Fur4 mediated uracil-scavenging to screen for surface protein regulators

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ABSTRACT

Cell surface membrane proteins perform diverse and critical functions and are spatially and temporally regulated by membrane trafficking pathways. Although perturbations in these pathways underlie many pathologies, our understanding of these pathways at a mechanistic level remains incomplete. Using yeast as a model, we have developed an assay that reports on the surface activity of the Fur4 uracil permease in uracil auxotroph strains grown in the presence of limited uracil. This assay was used to screen a haploid deletion library that identified mutants with both diminished and enhanced comparative growth in restricted uracil media. Factors identified, including various multi-subunit complexes, were enriched for membrane trafficking and transcriptional functions, in addition to various uncharacterised genes. Bioinformatic analysis of expression profiles from many strains lacking identified transcription factors required for efficient uracil-scavenging revealed they control expression of other uracil-scavenging factors, in addition to membrane trafficking genes essential for viability, and therefore not represented in the screen. Finally, we performed a secondary mating factor secretion screen to functionally categorise factors implicated in uracil-scavenging, most of which are conserved throughout evolution.

INTRODUCTION

Cell surface membrane proteins are regulated by a variety of overlapping and often co-regulated membrane trafficking pathways. Surface cargoes are co-translationally imported into the endoplasmic reticulum (ER) before transiting the secretory pathway to the plasma membrane (PM) for function. Surface proteins are internalised via clathrin mediated endocytosis, followed by recycling back to the PM or entering the lysosomal degradation pathway. These pathways allow surface localisation and activity of myriad proteins to be precisely controlled to meet cellular demands, for example during the cell cycle or in response to reduced nutrient availability; however, these pathways remain incompletely characterised. The budding yeast system has been useful to discover and define membrane trafficking mechanisms. Yeast cells uptake nutrients from their external environment by a variety of transporters that localise to the PM, such as transporters for sugars, metal ions and vitamins. Amino acids and nucleosides are also actively imported via nutrient transporters, for example Gap1 broadly uptake amino acids, Mup1 uptakes methionine and Fur4 uptakes uracil. Permease activity can be controlled by changes in transporter expression and the rate of turnover by ubiquitin mediated vacuolar degradation, in addition to spatiotemporal control between eisorsomes and other regions of the PM. To develop an assay that reports on nutrient uptake via surface transporters, we focussed on the uracil permease Fur4, which is controlled by the above-described trafficking pathways and regulatory mechanisms. For example, the presence of uracil downregulates expression of FRU4 whilst also triggering endocytosis and Rsp5-mediated ubiquitination and degradation of Fur4. Furthermore, Fur4 activity is regulated in response to metabolic stress via storage in eisosomes. Fur4-mediated uptake of uracil might be considered particularly important for many lab strains that cannot synthesise uracil biosynthetically, due to disruption of the orotidine-5'-phosphate decarboxylase URA3 gene (e.g. ura3-52 or ura3Δ), which impose the useful selection characteristics of auxotrophy and resistance to 5-Fluoroorotic Acid. Indeed, many genome-wide libraries have been created from designer parental strains. We therefore chose Fur4-mediated uptake to develop a simple and cost-effective growth assay that indirectly reports on the surface-mediated uptake of uracil, which we have used to screen a haploid library of deletion mutants for factors that regulate Fur4 trafficking, many of which are conserved across evolution.

RESULTS & DISCUSSION

Uracil-scavenging yeast growth assay

The uracil permease Fur4 is dispensable for growth in rich media but is critically required when uracil-auxotroph cells are grown in synthetic defined media containing replete (4 mg/L) uracil (Figure 1A - 1C). Importantly, the robust Fur4-dependent growth in BY4742 cells, which harbour a ura3Δ mutation herein referred to as wild-type, corresponds to the concentration of available uracil. There is a significantly reduced rate of growth when wild-type cells are grown in media containing only 0.1 mg/L uracil, but Fur4...
dependent uracil-scavenging supports growth (Figure 1B, 1C). Fur4 has been previously shown to respond to extracellular uracil\textsuperscript{14}, and we confirm steady state surface localisation of Fur4 tagged with mNeonGreen (mNG) is observed, unlike Mup1-GFP that is trapped by Snf7-oligomers\textsuperscript{33}. In contrast, surface levels of Fur4-mNG was greatly reduced in both rcy1Δ or nhx1Δ mutants, (Figure 2A), which lack factors required for efficient endosomal recycling\textsuperscript{34–36}. Therefore, we compared growth of rcy1Δ and nhx1Δ mutants with wild-type cells on plates of varying uracil concentrations. There was no statistically significant difference in growth between wild-type and trafficking mutant cells in the range of 1 mg/L - 32 mg/L uracil, but at lower uracil concentrations the rcy1Δ and nhx1Δ mutants, which have reduced surface Fur4, exhibit a low-uracil specific growth defect (Figure 2B, 2C). Although significant defects were observed at 0.5 mg/L and 0.25 mg/L uracil, we selected 0.1 mg/L and 0.05 mg/L uracil for optimal resolution scavenging conditions, as wild-type cells grow efficiently but rcy1Δ or nhx1Δ both show dramatically reduced growth. We tested this concept with another surface-localised nutrient transporter, the methionine transporter Mup1 grown in methionine auxotroph (met15Δ) cells but no methionine concentration that supports growth could distinguish trafficking mutants (Figure S1). We assume differences in steady state surface levels, substrate affinity and uptake pathways\textsuperscript{8} account for this.

**FIGURE 1:** Low uracil growth relies on the Fur4 transporter

A: Schematic illustration showing uracil-auxotroph cells (ura3Δ) with (left) and without (right) the uracil permease Fur4. B: Indicated yeast strains were grown to mid-log phase before plating on rich (YPD) and synthetic complete (SC) media containing either 4 mg/L or 0.1 mg/L uracil. C: Quantification of yeast growth from B, asterisks (*) indicate Student’s t-test comparisons p < 0.001. D: Wild-type cells expressing Fur4-mNeonGreen (Fur4-mNG) from its endogenous promoter were labeled with FM4-64 for 1-hour, grown to mid-log phase in SC-Ura media (upper) or in the presence of 40 µg/ml uracil for 1 hour (lower) prior to confocal microscopy. White arrow heads indicate plasma membrane (PM) signal. Scale bar = 5 µm.

**FIGURE 2:** Surface localization of Fur4 is required for growth in low-uracil

A: Indicated strains expressing Fur4-mNeonGreen (mNG) were labelled with a 1-hour FM4-64 pulse prior to growth in label-free chase media for 2 hours and preparation for confocal imaging. Scale bar = 5 µm. B: Wild-type, rcy1Δ and nhx1Δ cells grown to mid-log phase were spotted in a 10-fold serial dilution onto plates titrated with indicated concentrations of uracil and grown at 30°C for 3 days. C: Growth of strains from B were quantified, asterisks (*) indicate Student’s t-test comparisons of mutants with wild-type cells, p < 0.005. D: Schematic diagrams showing the predicted effects on Fur4 following different trafficking pathway perturbations.
A genetic screen for uracil-scavenging mutants

We hypothesised that mutants with growth similar to wild-type in replete uracil but differences specifically at low uracil could be used to identify mutants from a non-essential haploid deletion library with perturbed surface levels of Fur4 (Figure 2D). Cultured yeast strains representing 5132 different mutants were diluted and spotted out (16 replicates of each) on to solid agar media containing replete (4 mg/L) and limited (0.1 mg/L and 0.05 mg/L) uracil concentrations (Figure 3A). As expected, mutants with growth differences in uracil-replete media were observed, but the screen was specifically focused on differences in growth between high and low uracil concentrations. This is exemplified by the mutants used to calibrate the assay, rcy1∆ and nhx1∆, which both exhibit growth defects specifically in low-uracil when compared with neighboring mutants (Figure 3B). A low stringency scoring system was used to identify 208 null mutants for follow up analysis. Candidates were grown to mid-log phase then serially diluted and spotted on 4 mg/L, 0.1 mg/L and 0.05 mg/L uracil media. Growth was quantified for the 208 mutant strains compared to a wild-type control from the same plate, and then values were used to compare growth across uracil concentrations (Table S1). Statistical comparisons of mutants from these optimized growth assays revealed 58 mutants, such as cla4∆ (Figure 3C), that did not show a significant difference in growth compared to wild-type. However, 126 mutants with growth defects specifically in uracil-scavenging conditions were identified (Figure 3D), ranging from relatively subtle defects (e.g. vps74∆ at 0.1 mg/L = 0.78 ± 0.04) to extreme (e.g. vps3a∆ at 0.1 mg/L = 0.004 ± 0.003). Furthermore, although the assay was calibrated for mutants with defective growth in low-uracil, 24 mutants with enhanced growth compared to wild-type were validated. We note many of these strains exhibit growth defects in uracil replete media, such as ipk1∆ (Figure 3C), allowing for benefits to be observed at low uracil (Table S1).

Screen enriched for molecular complexes

A comparative orthologue search of uracil-scavenging mutants identified 94 highly conserved genes, corresponding to 267 human orthologues associated with 87 distinct diseases (Table S2). Gene Ontology (GO) enrichments were performed to reveal a number of cellular component enrichments of molecular complexes (Figure S2), including the GET complex and the prefoldin complex (Figure 4A). The identification of multiple complex members suggests the screen was

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**Figure 3: A genetic screen for mutants that affect uracil-scavenging**

A: Yeast strains grown overnight in 96-well plates were diluted 20-fold in water then replicated 16 times onto solid agar plates containing varying uracil concentrations. An example 4 mg/L uracil plate shows identifier wells (yellow), alongside strains with defect (white) and accelerated (blue) growth. B: Example of screen data showing indicated mutants grown on media containing 4 mg/L, 0.1 mg/L and 0.05 mg/L uracil, including known Fur4 trafficking mutants (red). C: The screen identified 208 candidates that were subsequently grown to mid-log phase and spotted out over a 6-step, 10-fold serial dilution onto high (4 mg/L) and lower (0.1 mg/L and 0.05 mg/L) uracil containing media. Uracil-related cellular growth relative to wild-type was quantified and categorized as defective (e.g. pmp22), advantageous (e.g. ipk1∆) or not significantly altered (e.g. cla4∆). D: Ratio of relative growth between 4 mg/L and 0.1 mg/L uracil from C was plotted for all candidates.

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**Figure 4: Uracil-scavenging screen identifies multi-subunit complexes**

A: Gene Ontology enrichment analysis for cellular component of the 150 factors identified from the screen. B: Ratio of growth compared to wild-type (WT) cells at 4 mg/L, 0.1 mg/L and 0.05 mg/L uracil for GET complex mutants: get1∆, get2∆ and get3∆. Asterisks (*) indicate Student’s t-test comparisons p < 0.001. C: Ratio of growth compared to wild-type (WT) cells at 4 mg/L, 0.1 mg/L and 0.05 mg/L uracil for identified Prefoldin complex mutants: gim4∆, pac10∆, pff61∆ and yke21∆. Asterisks (*) indicate Student’s t-test comparisons p < 0.01. D: Confocal microscopy of wild-type (WT) and prefoldin mutants fixed with 4% paraformaldehyde and stained with Phallloidin-594 and DAPI. Scale bar = 5um.
stringent and robust. For example, deletion of GET1, GET2, or GET3 results in low-uracil growth defects (Figure 4B). As the GET complex is required for sorting of tail-anchored single-pass membrane proteins, many of which are important factors in secretory pathway trafficking, we presume the role of the GET complex in efficient surface trafficking of Fur4 is via an indirect membrane trafficking mechanism. Various prefoldin complex members were also identified as having defective growth specifically on low uracil (Figure 4C), implicating it as a potential regulator of Fur4 trafficking. This could be explained by prefoldin-mediated assembly of cytoskeleton proteins. Indeed, actin filament structures observed in wild-type cells are absent in prefoldin mutant cells pdf1Δ and pac10Δ (Figure 4D), indicating impaired microtubule function that could adversely affect correct trafficking of Fur4 to the surface. However, the prefoldin complex is also involved in transcriptional elongation, so its role in uracil-scavenging could be indirect.

**Screen enriched for trafficking and transcriptional machinery**

GO enrichments for biological process revealed almost a third (44/150) of annotations were for machinery associated with membrane trafficking and signalling (Figure 5A, Table S3), including the blind identification of rcy1Δ and nhx1Δ mutants that were used to calibrate the assay (Figure 2). The other biological process significantly enriched was transcription, including 3 prefoldin subunits annotations (Figure 5A, Table S3). Physically interacting transcription factors were identified (Figure S2), such as members of the COMPASS complex, Swd1, Swd3, and Sdc1Δ alongside the Swi3/Snf5 pair amongst others, all of which exhibit significant defects in low uracil (Figure 5B). We reasoned transcriptional regulators could be indirectly involved in Fur4 membrane trafficking, controlling gene expression of either essential genes not tested in the primary screen or mutants identified from the screen itself. To explore transcription factors (TFs) implicated in uracil-scavenging, we assembled genome-wide expression datasets for wild-type cells versus 28 TF-null mutants from a large-scale microarray analysis. A matrix of all mutants showed high correlation of associated factors, such as known complex members (Figure S3). Cross-referencing expression data for 1183 genes that are essential for viability (Table S4), followed by hierarchical clustering was used to generate a heat map of related gene expression changes (Figure 5C). Strains such as ies2Δ, bre1Δ and uba4Δ created distinct expression signatures, but others were similar, such as each of the COMPASS complex mutants swd1Δ, swd3Δ, and sdc1Δ. Particularly modulated clusters of genes were identified from this analysis (Table S5), including genes associated with membrane trafficking that we chose for experimental testing (Pma1, Gpi8, Mrs6, Gpi12 and Sec62). To achieve this, we performed uracil-scavenging assays with strains containing Decreased Abundance by mRNA Perturbation (DAmP) cassettes at the 3’ UTR of each candidate. This analysis revealed sec62-DAmP cells, which have very low protein levels of Sec62246, have specific growth defects in low uracil (Figure 5D). SEC62 expression was greatly reduced upon deletion of several TFs from the screen, for example uba4Δ and met18Δ mutants (Figure 5E). The uracil-scavenging defects in sec62-DAmP cells can be explained in the context of reduced Fur4 trafficking to the surface, as shown by localization defects of Fur4-mNG, and an unrelated transporter.
Mup1-GFP (Figure 5F). Collectively this example suggests that Uba4 and Met18 regulate expression of sufficient levels of Sec62, which are all required for proper trafficking of cargoes to the PM (Figure 5G).

**Uncharacterized factors are controlled at the transcriptional level**

The uracil-scavenging screen identified ten uncharacterized candidates (Figure 6A). In an effort to understand whether these were also regulated by the TFs from the screen, we used a similar approach to cross-reference gene expression profiles of the TF-deletion strains against the 150 candidates identified in the screen. Again, hierarchical clustering revealed many gene profiles share signatures across different TF deletion experiments (Figure 6B). We were particularly intrigued by ydr222wΔ mutants defective in growth from the screen (Figure 6A) as YDR222W was greatly downregulated in a large number of the transcription factor null mutants, including the swi3Δ, spt3Δ and COMPASS complex mutants, but upregulated in other mutants not associated with low-uracil growth (Figure 6C, Figure S4). To test whether Ydr222w has a role in general surface protein trafficking, we expressed the distinct Mup1-GFP transporter in wild-type and ydr222wΔ mutants to reveal mislocalization phenotypes (Figure 6D) at both mid-log phase and late-log phase, the latter of which induces vacuolar sorting of Mup1-GFP. Therefore, this bioinformatic approach helps prioritize factors, like the uncharacterized protein Ydr222w, for follow up testing and can be applied to other genetic screens, even retrospectively, that identify transcriptional regulators. To initiate functional categorisation of all the 150 factors, in particular where uncharacterised factors like Ydr222w might function, a secondary screen was performed based on the trafficking of α-factor through the secretory pathway to induce cell cycle arrest on a lawn of MatA yeast, which are sensitised to arrest through a mutation in the gene that encodes the Bar1 mating factor protease (bar1-1). We hypothesised that mutants with defects in the secretory pathway leading to reduced alpha factor secretion would result in a reduction in the growth arrest response seen when MatA cells are spotted onto a lawn of Matα cells (Figure 6E).

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**FIGURE 6**: Previously uncharacterised proteins implicated in membrane trafficking. A: Ratio of growth compared to wild-type cells at 4 mg/L, 0.1 mg/L and 0.05 mg/L uracil for indicated uncharacterised mutants identified in the screen. Asterisks (*) indicate Student’s t-test comparisons p < 0.001. B: Hierarchical clustering of genes identified from the screen, plotting expression profiles as a heat map based on fold changes following deletion of 28 different transcriptional regulators. C: Volcano plots constructed for log2 fold changes and their corresponding p-values for genes in microarray analyses comparing wild-type cells to swi3Δ (left) and sdc1Δ cells (right). Value for YDR222W is shown in each plot (red). D: Wild-type and ydr222wΔ mutants were grown in serial dilution overnight and images at mid-log phase (OD600 = 1.0) or late log phase (OD600 = 2.0 + 2 hours) prior to confocal imaging. E: Schematic showing basis of α-factor induced secondary screen growing Matα mutants from the primary screen on a lawn of bar1-1 MatA mutants (left), with representative examples shown (right). F: Quantification of growth inhibition surrounding spots of Matα cells from screen described in C, shown relative to wild-type controls from same plate (n = 3). Asterisks (*) indicate Student’s t-test comparisons p < 0.01.
Conversely, mutants with no halo phenotype could act downstream of the PM, or indirectly, to explain uracil-scavenging phenotypes. We observed mutants to have a reduction in halo size (eg. *bud16Δ*) or a halo comparable to WT cells (eg. *adk1Δ*). We then performed growth arrest experiments in triplicate for all 150 screen candidates and found approximately half (76 / 150) had statistically significant reduction in halo diameter (*Table S6*). Mutants of proteins well characterized to act in the early stages of the secretory pathway such as GET-complex nulls (discussed above) and *apl2Δ* were seen to display a significantly reduced halo phenotype. Whilst mutants of factors acting downstream of the secretory system in the endosomal system such as *rcy1Δ* and *nhx1Δ* were seen to display a halo phenotype not significantly different from WT (*Figure 6F*). Interestingly, uncharacterized mutant *ydr222wΔ* gave a halo type not significantly different to WT cells.

**Summary**

In conclusion, we report a simple growth assay that indirectly reports on surface protein trafficking via nutrient transporter activity of uracil auxotroph yeast strains. The assay relies on comparison of growth efficiency of yeast cells on relatively high and low uracil media to infer the capacity of the Fur4 transporter to scavenge uracil required for growth. It is therefore cheap, simple and easy to perform at high throughput, as demonstrated by testing a haploid deletion library of over 5000 yeast strains. This genetic screen identified many novel candidates as potential Fur4 regulators and was particularly enriched for membrane trafficking and transcriptional machinery. By cross-referencing essential genes and factors identified from the screen, with genome-wide expression patterns in the majority of these transcriptional regulators, we were able to identify connections between TFs and the genes they regulate, both of which relate to uracil-scavenging. As an example, we document the essential gene *SEC62* and the uncharacterised gene *YDR222W*, as repressed in many of the TFs mutants identified from the screen. Although we cannot exclude the possibility that mutants have indirect effects on uracil scavenging, for example via the biosynthetic or metabolic processes. However, as membrane trafficking and transcription factors were significantly enriched, and many of the TFs can be functionally explained in the context of membrane trafficking, this suggests the bulk of mutants reported likely affect trafficking pathways used by Fur4. Furthermore, both factors prioritized by bioinformatics, Sec62 and Ydr222w, are shown to be required for proper sorting of fluorescently labelled cargoes. In addition to fluorescently labelled Fur4-mNG (*Figures 1, 2, 5*), and other surface cargoes affected by mutants from the screen, like Mup1-GFP and the G-protein coupled receptor Ste3 tagged with mCherry (*Figure S5*), we created mGFP tagged versions of both full length Fur4, or a mutant lacking its N-terminal 60 residues under copper inducible *CUP1* promoter, to allow temporal control of trafficking (*Figure S6*). We reasoned Fur4⁲-mGFP would be a suitable reporter for secretory pathway mutants, as it cannot be endocytosed, however localisation in *get1Δ*, *get2Δ* and *get3Δ* was indistinguishable from wild-type cells.

This might suggest the GET complex exhibits a distinct function in uracil-uptake. However, given the GET complex is known to be involved in secretory trafficking and not only were all 3 members identified from a blind screen of >5000 mutants, but *get1Δ* *get2Δ* and *get3Δ* cells were all defective in an assay reporting on secreted mating factor (0.53 ± 0.28, 0.80 ± 0.15, and 0.76 ± 0.24 respectively), we favour the explanation that the uracil-scavenging assay is sensitive enough to reveal a phenotype that is not apparent from steady state localization experiments. Therefore, we propose this uracil-scavenging assay, used in combination with fluorescently tagged cargoes, the mating factor secretion assay and bioinformatic approaches all documented herein serve as useful tools to study surface protein trafficking, in addition to the mutants identified and characterized, many of which are novel and evolutionarily conserved, that can inform future studies.

**METHODS**

**Cell culture**

Yeast cells were grown in rich yeast extract peptone dextrose (YPD) media (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) D-glucose) or synthetic complete (SC) minimal media (0.675% (w/v) yeast nitrogen base without amino acids, 2% (w/v) D-glucose, plus the appropriate amino acid or base drop-outs for selection). Standard SC media contained 4 mg/L uracil and lower concentrations used, typically 0.1 mg/L and 0.05 mg/L for uracil stress conditions listed throughout. Cells were routinely cultured overnight to early/mid-log phase (OD<sub>600</sub> < 1.0) prior to experimental procedures. Yeast strains used in this study are listed in *Table S7* and plasmids used are itemised in *Table S8*.

**Yeast growth assays**

For the primary screen, 10µl culture of each mutant strain was grown in the well of a 96-well plate containing 150µl of YPD media with 250 µg/ml G418 overnight at 30°C in a humidified incubator. The bulk of YPD was then removed, followed by resuspension in water, and transfer of 10µl to a fresh 96-well plate containing 200µl sterile water. Dilutions were then mixed with a 96-pin replicator and pinned onto solid agar in 1.536 format using a ROTOR-HDA (Singer Instruments). Each mutant was pinned 16 times on solid media containing varying concentrations of uracil (4 mg/L or 0.1 mg/L or 0.05 mg/L) incubated at 30°C until sufficient growth was observed, followed by Phenobooth image capture (Singer Instruments) to record yeast growth. For follow up growth assays, the principle was the same but cells were cultured in 5ml serial dilutions to capture mid-log phase cells, which were then harvested with equivalent volumes to other strains, including a wild-type control, to be plated together. 6-step serial dilutions (10-fold) of each strain was generated in sterile water, followed by plating on SC plates containing of 4 mg/L, 0.1 mg/L and 0.05 mg/L uracil. Plates were then incubated at 30°C,
growth recorded and intensity quantified after normalising for background (ImageJ; NIH).

Confocal microscopy
Yeast cells expressing GFP and/or mCherry tagged proteins were grown to mid-log phase and then viewed in kill-buffer (50mM Tris pH 8.0, 10mM NaNO₃, 10mM NaF) or water (dH₂O) at room temperature on Zeiss laser scanning confocal instruments (LSM710 or LSM880 equipped with Airyscan) with a Plan-Apochromat 63x/1.4 Differential Interference Contrast (DIC) objective lens. Images were captured using Zen Black Imaging software and modified using Image J (NIH). Yeast vacuoles were labelled with 1.6µM FM4-64 (ThermoFisher) in YPD media, followed by 3x washes with SC media and further growth in SC media for 1-hour prior to imaging. Where stated cells were fixed for imaging by spinning down mid-log cultures and washing with 100 mM potassium phosphate buffer (pH 8) at 7000rpm. Cells were resuspended and incubated for 10 minutes at room temperature in 4% paraformaldehyde (950µl K-Phos, 50 µl PFA) before spinning at 7000rpm and resuspending in 1 x PBS. Fixed cells were stained with Rhodamine-phalloidin (Phalloidin-594) and DAPI. When under the control of a copper inducible promoter

α-factor induced arrest 'halo' assay
Wild-type and mutant Matα cells were grown to saturation overnight and then diluted back and grown for 4-6 hours in YPD media. Equivalent volumes of cells were harvested and spun down and brought up in 50 µl sterile water before spotting on low density lawns of MatA bar1-1 cells created from mid-log phase cells on YPD plates. Plates were incubated at 30 degrees overnight. Regions of growth inhibition for mutants relative to 2x wild-type controls from the same plates were determined using ImageJ (NIH), with mean and standard error values calculated from three independent biological replicates.

Gene Ontology analyses
All GO enrichments were acquired using the GO Term Finder v0.8658, using the default settings. The default background was used for all analyses except those for clusters derived from essential gene expression analysis, which instead used a background of essential genes listed in Table S4.

Hierarchical clustering and gene expression analyses
Microarray data documenting changes in gene expression in mutant strains lacking transcriptional regulators compared to wild-type46 were assembled, representing 6123 genes. The data were read into R52 then processed using the dplyr v1.0.453 and janitor v2.0.154 packages to include only 28 strains lacking transcription factors identified in the uracil-scavenging genetic screen. For downstream bioinformatic analyses, two gene reference subsets were generated: the first included all 147 verified candidates from the screen, and the second included 1183 genes denoted as essential for viability. For all analyses, hierarchical clustering was performed using the pheatmap package v1.0.1255 using complete linkage. Elbow and silhouette analyses were performed using the factoextra package v1.0.756 to determine the optimal number of clusters to guide further GO enrichment analyses. The Pearson correlation matrix of the whole genome expression data was produced with base R and visualised with the pheatmap package.

String analysis
Interactome analysis of physical interactions was carried out using STRING software57, with a minimum required interaction score of 0.400 and FDR stringency of 5%. The full interaction network was clustered using k-means clustering (k=15).

Statistical analysis
Statistical significance for experimental conditions were calculated using a student’s t-test/Bonferroni-Dunn method in GraphPad prism v8. Asterisks were used to denote significance on scatter plot histograms with p values documented in Table S9.

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DECLARATION OF INTERESTS
The authors declare no competing interests.
REFERENCES


