher the number of reads at a few jackpot sites, the lower the number of informative hits and reads. Avoiding the selection of cells in which a transposon was already mobilized is key to ensuring that the number of hits and reads provide good genome coverage. Dividing the cultures into dozens of small cultures and then re-pooling these sub-cultures after transposase induction can effectively dilute out most jackpot events. Preparing several independent libraries and sampling a few sequences in each may also be worthwhile. For example, if a tested library shows one sequence twice in one hundred colonies, it is likely to be a >1M jackpot event.

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Insight #3. Consider which features are most important in the analysis of a given transposon

In decision tree based algorithms, such as Random Forest[25], every node is a condition of a split of the data by a single feature. The splitting process continues until it reaches a stop condition such as: all the features have been used, the obtained subset is very small or the training labels are the same for the obtained subset. The goal is to reduce entropy (uncertainty) in the data. Entropy is zero when all the labels in the obtained subset are the same; and is maximum when half of the labels are the same in the obtained subset (in a binary classification). Each split of the data by a given feature (node in the tree) reduces the entropy. The importance of a given feature in the Random Forrest classifier is the calculated decrease in entropy contributed by that feature. Here we describe the features of the classifiers, and discuss their relative importance.

For each ORF, we calculated predictive features including the number of hits, number of reads and the length of each ORF, a neighborhood index, which normalizes for insertion bias due to genomic position (e.g., proximity to the initial excision site in the genome) and a freedom index, which reports the proportion of an ORF that is hit-free. The freedom index is especially useful for identifying genes with essential domains, that are able to tolerate insertions outside of the essential domain. The number of the transposon hits per transposon target sequences in an ORF was an additional feature used in the analysis (Figure 3), where applicable (in *PB* and *Hermes* studies). Furthermore, we calculated the number of hits and the number of reads normalized by the length of each ORF. We compared the 'feature importance' for each library to ask whether specific features were more important for the different classifiers and whether feature importance was characteristic for a given transposon or yeast species.

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The number of hits per ORF played an important role in determining essentiality, with essential genes having far fewer hits than non-essential ORFs (~7 times less, on average, across the 6 datasets), consistent with the strong correlation between number of hits and the AUC (Fig. 2A). The number of reads per ORF played a lesser role in these classifications, also consistent with the correlation above (Fig. 2B). Gene length also affected the probability of transposon insertion in a gene, and thus was a crucial normalization parameter for the numbers of hits and reads for every ORF.

The Neighborhood Index (NI) feature made important contributions in all of the classifications (except *SpPB*, which had far less data). Importantly, the NI did not differ considerably between the different transposons, consistent with the idea that chromatin accessibility, 3D chromosome organization and other factors that may bias the insertion site frequency in a given organism affect the frequency of insertion of different transposons in a similar manner.

The Freedom Index (FI) was a major contributor to both *ScAcDs* and *CaAcDs* predictions while results with the *PB* and *Hermes* datasets were mixed (Fig. 3). This is consistent with the idea that *AcDs* does not have a specific target sequence and thus inserts throughout ORFs, while *PB* and *Hermes* have fewer target sequences within ORFs. Thus, the FI more important in *AcDs* experiments because hits occur more randomly throughout an ORF.

The importance of the number of hits in the proximal regulatory sequences (100 bp upstream to the start codon) to the essential/non-essential predictions was only minor, but is highly variable. For example, the impact of *ScHermes* was nearly twice that of *ScAcDs* for this feature. As

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described further below, we posit that this difference is due to cryptic enhancer/promoter activity in the miniDs transposon in *S. cerevisiae* that not seen with the *Hermes* transposon.

Insight #4: Consider the effect of transposon-specific target sequence specificity

Some transposons have preferred sites of insertion: *Hermes* prefers TnnnnA and *PiggyBac* inserts primarily at TTAA sequences; *AcDs* does not have an insertion site preference. Theoretically, the length of the insertion site sequence necessarily scales inversely with the number of potential unique hit sites. However, it was not clear at what insertion sequence length the resolution of studies of gene essentiality becomes limiting.

The feature importance of the number of hits per transposon target sequence in an ORF, which should be a measure of library saturation, showed a varying degree of importance in the *PB* and *Hermes* studies. Curiously, its importance wasn't dependent on the type of the transposon or the target sequence prevalence in the genome. This likely because target sequences are preferred sites of insertion, yet are not exclusive or absolute. For example, *PiggyBac* in *C. albicans* had 1.6-fold more unique insertion sites than the theoretical number of target sequences in the *C. albicans* genome. By contrast, for both *ScHermes* and *SpHermes*, the number of target sequences available far outnumbered the number of unique hits. The proportion of target sequences not hit ranged from 8.9% for *CaPB* to 85% for *SpHermes* (Table 1) and the proportion of hits not in target sequences ranged from 14% in *SpPB* to ~50% in *CaPB* as well as both *Hermes* data sets. Thus, we surmise that the preference for target sequences is only a minor limitation for both of these transposons, except when the total number of hits is very low as in *SpPB*.

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Another critical issue is the number of genes that lack any preferred target sequences within the ORF; there are 228 and 185 ORFs without a single TTAA sequence in *C. albicans* and *S. pombe,* respectively. These ORFS have a lower probability of acquiring insertions and, if the genes are non-essential, they are much more likely to give false positive information (be predicted essential for lack of insertions). Indeed, 155 ORFs without TTAA sequences were predicted essential in the *CaPB* data and yet are predicted non-essential in the *CaAcDs* study. Similarly, 118 of the 185 ORFs lacking TTAA sequences were predicted essential from the *SpPB*study, but were non-essential in the *SpHermes* study. We assume that many of these genes are false positives, especially given that 127 of the 185 ORFs lacking TTAA, including 95 of the 118 aforementioned ORFs, were non-essential in classical genetics studies of *S. pombe.*

Next, we asked if the number of target sequences within an ORF affected the *CaPB* classification performance for that ORF. To address this, we compared the performance (AUC) to sets of ORFs filtered to exclude ORFs with different numbers of target sequences (from 0 to 10) from the training set used to train the classifier (Fig. 4). The AUC increased from ~0.94 for the entire training set to >0.98 for the training set containing only genes with 10 or more target sites (~50% of the genes in the training set). This suggests that studies using the *PiggyBac* transposon may struggle to correctly infer gene essentiality for ORFs with low numbers of target sites.

Insight #5. Consider whether the transposon can activate as well as disrupt gene expression.

The prediction of essentiality was based upon the assumption that transposon insertion into an ORF disrupted gene expression and produced loss-of-function allele. However, this is not

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necessarily the case for all genes. For example, if an insertion allele removes a regulatory domain from a protein, the protein may become hyperactive, and thus the result would be a gain-of-function allele. Additionally, some transposons may introduce enhancer and promoter activities that could increase gene expression in some species. The miniDs transposon used in the *ScAcDs* data is likely to contain such activities.[10] Consistent with this idea, the *ScAcDs* dataset contains an average of 1.89 insertions within the first 100 bp of essential genes whereas the other datasets including *CaAcDs,* which has a transposon modified from the *miniDs,* contain significantly fewer (0.82 insertions in the first 100 bp of Ess genes). Additionally, many essential genes of *S. cerevisiae* appeared to tolerate miniDs insertions, but not *Hermes* insertions, at sites in the 5' UTR that are very close to the start codon. Thus, the *miniDs* transposon in *S. cerevisiae* may facilitate inappropriate activation of gene expression when inserted upstream or within certain genes.

Cross-study analysis

Knowing the full set of essential and non-essential genes in eukaryotic microbes, including pathogens of humans, animals and plants, will improve our understanding of common and species-specific properties of these understudied organisms. Furthermore, once a transposon library has been collected, it can be screened under many other growth conditions to reveal genotype/phenotype relationships. *In vivo* transposon analysis of gene essentiality is a practical and feasible approach, because the cost in time and resources for obtaining libraries is far lower than that for producing engineered deletion mutants, especially given that the amount of baseline information (other than the genome sequence) about the organisms may be minimal. The only technical hurdle is to introduce the heterologous transposon of interest, either on a plasmid (where feasible) or into a useful locus within the genome of the relevant organism.

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An additional challenge is that ML approaches require a high-quality training dataset (of gold-standard essential and non-essential genes). For many non-model organisms, such training data is too sparse to build a robust training set. For *C. albicans,* we circumvented the low numbers of genes already known to be essential by relying upon genes that had been determined to be essential from comprehensive classical genetic deletion studies in both model yeasts (*S. cerevisiae* and *S. pombe)* and that had orthologs in *C. albicans.* Training on *S. cerevisiae* or *S. pombe* orthologs with consistently essential orthologs yielded good performance predictions for *C. albicans* (AUC: 0.940 to 0.993, Table 1). *CaAcDs* performance was lower when training only on the 66 genes known to be essential plus the set of presumed non-essential genes (those that had been successfully deleted in *C. albicans* studies, AUC of ~0.92)[21].

Next, we considered the quality of the learning performance for each dataset, if we trained on orthologs from one species and predicted essentiality of genes in a different organism (Figure 5). The transfer learning performance of the classifications was of a comparable quality to the single study classifiers for most *AcDs* and *Hermes* cases (Figure 5a and Figure 2). Furthermore, it displayed a somewhat symmetrical property: in most cases, there were minor differences in performance between train/test and test/train pairs (reducing the quality by ~ 0.5% to 5.7%) when the tests were between or among *AcDs* and *Hermes* experiments. Conversely, when testing for predictions from *PB* data that were trained on either *AcDs* or *Hermes,* the AUCs dropped more dramatically (up to ~21.9%). Thus, *PB* data was less transferable than the *Hermes* and *AcDs* data.

The low *PB* transferability between *SpPB* and *CaPB* is likely due to the sparser target sequence distribution relative to either *Hermes* or *AcDs*, which causes *PB* studies to produce false positives

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as noted above, and thus might contribute to reduced performance in cross-study analyses. The lower performance of the classifiers in the original *PB* single studies (Table 1), also may have contributed to the reduced ability to predict essentiality in pools of *PB* mutants using cross-species models.

Reduced differences cross-study performance could also be due to differences between the importance of different features in the classifiers for the different datasets. To test this possibility, we correlated the vector of the relative feature importance for each study with the feature importance in all the other studies (Figure 5b). The analysis distinguished 3 groups within the 6 studies, based on the correlation coefficient values for feature importance between members of the group: *CaAcDs* and *ScAcDs* (Pearson's r = 0.902); *Sp Hermes* and *CaPB* (Pearson's r = 0.898); and *SpPB* and *ScHermes* (Pearson's r = 0.929). Notably, the quality of the transfer learning predictions appears to be independent of both the transposon type and the organism studied, with the exception of the *AcDs* studies. We presume that this is due to the lack of a specific target sequence for the *AcDs* transposon system.

Insight #6: As necessary, construct training sets using genes with orthologs in models where essentiality is known and then validate the training set manually.

We suggest that an initial training set of orthologous genes known to be essential and non-essential in related model organisms can be used to facilitate analysis of a transposon insertion study in a non-model organism with sparse essentiality information. An important caveat is that differences between gene function in different species can alter gene essentiality of a small number of these orthologs; thus, it is important to visually inspect this orthologous training set

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before applying it. The goal is to remove any genes with insertion patterns that are highly contradictory to the 'essentiality label' that the orthologs provided. For example, for *C. albicans*, the entire orthologous training set was reviewed in an unprejudiced fashion by three independent inspectors, who visually reviewed the insertion patterns in the *CaAcDs* data and manually labeled each gene as essential, non-essential or ambiguous. When all three inspectors classified a gene as non-essential (e.g., many insertions throughout an ORF within a genome region that had many insertions outside of that ORF) and the orthologs were labeled 'essential' in the two model yeasts, we removed that gene from the training set.

Once a training set has been established, and the features for the ORFs have been calculated, the Random Forest classifier can be run in a cross-validation scheme and the AUC can be calculated using the essentiality labels. This provides an efficient approach to obtain information about all of the genes in a species that has been sequenced but not subjected to much molecular manipulation. Clearly, the same approach can be used to compare the essentiality of the same sets of genes grown in different conditions as well, potentially providing large amounts of phenotypic data across an entire set of ORFs. If applied to a species that had not been the subject of many genetic studies, such data would represent a treasure-trove of information about genes that had not been previously studied and the phenotypes associated with loss-of-function of those genes.

A Comparative Analysis of Gene Essentiality Predictions

An important issue is whether different transposon insertion studies in the same organism had similar or different predictions from one another and from the known essentiality status of

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deletion mutants, which are by definition 'loss-of-function' null alleles. For *S. cerevisiae,* the classifiers displayed a high degree of agreement on the final verdicts of gene essentiality (Figure 6a), while more discrepancies were evident for the *C. albicans* and *S. pombe* studies. Both *PiggyBac* studies predicted a much higher number of essential genes than the *AcDs* or *Hermes* studies (Figure 6b and 6c) as expected from the paucity of target sequences that are likely to give false positive predictions discussed above. For example, the *CaPB* study had an average 5.84 target sites per kb in genes likely to be false positives vs. 10.62 target sites per kb in all the genes (Mann Whitney U: p-value < $2.38*e^{-78}$). Importantly, when compared to the set of essential genes for each species determined by deletion analysis, the transposon studies also did quite well, with only 20 to 35% of the genes in disagreement. In some cases, such discrepancies were found to be due to issues with the deletion collection isolates. For example, ~8% of the original *S. cerevisiae* deletion collection carried aneuploidies or gene amplifications,[26] and ~10% of *S. pombe* deletion strains retained a wild-type copy of the ORF that had been targeted for deletion. Extra copies of the 'deleted' gene reduces the apparent number of essential genes.

Gene essentiality in haploid versus diploid strains of *S. cerevisiae*

S. cerevisiae is readily grown in both the diploid and haploid states, which allows identification of the haplo-insufficient subset of genes among the set of essential genes. Based on gene knockout studies, only 2 genes (*NDC1*, *MLC1*) were classified as haplo-insufficient,[27, 28] while all other essential genes were haplo-proficient (i.e. heterozygous knockouts in diploids were viable). To determine whether additional haplo-insufficient genes exist in *S. cerevisiae*, we collected *ScHermes* insertions in diploid strain BY4743 and compared them to haploid strains BY4741 and BY4742. *Sc Hermes* transposon mutagenesis libraries were used with the classifier that had been

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trained on the *SpHermes* haploid training set data, applying the same threshold for classification (Figure 8). The classifier identified 155 genes as "essential in both haploid and diploid", a number far higher than expected. Upon closer analysis, 98 contained regions of poor mapping due to duplications elsewhere in the genome, 50 were categorized as dubious in the Saccharomyces Genome Database (yeastgenome.org), one (*LEU2*) had been deleted in the strains studied, and the two known haploinsufficient genes (*NDC1, MLC1*) had been identified, providing support for this approach. Upon visual inspection of data of the remaining four genes, one essential gene (*BCY1*) appeared haploinsufficient, whereas another (*RPC10*) contained numerous insertions in its 5' UTR in diploids but not haploids, suggesting that it might not be essential (Fig. 9). The other two ORFs predicted to be haploinsufficient are very small (165-225 bp) and also are within regions of sparse insertion density. Thus, we have lower confidence in the data for these two genes. Thus, transposon mutagenesis of a diploid strain successfully revealed the two known haploinsufficient genes and one new one (*BCY1*), which is known to be essential in the conditions employed in the screen, but not essential in other culture conditions[29].

The classifier also identified 29 genes as "haploinsufficient" in diploids and not essential in haploids. Of these, 20 could be dismissed based on their annotation as dubious or the presence of duplicated (unmappable) segments. All of the remaining 9 genes were small (87-528 bp) and were found in regions of sparse insertion density. These genes are annotated in SGD as non-essential and are probably false positives.

These observations raise an important issue about data quality control. It is important to filter dubious and uninformative ORFs from the data set (as was done in the analysis of *CaAcDs*[21]. This includes genes with repeated domains or duplicate copies in the genome that prevent

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unambiguous mapping of short Illumina reads. Furthermore, predicting the essential/non-essential status for short ORFs and especially those located in regions with sparse intergenic insertions is more likely to be problematic.

Insight #7: Prediction quality increases considerably when uninformative data such as mitochondrial genome sequences and duplicated genes that are difficult to map.

In summary, we suggest a number of metrics for the inference of gene essentiality using *in vivo* transposon mutagenesis studies in yeasts, including those with little available genetic data. Maximizing the total number of unique transposon insertions is the most critical factor in achieving optimal performance of the classification. It can be attained by collecting many independent insertion clones, striving to reduce the possible jackpot events in the study, maximizing the depth of the coverage and by utilizing a transposon with a fairly permissive target sequence or no preferred target sequence. Furthermore, while transposons with relatively stringent target sequences have some advantages for screens that identify individual mutants, for determining gene essentiality they are less robust, as the low number of potential target sequences, and especially the lack of any target sequences, in certain ORFs will increase the likelihood of falsely classifying non-essential genes as essential. Additionally, we think that transposon mutagenesis is an ideal approach to gain large amounts of useful genotype/phenotype data understudied organisms: the cross-species learning methodology allows inference of gene essentiality based on conserved orthologs, especially when coupled with visual screening of the data. Finally, *in vivo* transposon mutagenesis is an incredibly useful tool for high throughout genomic studies, not only of gene essentiality *per se,* but also of genes required under specific

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selective conditions. We hope that the recommendations provided here will facilitate future work to understand gene in a wide range of yeast species.

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MATERIALS AND METHODS

Data Acquisition

Experimental

Sc Hermes data was obtained as follows: The haploid and diploid strains of *S. cerevisiae* were transformed with plasmid pSG36.[30] A single colony was suspended in 100 mL synthetic complete (SC) medium lacking uracil and containing 2% galactose, divided into twenty 16 x 150 mm glass culture tubes, and shaken for 3 days at 30°C. This protocol yielded ~5 x 10^6 cells bearing transposon insertions per mL (~3% of all cells). To enrich for cells bearing transposon insertions, the twenty cultures were pooled, centrifuged, and the cell pellet was resuspended in 600 mL SC medium containing 2% glucose, 0.1 mg/mL nourseothricin, and 1 mg/mL 5-fluoroorotic acid, and then shaken overnight at 30°C. The cells were pelleted, resuspended in 600 mL of the same medium, and cultured as before. Finally, 60 mL of these enriched cells were pelleted, resuspended in 600 mL of the same medium, and cultured as before. These highly enriched cells were pelleted, resuspended in 15% glycerol, and frozen in aliquots at -80°C. To extract genomic DNA, 100 mg of thawed cell pellets were washed three times in 1 mL deionized water and extracted using Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research). A total of 2.4 µg of purified gDNA was fragmented by sonication in four separate tubes using a Diagenode Picoruptor. The fragmented DNA was then end repaired, ligated to splinkerette adapters, size selected with AMPure xp beads, and PCR amplified in separate reactions using transposon-specific and adapter-specific primers as detailed previously.[31] Samples were then PCR amplified to attach Illumina P5 and P7 (indexed) adapters, purified with AMPure xp beads, mixed with phiX-174, loaded into MiSeq instrument (Illumina) and 75 bp of each end was sequenced using primers specific for Hermes right inverted repeat and P7. Detailed protocols and primer sequences are available upon request. De-multiplexed reads were mapped to the *S. cerevisiae* S288C reference genome using

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Bowtie2, and any mapped reads with a quality score < 20 or a mismatch at nucleotide +1 were removed. This process was repeated a total of 3 times in diploid strain BY4743, 2 times in haploid strain BY4741, and 1 time in haploid strain BY4742. The diploid and haploid datasets were combined prior to analyses. The *S.cerevisiae Hermes* data (mapped reads and counts) are available at http://genome-euro.ucsc.edu/s/CunninghamLab/Hermes%20Vs%20AcDs . FastQ files are available from Sequence Read Archive (SRA) and the ArrayExpress Experiment Archive (ArrayExpress), which are core repositories of the European Nucleotide Archive (ENA) at accession number [XXXX] https://www.ebi.ac.uk/ena/about/data-repositories (to be updated when accession number is issued).

Publicly available databases

The rest of the datasets analyzed here were obtained from previously published studies. *ScAcDs* was published by Michel et al, 2017[10], from which both WT1 & WT2 were combined for the analysis. The data was downloaded: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-4885/samples/. *SpPB* was published by Li et al, 2011[20] and the data was obtained from the SRA database: SRR089408. *Sp Hermes* was published by Guo et al, 2013[22] and the data was obtained from the SRA database: SRR327340. *CaAcDs* was published by Segal et al, 2018[21] and the data was obtained from the SRA database, where SRR7824843, SRR7824841 and SRR7824838 files, were combined for the analysis. *CaPB* was published by Gao et al, 2018[22] and the data was obtained from the SRA database, where all the following files were for the analysis: DMSO (untreatment): SRR7704188, SRR7704193, SRR7704196; 5-FOA (untreatment: SRR7704189, SRR7704194, SRR7704200; No drug screen: SRR7704195. All the SRR files were obtained using

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fastq-dump, with the following bash command: fastq-dump --gzip --skip-technical --readids --dumpbase --split-files --clip <SRR*******>

Data Processing

The .fastq files downloaded with fastq-dump, were processed using cutadapt to filter out reads not containing partial transposon sequences. Reads with transposon sequences were trimmed to remove the transposon sequences for alignment purposes, as follows: cutadapt --cores=8 -m 2 -g <primer sequence> <input fastq filename> -o <output fastq filename> --discard-untrimmed --overlap <overlap length>. In the analysis of the *Sc Hermes* study all the sequencing reads contained the transposon and the reads start at the first genomic base, thus required no filtering. In the analysis of the *Sp PiggyBac* we filtered the reads containing the transposons from the rest, by identifying the ACGCAGACTATCTTTCTAGGG sequence, cutting it out and aligning only the remaining part of the relevant reads. In the analysis of the *Ca AcDs* we filtered the reads containing the transposons from the rest, by identifying the GTATTTTACCGACCGTTACCGACCGTTTTCATCCCTA sequence, cutting it out and aligning only the remaining part of the relevant reads, starting 37bp downstream (Segal et al, 2018[21]). In the analysis of the *Ca PiggyBac,* we filtered the reads containing the transposons from the rest, by identifying the TGCATGCGTCAATTTTACGCAGACTATCTTTCTA sequence, cutting it out and aligning only the remaining part of the relevant reads, starting 3bp downstream. In the analysis of the *Sc AcDs* study we used the published transposon hitmaps of WT1 and WT2. In the analysis of the *Sp Hermes* study we used the published transposon hitmaps (Segal et al, 2018[21]).

Alignment of reads and mapping the transposon hits

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bowtie2 indices were created for each organism and gffutils databases were created for each organism's genetic features, using the latest versions of the reference genomes (fasta) and the genomic feature files (gff), which were downloaded from the respective official sources for the three organisms: *S. cerevisiae* from https://downloads.yeastgenome.org, *S. pombe* from ftp://ftp.pombase.org/pombe/ and *C. albicans* http://www.candidagenome.org/download/. Sequencing reads were aligned using bowtie2 with the default settings. The resulting sam files were converted to bam using samtools. bam files were sorted using samtools and indexed via pysam. Transposon hits and their corresponding reads were mapped to the respective genomes and counted in each genomic feature. Transposon target sites were found in every genome using Biopython and counted in each genetic feature.

Gene essentiality classification

Table 2 summarizes the features for machine learning classification that were engineered from the mapped transposon hits, reads and the transposon target sequences in the genomes. Random Forrest classification was performed, using Python's scikit-learn library with the default parameters, except the n_estimators parameter that was increased to 200, and the random_state parameter was fixed at 0, for reproducibility purposes. The results were validated using a 5-fold cross-validation. Essentiality labels for the training set of each organism were obtained previously (Shiftman et al, 2018) and are provided in Table S1.

Thresholds for the essentiality predictions in each classification were chosen as follows: Two metrics were evaluated (Figure 8): 1) Minimum of the Euclidean distance between (0, 1) and the receiver operating characteristic (ROC) curve. 2) Maximum of the vertical distance between the line describing a random choice (a straight line from (0, 0) to (1, 1)) and the ROC curve. The first method was chosen, and we verified that the second metric is reasonably close, to eliminate any

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possible artifacts. We predicted the essentiality of all the available genes for each organism based on their respective features, and using the aforementioned method to choose the threshold for each binary classification.

Figures were generated using Python's matplotlib and seaborn libraries. The schematics were drawn using Inkscape. Mann Whitney U p-values and Pearson's correlation coefficients and p-values were calculated using Python's Scipy.

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Figure Legends

Figure 1. Overview of data acquisition and analysis: transposition events to gene essentiality.

a. Three yeast species analyzed (Sp, *S. pombe*; Sc, *S. cerevisiae*; and Ca, *C. albicans*) by in vivo transposition in this study and which transposons (PB, PiggyBac; AcDs and Hermes) were used to mutagenize which species. Note that each species was analyzed with two different transposon systems.

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b. Comparison of genome insertion sites for transposition events that initiate from an extrachromosomal plasmid (left, red region of plasmid circle) or a specific chromosomal locus (right, red bar on a given chromosome). Horizontal lines represent multiple copies of the same genome, each of which underwent a single insertion event (green arrow) per genome. While transposition is generally random, a bias for loci in close proximity to the initial transposon insertion site demands normalization of the final data.

c. Mapped Tnseq analysis of the pool of transposition events yields the chromosomal insertion sites (brown vertical lines) in the reference chromosomes relative to the ORFs (purple regions). A close up of a small region of a single chromosome (olive horizontal line) including 5 ORFs is illustrated.

d. A training set is constructed using known or inferred labels (non-essential, blue; essential, red) together with extracted features calculated from the data and its position relative to ORFs. Features are defined in Table 2.

e. The training set features, as well as features for all ORFs are used as input for Random Forest classification (black rectangle); output is a prediction of essentiality (red or blue as in d), for which an optimal threshold is determined and applied to designate all genes in one of the two categories.

Figure 2. Contribution of unique hits and total number of reads to the quality of ML predictions for gene essentiality/non-essentiality.

Performance of the classifier vs (a) the total number of unique insertion sites (hits) and (b) the total number of sequencing reads, in each study (organism abbreviations as in Fig. 1a; Ac, AcDs; H, Hermes; PB, PiggyBac).

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Figure 3. Feature importances in the different classiffiers.

Importance of each feature used in the Random Forest classifier of essentiality for each dataset. Features are described in Table 2; Neighborhood index generally normalizes for non-random insertion frequencies across the genome; Freedom index reports on the largest proportion of an ORF that has no hits, which is a measure of domains that may be essential.[21]

Figure 4. Analysis of the ability of the CaPB classifier to infer gene essentiality in genes with increasing number of target sequences.

When only ORFs with a specific number of target sites are considered (>= x-axis), AUC rises accordingly (red), but the number of ORFs that can be analyzed necessarily decreases (numbers above red dots). This demonstrates the importance of the prevalence of the transposon target sequences in ORFs, for the quality of gene essentiality inference, using in-vivo transposon mutagenesis studies. X axis: Minimum number of target sequences per ORF needed for inclusion in the classification. Y-axis (red): CaPB classifier AUC.

Figure 5. Analysis of ROC AUC values for Random Forest classification trained on data from a different organism and/or transposon in all possible combinations.

a. For each ROC AUC value in the table, training was performed on 80% of the original training set used in the training species/transposon described in the rows. This training data was then used to predict the essentiality of the remaining 20% of the training set in the species/transposon described in the columns. The train/test split ratio was similar to the 5-fold cross-validation performed in the single study analyses.

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b. For each study, the vector of the relative feature importance was correlated with the feature importance in all the other studies. Pearson r correlation coefficient values are presented.

Figure 6: Comparison with known essentials genes.

a. Comparison of the essentiality verdicts in *S. cerevisiae*, based on the known essential genes from the literature, ScAcDs and ScHermes classifiers.

b. Comparison of the essentiality verdicts in *S. pombe*, based on the known essential genes from the literature, SpPB and SpHermes classifiers.

c. Comparison of the essentiality verdicts in *C. albicans*, based on the known essential Sp and Sc orthologs from the literature, CaAcDs and CaPB classifiers.

Classification thresholds differ slightly from the previously published analyses [21] based on threshold selection applied systematically to all 6 studies (described in detail in Methods).

Figure 7. Gene essentiality in haploid and diploid *S. cerevisiae*.

Comparison of essential genes in haploid and diploid *S. cerevisiae* analyzed with Sc Hermes. RF classifier was trained on the haploid ScHermes study and predicted gene essentiality in a diploid strain, using the same threshold for the final verdict. Mitochondrial genes were not considered.

Figure 8. Threshold optimization.

Two metrics were evaluated: 1) Minimum of the Euclidean distance between (0, 1) and the receiver operating characteristic (ROC) curve. 2) Maximum of the vertical distance between the line describing a random choice (a straight line from (0, 0) to (1, 1)) and the ROC curve.

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Figure 9. Suspected haploinsufficient genes in *S. cerevisiae*.

Four genes suspected to be haploinsufficient in *S. cerevisiae: NDC1, MLC1, RPC10 and BCY1*, as they appear in the UCSD genome browser [32]. *NDC1* and *MLC1* were known to be haploinsufficient. *BCY1* appears to be a previously unknown haploinsufficient gene. *RPC10* might not be essential as it sustained hits in the 5' UTR in diploids but not haploids.

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Click here to access/download Table Table 1 Sequencing data summary.docx

Table 1. Statistics of the transposon data sets

	Ca AcDs	Sc AcDs	Sc Hermes	Sp Hermes	Ca PiggyBac	Sp PiggyBac
Transposon target sequence	-	-	TnnnnA	TnnnnA	TTAA	TTAA
Initial transposon insertion	Genome	Plasmid	Plasmid	Plasmid	Genome	Genome
Number of target sequences (10^3)	-	-	1154.84	1302.41	120.27	111.37
Total number of unique hits (10^3)	588.97	514.89	444.41	382.82	191.49	37.5
Target Sequences without a hit (10^3)	-	-	924.56	1110.2	10.69	79.09
Percent of target sequences without a hit	-	-	80.06%	85.24%	8.89%	71.02%
Percent of hits in target sequences	-	-	51.82%	50.21%	56.74%	86.06%
Total number of reads (10^6)	84.16	47.1	18.22	23.92	32.58	6.14
Average number of reads per hit	143	91	41	62	170	164
Standard deviation reads per hit	9250	4357	137	5069	4584	481
Highest reads per hit (10^3)	3254.83	2210.55	11.72	2355.61	1301.82	48.31
Highest reads per hit / average reads per hit	22761	24292	286	37994	7658	295
Number of over 106 reads per hit	10	2	0	2	1	0
Average number of hits per gene	29.11	44.05	30.36	24.64	11.81	1.32
Number of genes with 0 hits	300	261	332	99	397	2580
Average number of target sequences per gene	-	-	123.64	131.44	10.62	8.39
Number of genes with 0 target sequences	-	-	0	0	228	186
ROC AUC	0.993	0.985	0.972	0.962	0.94	0.785

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Click here to access/download Table Table 2 Classification features.docx

Table 2. Classification features

Feature	Description
Hits	Number of transposon hits within the ORF
Reads	Number of reads associated with the transposon hits within the ORF
Neighborhood Index (NI)	Number of transposon hits within the ORF, normalized by length of the ORF and the surrounding 10 kbp
Freedom Index (FI)	Length of the largest hit-free region in the ORF, divided by the ORF's length
Hits 100 upstream	Number of transposon hits within the upstream region of the ORF
Hits per Target Seqs	Number of transposon hits divided by the number of transposon target sequences within an ORF
Reads per Length	Number of transposon hits divided by the length of the ORF
Hits per Length	Number of reads associated with the transposon hits divided by the length of the ORF

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Click here to access/download Figure Figure 1 Pipeline overview.docx

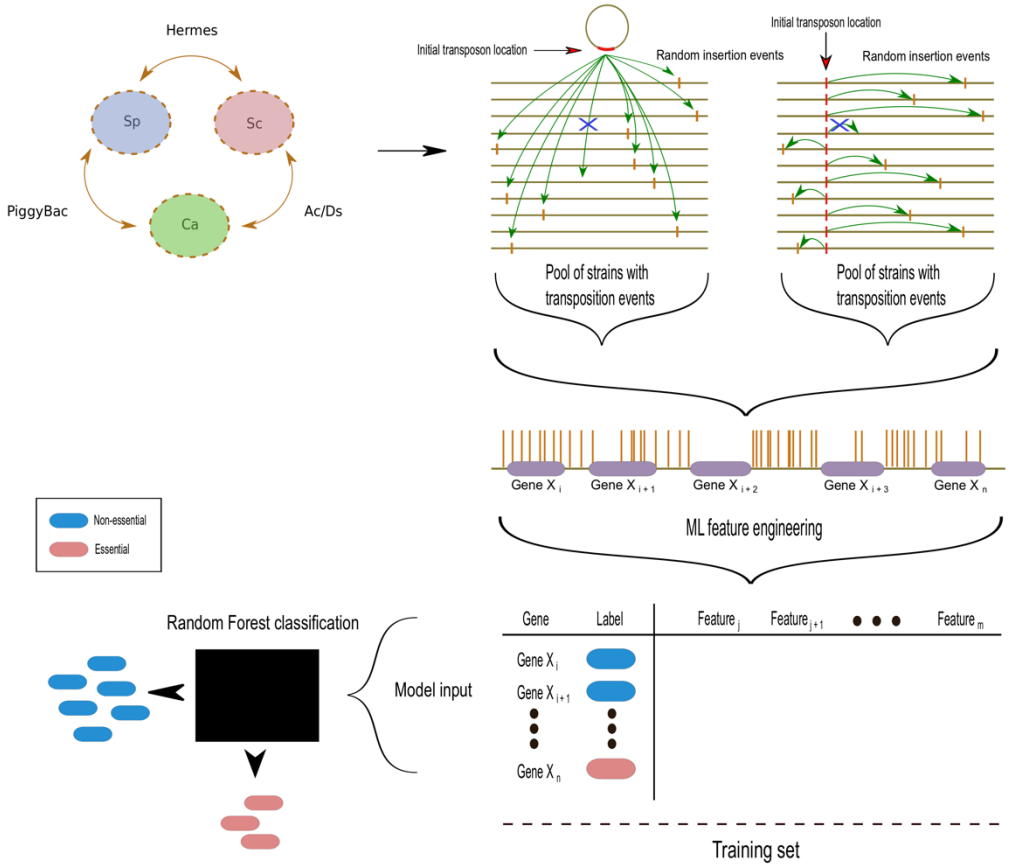

Figure 1. Overview of data acquisition and analysis: transposition events to gene essentiality.

A. Three yeast species analyzed (Sp, *S. pombe*; Sc, *S. cerevisiae*; and Ca, *C. albicans*) by in vivo transposition in this study and which transposons (PB, PiggyBac; AcDs and Hermes) were used to mutagenize which species. Note that each species was analyzed with two different transposon systems.

B. Comparison of genome insertion sites for transposition events that initiate from an extrachromosomal plasmid (left, red region of plasmid circle) or a specific chromosomal locus (right, red bar on a given chromosome). Horizontal lines represent multiple copies of the same genome, each of which underwent a single insertion event (green arrow) per genome. While transposition is generally random, a bias for loci in close proximity to the initial transposon insertion site demands normalization of the final data.

C. Mapped Tnseq analysis of the pool of transposition events yields the chromosomal insertion sites (brown vertical lines) in the reference chromosomes

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relative to the ORFs (purple regions). A close up of a small region of a single chromosome (olive horizontal line) including 5 ORFs is illustrated.

D. A training set is constructed using known or inferred labels (non-essential, blue; essential, red) together with extracted features calculated from the data and its position relative to ORFs. Features are defined in Table 2.

E. The training set features, as well as features for all ORFs are used as input for Random Forest classification (black rectangle); output is a prediction of essentiality (red or blue as in d), for which an optimal threshold is determined and applied to designate all genes in one of the two categories.

F.

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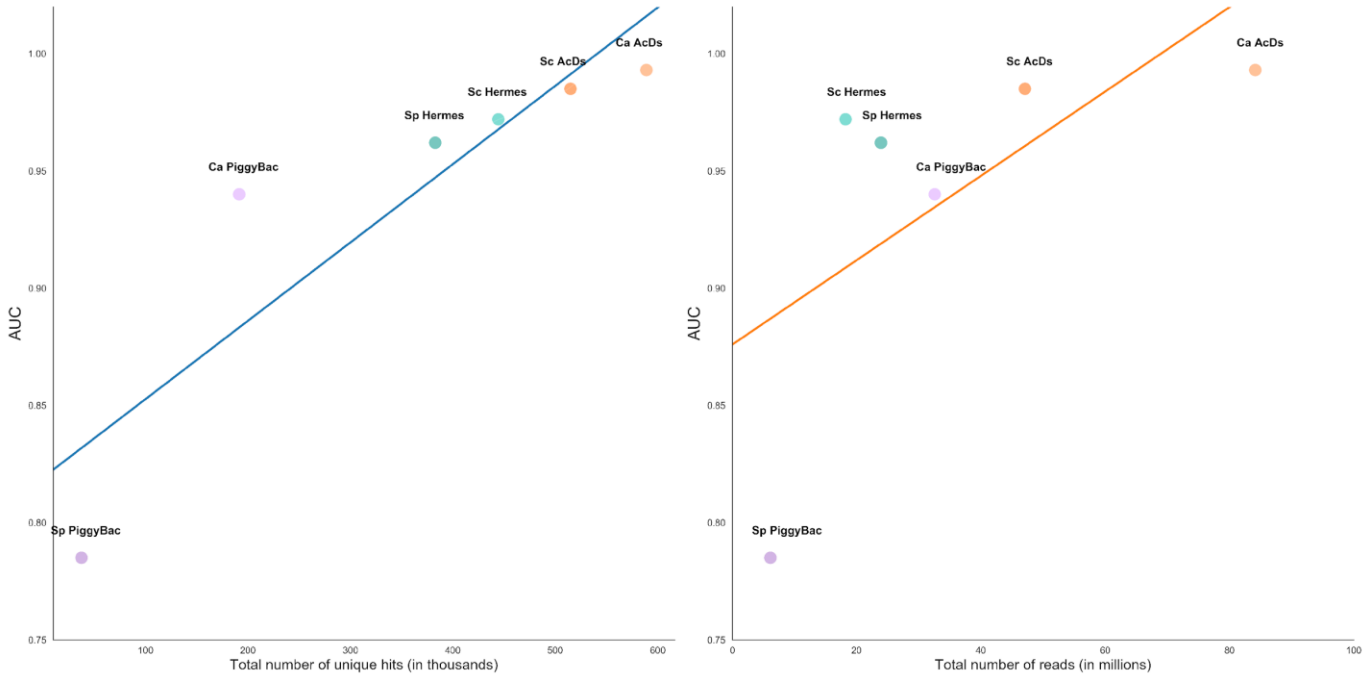
Click here to access/download Figure Figure 2 hits or reads vs AUC.docx

Figure 2. Contribution of unique hits and total number of reads to the quality of ML predictions for gene essentiality/non-essentiality.

- Performance of the classifier vs (a) the total number of unique insertion sites (hits) and (b) the total number of sequencing reads, in each study (organism abbreviations as in Fig. 1a; Ac, AcDs; H, Hermes; PB, PiggyBac).



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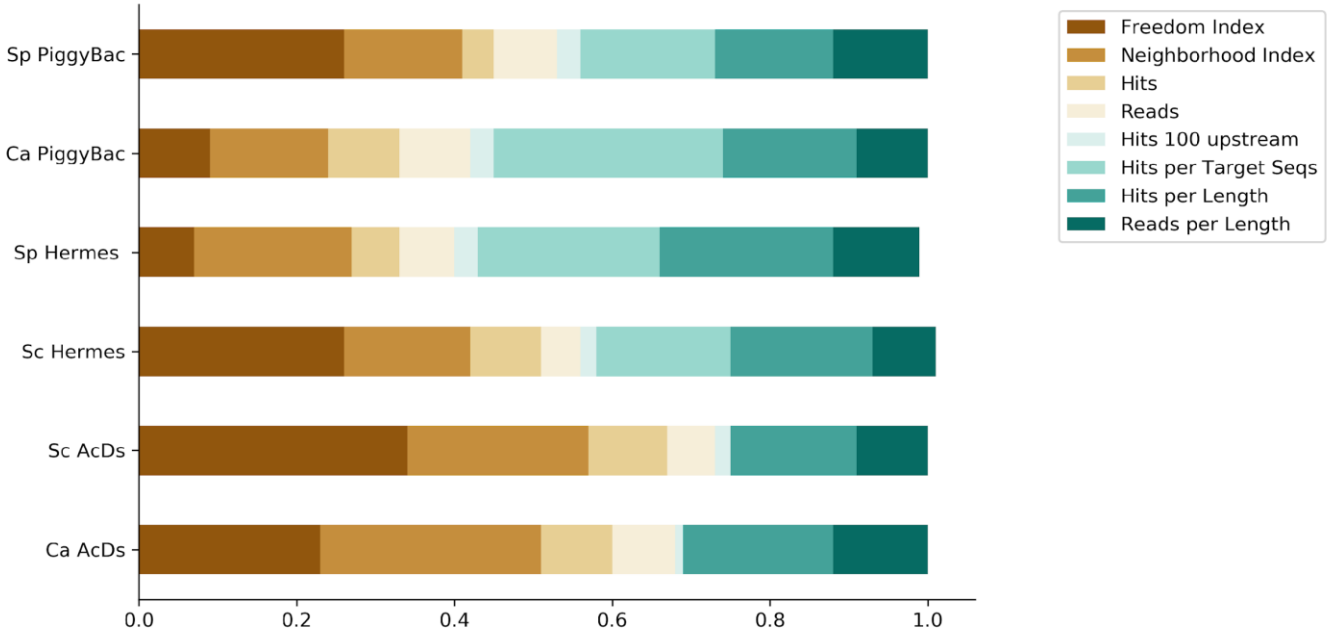
Click here to access/download Figure Figure 3 feature importance of the classifiers.docx

Figure 3. Feature importances in the different classifiers.

- Importance of each feature used in the Random Forest classifier of essentiality for each dataset. Features are described in Table XXX; Neighborhood index generally normalizes for non-random insertion frequencies across the genome; Freedom index reports on the largest proportion of an ORF that has no hits, which is a measure of domains that may be essential.[21]



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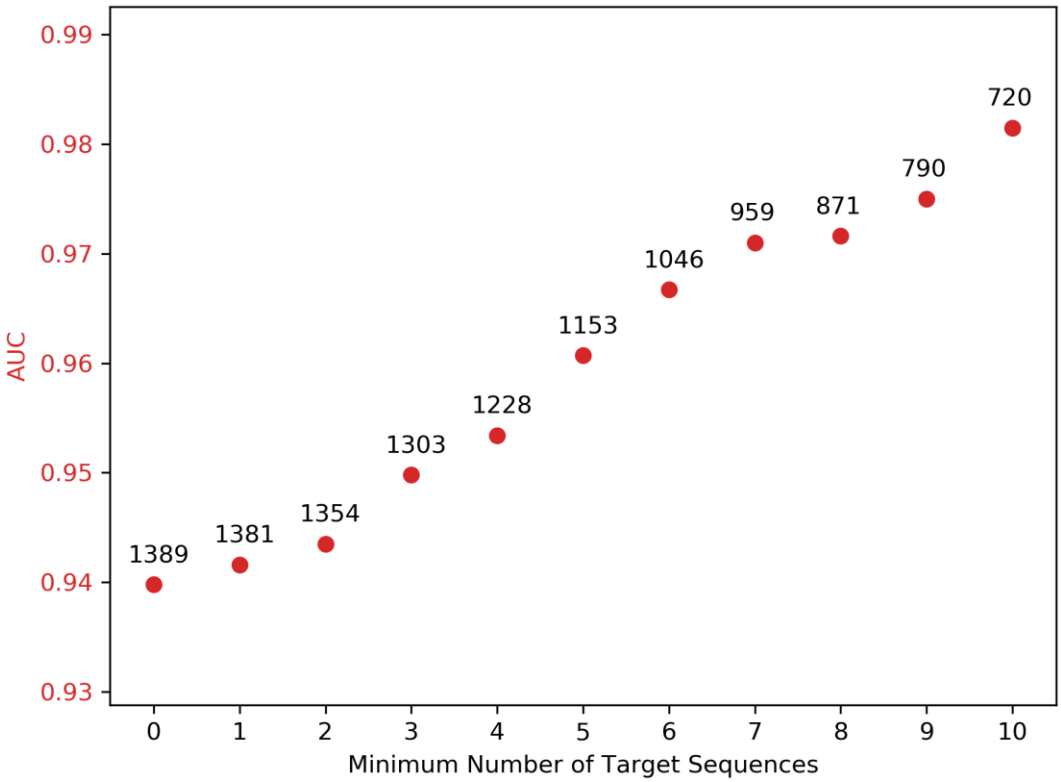
Click here to access/download Figure Figure 4 CaPB f of target sequences.docx

Figure 4. Analysis of the ability of the CaPB classifier to infer gene essentiality in genes with increasing number of target sequences.

- When only ORFs with a specific number of target sites are considered (>= x-axis), AUC rises accordingly (red), but the number of ORFs that can be analyzed necessarily decreases (numbers above red dots). This demonstrates the importance of the prevalence of the transposon target sequences in ORFs, for the quality of gene essentiality inference, using in-vivo transposon mutagenesis studies.
- X axis: Minimum number of target sequences per ORF needed for inclusion in the classification.
- Y-axis (red): CaPB classifier AUC.



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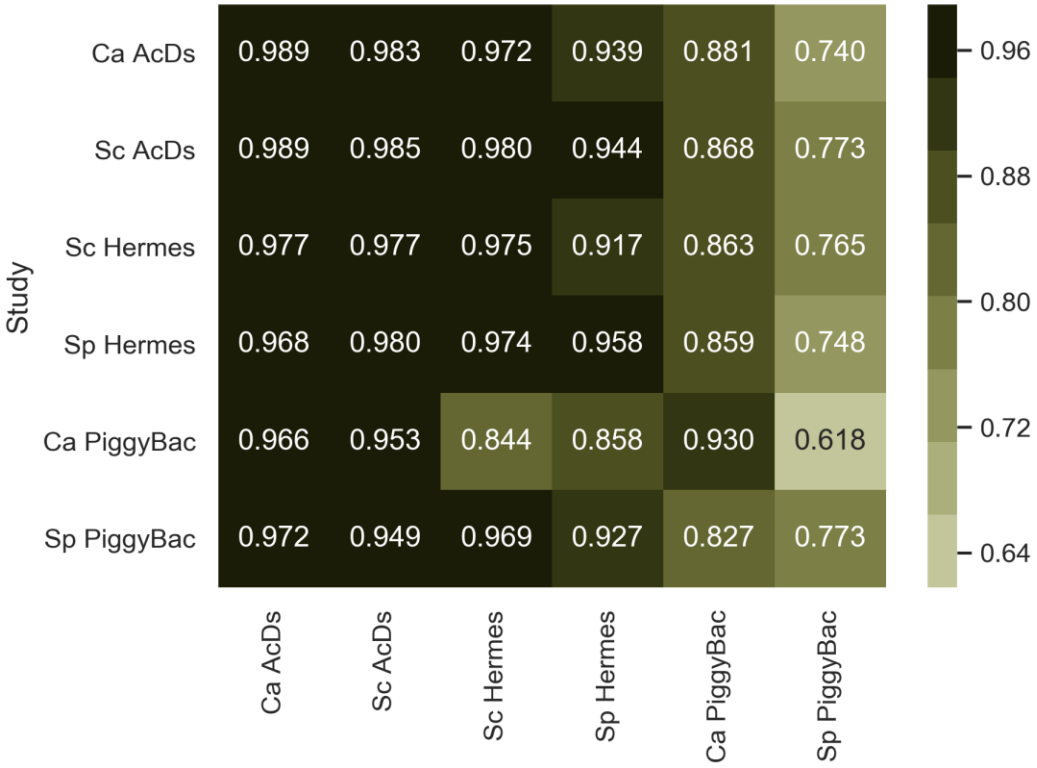
Click here to access/download Figure Figure 5 Cross study classifications.docx

Figure 5. Analysis of ROC AUC values for Random Forest classification trained on data from a different organism and/or transposon in all possible combinations.

A. For each ROC AUC value in the table, training was performed on 80% of the original training set used in the training species/transposon described in the rows. This training data was then used to predict the essentiality of the remaining 20% of the training set in the species/transposon described in the columns. The train/test split ratio was similar to the 5-fold cross-validation performed in the single study analyses.

B. For each study, the vector of the relative feature importance was correlated with the feature importance in all the other studies. Pearson r correlation coefficient values are presented.

a. Transfer learning AUCs



b. Correlation of feature importance vectors

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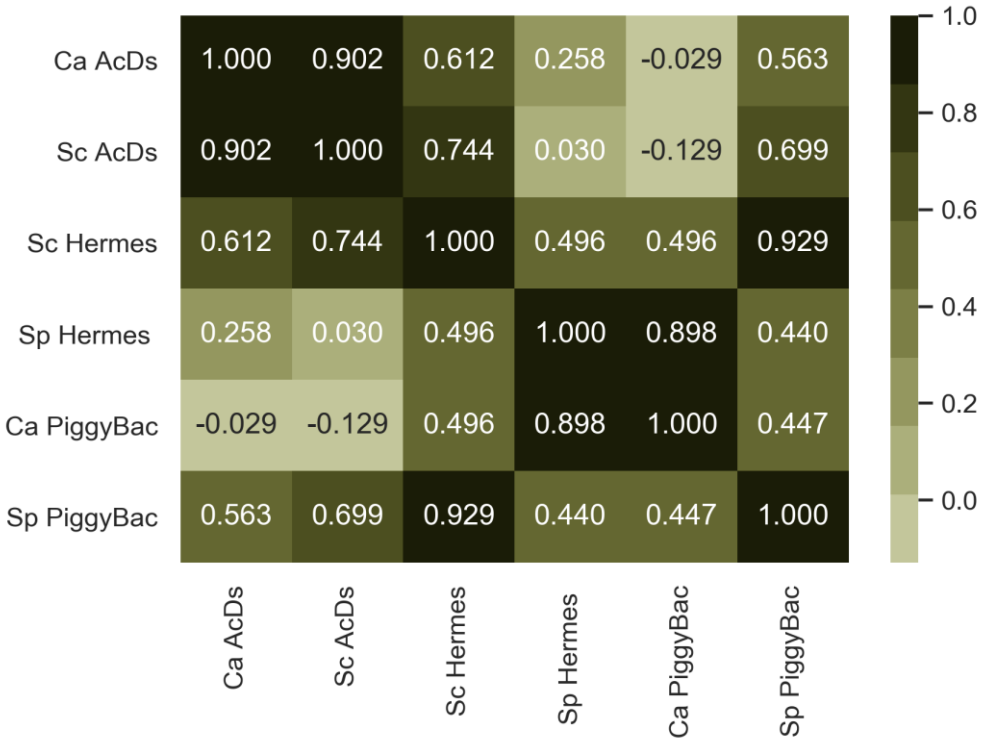


C.

Click here to access/download;Figure;Figure 6 Comparative analysis of essentiality verdicts.docx

Figure 6: Comparison with known essentials genes.

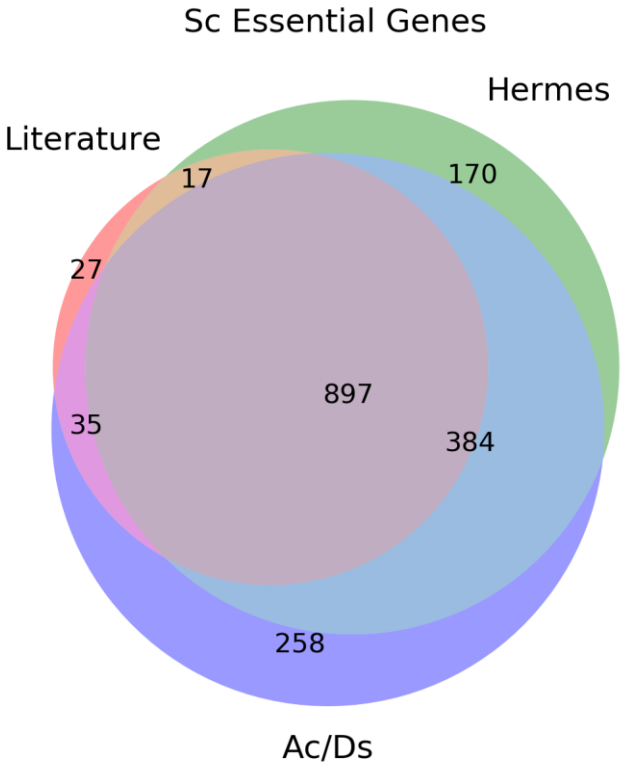
A. Comparison of the essentiality verdicts in *S. cerevisiae*, based on the known essential genes from the literature, ScAcDs and ScHermes classifiers.

B. Comparison of the essentiality verdicts in *S. pombe*, based on the known essential genes from the literature, SpPB and SpHermes classifiers.

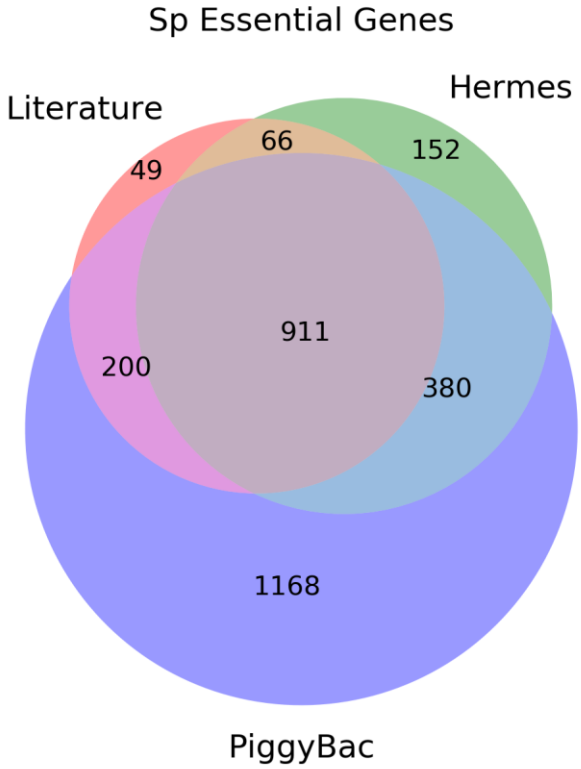
C. Comparison of the essentiality verdicts in *C. albicans*, based on the known essential Sp and Sc orthologs from the literature, CaAcDs and CaPB classifiers.

- Classification thresholds differ slightly from the previously published analyses[21]based on threshold selection applied systematically to all 6 studies (described in detail in Methods).

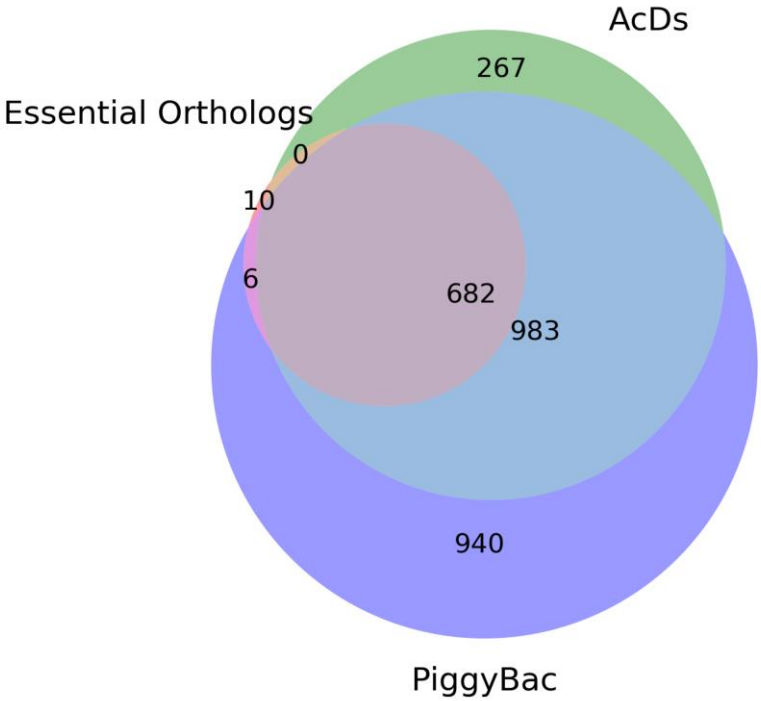
a.



b.


Sp Essential Genes
Literature
Hermes
66
152
49
911
200
380
1168
PiggyBac

c.


AcDs
267
Essential Orthologs
0
10
6
682
983
940
PiggyBac

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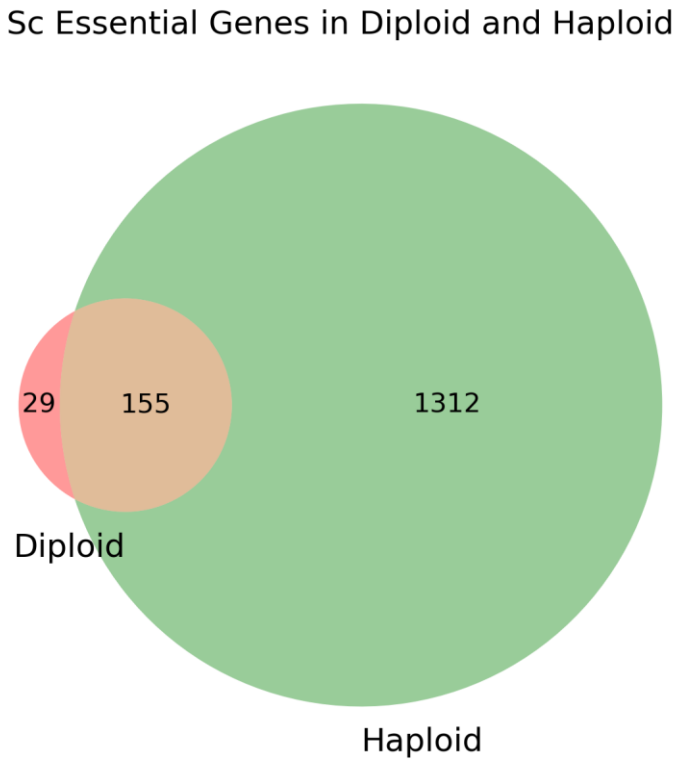
Click here to access/download Figure Figure 7 Comparison of haploid and diploid SC.docx

Figure 7. Gene essentiality in haploid and diploid *S. cerevisiae*.

- Comparison of essential genes in haploid and diploid *S. cerevisiae* analyzed with Sc Hermes. RF classifier was trained on the haploid ScHermes study and predicted gene essentiality in a diploid strain, using the same threshold for the final verdict. Mitochondrial genes were not considered.



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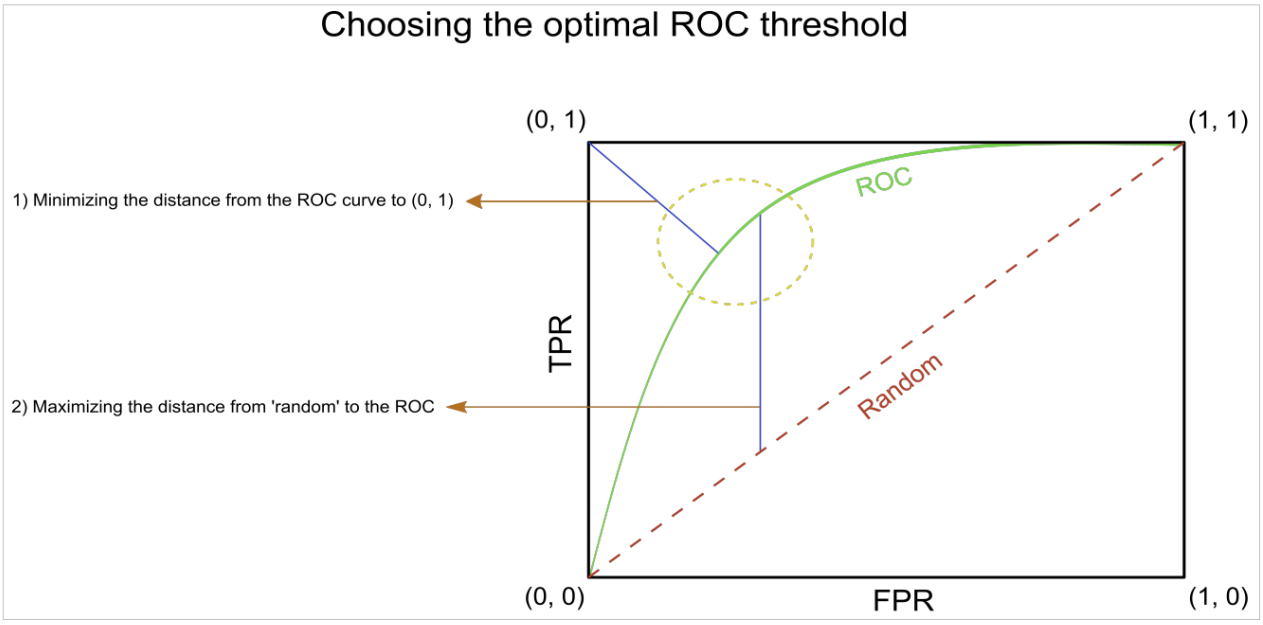
Click here to access/download Figure Figure 8 Threshold optimization.docx

Figure 8. Threshold optimization.

- Two metrics were evaluated: 1) Minimum of the Euclidean distance between (0, 1) and the receiver operating characteristic (ROC) curve. 2) Maximum of the vertical distance between the line describing a random choice (a straight line from (0, 0) to (1, 1)) and the ROC curve.



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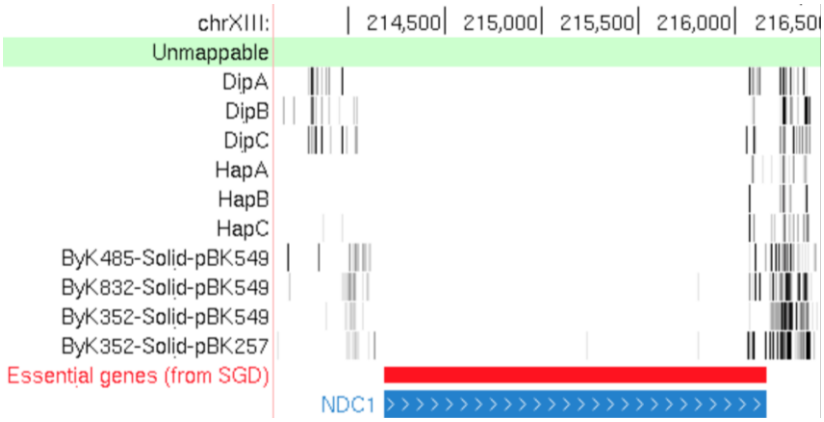
Click here to access/download;Figure;Figure 9 Suspected haploinsufficent genes in Sc.docx

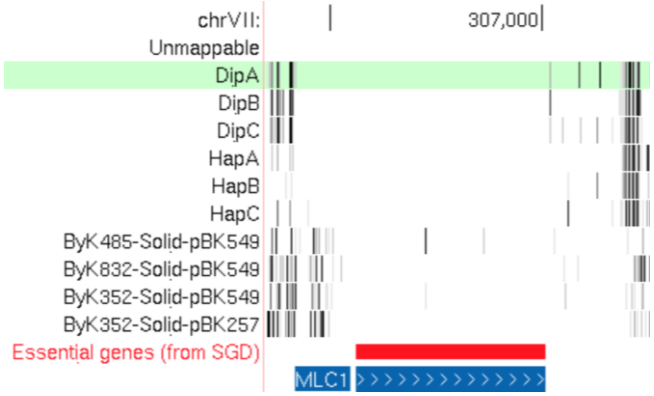
Figure 9. Suspected haploinsufficient genes in *S. cerevisiae*.

- Four genes suspected to be haploinsufficient in*S. cerevisiae*: NDC1, MLC1, RPC10 and BCY1, as they appear in the genome.[32]NDC1 and MLC1 were known to be haploinsufficient. BCY1 appears to be a previously unknown haploinsufficient gene. RPC10 might not be essential as it sustained hits in the 5' UTR in diploids but not haploids.

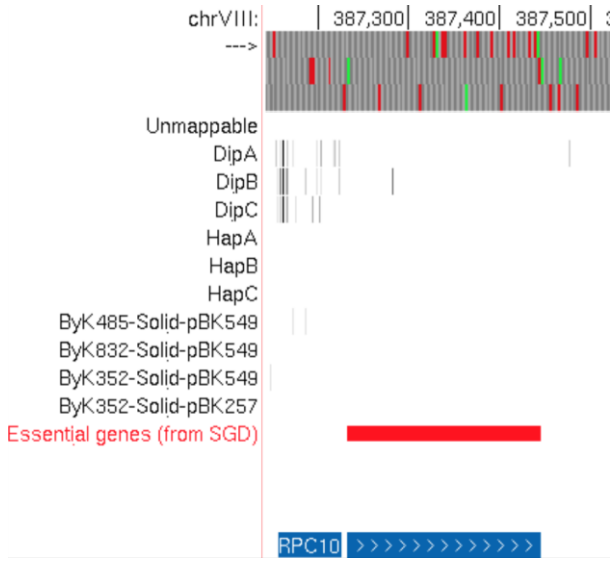
NDC1



MLC1



RPC10


chrVIII:
387,300
387,400
387,500
--->
Unmappable
DipA
DipB
DipC
HapA
HapB
HapC
ByK485-Solid-pBK549
ByK832-Solid-pBK549
ByK352-Solid-pBK549
ByK352-Solid-pBK257
Essential genes (from SGD)
RPC10

BCY1

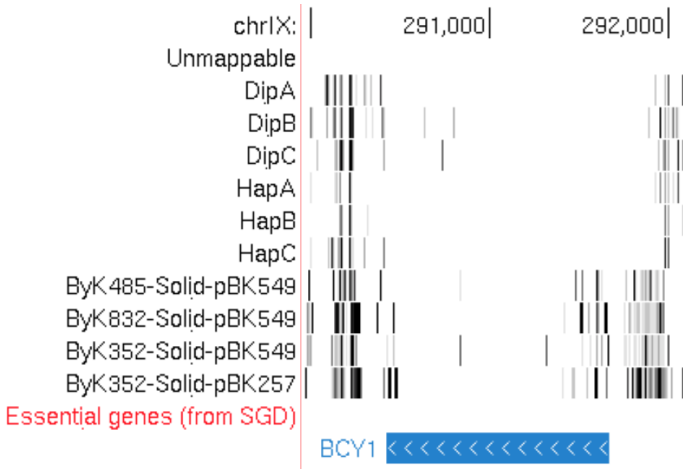

chrIX:
291,000
292,000
Unmappable
DipA
DipB
DipC
HapA
HapB
HapC
ByK485-Solid-pBK549
ByK832-Solid-pBK549
ByK352-Solid-pBK549
ByK352-Solid-pBK257
Essential genes (from SGD)
BCY1

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Table S1

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Table S1 Training Sets.csv

Table S2

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Table S2 CaAcDs ml predictions.csv

Table S3

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Supplementary Material
Table S3 ScAcDs ml predictions.csv

Table S4

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Table S4 ScHermes ml predictions.csv

Table S5

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Table S5 SpHermes ml predictions.csv

Table S6

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Table S6 CaPB ml predictions.csv

Table S7

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Table S7 SpPB ml predictions.csv

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Figure S1 Ca AcDs.png

Supp Fig S2

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Figure S3 Sc Hermes.png

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Figure S4 Sp Hermes.png

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Figure S5 Ca PB.png

Supp Fig S6

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Figure S6 Sp PB.png

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Figure S7 ScHermes target sequence frequency.png

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Figure S8 SpHermes target sequence frequency.png

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Figure S9 CaPB target sequence frequency.png

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Figure S10 SpPB target sequence frequency.png