Supplementary Experimental Procedures

**Purification of Yeast eIF4G1**

Full-length eIF4G fused with N-terminal GST and C-terminal 6xHis tags was purified as described (Mitchell et al., 2010), with the following modifications. The pGEX4T1-GST-eIF4G1-His6 plasmid was transformed into BL21 CodonPlus-RIL competent cells (Agilent) and grown in 4-6 liters of super broth (3.2% Tryptone, 2.0% yeast extract, 0.5% NaCl, pH 7.5.) to an OD600 of ~2.5 at 37°C. After 4 hours of induction by 1mM IPTG at 30°C, cultures were pelleted, resuspended in lysis buffer (20mM HEPES-KOH, pH 7.4, 100mM KCl, 10% glycerol, 0.1% Triton x100, 20mM Imidazole, 10mM 2-mercaptoethanol, 0.1mM PMSF, 1μg/ml pepstatin, aprotinin, leupeptin, 1 Roche EDTA-free protease inhibitor tab per 50ml), and frozen by dripping into liquid nitrogen. Cells were lysed by cryogenic ball milling in a Retsch Cryomill. Lysates were treated with DNase I (New England Biolabs), centrifuged to remove debris, brought to 0.5M KCl, and passed through 3-micron (Sterlitech) and 0.2 micron (VWR) filters. The protein was enriched from lysate on a HisTrap nickel column (GE Healthcare), eluted with 250mM imidazole, and exchanged into heparin buffer A (150mM NaCl, phosphate buffer pH7.3, 10% glycerol, 2mM DTT). The major fractions were brought to 2mM CaCl₂ and 1U/μl micrococcal nuclease (New England Biolabs), incubated for 30 minutes at room temperature, and then EGTA was added to 5mM on ice. The solution was filtered, passed over a HiTrap Heparin HP column (GE Healthcare), and eluted with a gradient of 0.5-2M NaCl. The eluate was loaded onto a GSTrap HP column (GE Healthcare), and eluted with 10mM glutathione in 50mM Tris-Cl, 10% glycerol, pH7.5. The eluate was concentrated
with Amicon Ultra 3.5kDa cutoff spin columns (Millipore) and passed over an S200 sizing column into storage buffer (20mM HEPES, pH 7.4, 100mM KCl, 10% glycerol, 2mM DTT). The protein eluted from the column in two distinct peaks. The early-eluting (higher molecular weight) peak had a higher A260/A280 ratio, suggesting that it consisted of RNA-bound protein. When run on SDS-PAGE gels, both peaks consisted of mostly full-length protein, with some proteolysis products (Figures S1A, S1B). The RNA-free peak was concentrated using Amicon Ultra 3K centrifugal filter units (Millipore) and used for subsequent experiments.

**RNA Bind-n-Seq Library Synthesis**

Randomized RNA libraries consisted of 20 nucleotides of randomized RNA sequence, followed by an adaptor sequence used for priming reverse transcription and Illumina library preparation. RNAs were transcribed from DNA oligos ordered from Integrated DNA Technologies with a 5’ T7 promoter sequence (Table S2), and gel purified. A complementary oligo (oBZ49) was annealed to the T7 promoter sequence and transcription carried out in as described in (Rio et al., 2011). The transcription products were filtered through G-50 size exclusion spin columns (GE healthcare), precipitated, and gel purified.

**RNA Bind-n-Seq**

1μM RNA pool was incubated with concentrations of eIF4G ranging from 0-1280nM for 30’ at 22°C in a 50ul volume of PBS, 0.1% TWEEN, 1mg/ml BSA, 3mM MgCl₂, 1mM DTT. The solution was then incubated with 50μl of MagneGST glutathione particle slurry (Promega, pelleted and pre-washed 5 times with PBST) for 10’ at 22°C. The supernatant was removed and washed once with 1ml PBST, then eluted by adding 100ul
elution buffer (50mM Tris 7.5, 1mM EDTA, 0.5% SDS) and heating at 70°C for 10 minutes. Eluates were phenol-chloroform extracted and ethanol precipitated. RNA was reverse transcribed at 48°C using SuperScript III (Life Technologies) and primer oTC225 (Carlile et al., 2014), and extended products were gel-purified. cDNA was circularized with CircLigase (epicentre) and PCR was performed with primers RP1 and barcoded reverse primers with Phusion polymerase (NEB) in 1x HF buffer for 8-10 cycles of (98° for 10 sec, 60° for 10 sec, 72° for 5 sec) with the addition of 1M betaine (Sigma) to the PCR reaction. Libraries were sequenced with 50nt single-end reads on an Illumina HiSeq 2000.

**Electrophoretic Mobility Shift Assays**

Homopolymeric U and A sequences were generated with polynucleotide phosphorylase in the presence of a single nucleotide diphosphate to eliminate the possibility of misincorporated nucleotides (Milligan and Uhlenbeck, 1989). 1 unit of polynucleotide phosphorylase (Sigma) in 1 ml of (10mM UDP/ADP, 50mM Tris pH9.0, 2mM MgCl₂) was incubated at 55°C (poly-A) or 37°C (poly-U) for 4 hours. Reactions were concentrated with the Zymo DNA Clean and Concentrator 25 kit and fragmented by incubation in 10mM ZnCl₂ for 4 minutes at 94°C. Fragmentation reactions were stopped by addition of EDTA to 40mM and were then ethanol precipitated. Fragmented RNA was run on a urea-PAGE gel to select the desired size. Poly-CA sequences (and those with homopolymer inserts) were created by T7 transcription (same as RNA libraries above) from oligos oBZ70-72 (Table S2). T7-transcribed RNA was dephosphorylated with Calf Intestinal Phosphatase (New England Biolabs) and cleaned with Oligo Clean & Concentrator kit (Zymo Research). RNA was end-labeled by reaction with T4
Polynucleotide Kinase (New England Biolabs) in the presence of $\gamma^{32}$P ATP (Perkin Elmer), and separated from unincorporated ATP and phosphate with G-25 or G-50 spin columns (GE healthcare).

Approximately 60 femtomoles of radiolabeled RNA was equilibrated at 22°C in 5μl 2xTHEM (35mM Tris, 57mM HEPES, 0.1mM EDTA, 2.5mM MgCl$_2$, 4mM DTT, 0.1-1mg/ml BSA, 10 units SUPERase•in (Life Technologies). eIF4G1 was diluted to 2x the assay concentration in enzyme storage buffer at 22°C. 5μl of protein mix was added to the RNA and equilibrated for 30’ at 22°C. Samples were loaded onto a 1xTHEM 7% acrylamide MIDI gel (pre-run for 1hr at 140 volts at 4°C) and run for 40-120’ at 140 volts. In some experiments, 2ul loading dye (1x THEM, 60% sucrose, xylene cyanol, bromophenol blue) was added before loading, which did not affect the results. Gels were wrapped in plastic, and exposed to phosphor screens for 5-50 minutes, and scanned on a Typhoon phosphorimager. Bands were quantified using ImageQuant TL software (GE Healthcare). Fraction bound was defined as the ratio of the upper band intensity to the total intensity of upper and lower bands. A $\gamma^{32}$P ATP-only control was run on each gel to differentiate full-length RNA from degradation products or unincorporated radioactive ATP or phosphate. Plots were generated in Prism 5 (GraphPad software) and fit to the hill equation with $K_d$, hill coefficient, and maximum fraction bound as free parameters.

**RNA Bind-n-Seq Sequence Enrichment Analysis**

RBNS data were analyzed using the code and algorithms developed in (Lambert et al., 2014), as well as custom Python scripts developed in-house. Although our binding reactions contained sufficient RNA molecules ($\sim$1.5x10$^{14}$) to capture the full theoretical complexity of the input RNA library ($4^{30}$,$\sim$10$^{12}$ sequences), our sequencing depth of
reads per concentration generally limited our analysis to motifs up to length 8, as some motifs of length 9 and longer had unreliably low counts in some of the libraries, most likely due to biases in the T7-transcribed input library. However, many motifs of longer length could be reliably quantified, such as the homopolymer motifs in figure S1.

**RBNS RNA Folding Analysis**

The RNA pairing probability analysis was modified from (Lambert et al., 2014). All reads in a given sequencing library containing a sequence of interest (such as U7 for Figure 4B) were folded by calling the partition fold function of the Vienna RNAfold package (Lorenz et al., 2011) from a custom Perl script modified from the one used in (Lambert et al., 2014). Calling RNA::pf_fld($seq) and RNA::get_pr($k,$m) computes the pairing probability between positions k and m in the sequence, where $seq is the full RNA sequence, including the common 3’ adaptor. The total pairing probability for a given position in the read is the sum of its pairing probabilities with all other positions in the RNA sequence. Average probability profiles were generated by averaging the pairing probabilities for a given position within the motif or surrounding sequence across all reads, then normalizing by the average pairing probability at the same motif position in the input library.

**Estimation of Cellular mRNA and eIF4G Content**

In order to estimate the number of mRNA molecules in a rapidly dividing yeast cell, we re-scaled existing RNA-seq RPKM data from (Subtelny et al., 2014), prepared by rRNA depletion, (GEO accession GSE53313) into per-cell mRNA counts. We performed a linear regression of this RNA-seq data against single-molecule FISH counts for 13 mRNAs (DOA1, KAP104, MDN1, POL1, PRE3, PRE7, PRP8, PUP1, RPB1, RPB3,
TAF12, TAF5, TAF6) from (Gandhi et al., 2010; Zenklusen et al., 2008). PDR5 data was also available, but omitted as an outlier. A linear fit of \( y = (0.0254\pm0.0073)x + (1.228\pm1.1516) \) was obtained. Rescaling and summing all RNA-seq RPKM values results in an mRNA content of \( \sim 26,400\pm11,400 \) molecules per cell (ranges indicate 95% confidence intervals). Total eIF4G content is estimated at 24,000 molecules per cell, based on the low-throughput value of 17,500 molecules per cell for eIF4G1 from (Haar and McCarthy, 2002), and assuming that eIF4G2 is present at 35% the level of eIF4G1 based on the high-throughput measurements of (Ghaemmaghami et al., 2003). Concentrations were computed assuming a cell volume of 42 femtoliters (Jorgensen et al., 2002).

**Conservation Analysis**

Transcript leader annotations were defined as the median length TL for each gene annotated in (Pelechano et al., 2013). 7-yeast alignment whole-genome alignments between *S. cerevisiae* (sacCer3), *S. paradoxus*, *S. Mikatae*, *S. kudriavzevii*, *S. Bayanus*, *S. castelli*, and *S. kluweri* were downloaded from the UCSC genome browser, along with the phylogenic tree and branch lengths. Sequence alignments for each TL were extracted from the alignments using custom Python scripts. Branch lengths were computed using the branch length scoring algorithm from [https://github.com/jakebiesinger/HTS-waterworks/blob/master/hts_waterworks/utils/BranchLengthScoring.py](https://github.com/jakebiesinger/HTS-waterworks/blob/master/hts_waterworks/utils/BranchLengthScoring.py) (Xie et al., 2009). The background set consisted of 7mers deviating from oligo(U) by the substitution of A nucleotides in 1, 2 or 3 positions. The choice of only substituting A was made to preserve the AU content of the sequences. The p values in Figure 2 B were generated as follows: for each conservation cutoff, the mean and standard deviation of conservation fractions...
for all background 7mers (the fraction of instances of each 7mer that were conserved at the given cutoff) was used to fit a normal distribution. P values were computed by integrating the resulting normal distribution from the value of the conservation fraction for U₇ to infinity (the probability of a background motif having a greater conservation fraction at that particular branch length cutoff).

**Western blotting**

For YPA samples, 2 OD600 units of culture were pelleted, precipitated with 5% TCA, washed with 10mM Tris pH7.5 and acetone, and lysed by bead beating. Equal volumes were mixed with SDS-PAGE loading dye for gel electrophoresis and western blotting. For SC samples, 7.5ul (approximately 1.9 A260 units) of the clarified lysate used for footprint profiling was loaded. eIF4G1 antibody (Clarkson et al., 2010) was used at 1:12000 dilution in 5% milk, and ASC1 antibody (Coyle et al., 2009) was used at 1:500,000. Horseradish peroxidase conjugated goat anti-rabbit antibodies and ECL reagent (GE life sciences) were used for visualization.

**Supplementary Figure Legends**

**Figure S1: Purification of eIF4G1, and oligo(U) binding specificity**

(A) Coomassie stained 7% SDS-PAGE gels of pooled eIF4G1 fractions from (A).

(B) Western blot of recombinant eIF4G1 using a polyclonal eIF4G1 antibody (Clarkson et al., 2010). Most bands visible in (B) are reactive to the antibody, indicating that the extra bands are eIF4G1 cleavage products.

(C) Illustration of the homopolymer overcounting problem. A U₁₀ sequence contains increasing numbers of 9mers, 8mer, 7mers and so on, which can lead to inflated
enrichment values for short oligo(U) sequences when longer ones are enriched. In this example, only the longest homopolymer ($U_{10}$) would be counted for panel B.

(D) For each concentration of eIF4G1, the enrichment relative to the input library for each length of oligo-U (1-13nt) is shown. For the purposes of this analysis, homopolymers that are part of a larger homopolymer sequence were excluded to eliminate over-counting of the shorter homopolymer.

(E) Comparison of eIF4G enrichment values to those of well-characterized sequence-specific RNA binding proteins. RBNS enrichments were re-scaled into B values (Lambert et al., 2014), which allow direct comparisons between experiments by accounting for differences in motif and RNA pool length. Shown are the top 10 most enriched motifs for eIF4G1 (320nM, 7mers as well $U_6$, $U_8$, $U_9$, $U_{10}$) as well as for RBFOX2 (6mers, 365nM), CELF1 (7mers, 64nM), and MBNL1 (7mers, 250nM) from (Lambert et al., 2014).

Figure S2: mRNAs with oligo-(U) in their TL are enriched in pulldowns of eIF4G and eIF4E-binding proteins.
(A) Scatterplot between eIF4G1/eIF4G2 and eIF4E RIP-seq enrichments from (Costello et al., 2015).

(B) Box plots of TL lengths from (Pelechano et al., 2013) for all mRNAs, as well as those containing oligo(U) in their TLs. The matched-length control sets (in blue) were chosen by fitting a Gaussian to the mean and standard deviation of a TL length set, and using the resulting probability distribution to pick TLs without oligo(U), without replacement, until an equal-sized gene set was achieved.
(C) Cumulative distribution plots of RIP-seq enrichments from (Costello et al., 2015) for indicated gene sets. 7xU indicates genes with an oligomer of length 7 or longer in their TL. P values for 7xU are for comparison of the indicated gene set with the set of all genes, as computed by Mann-Whitney U test. Control datasets are compared to the corresponding 7xU dataset.

(D) Cumulative distributions of RIP-seq enrichments from (Costello et al., 2015), for genes containing oligo(U) of length 7 or greater, grouped by the average PARS structural propensity of the oligo(U) from (Kertesz et al., 2010). P values are computed by Mann-Whitney U test.

**Figure S3: elf4G depletion and ribo-seq in synthetic media**

(A) Western blot of elf4G1 from WT and 4G-ts strains during depletion timecourse in SC. To exactly match the condition reported in (Park et al., 2011), cultures were grown at the permissive condition of SC + 2% raffinose + 0.1mM copper sulfate at 26°C (pre-shift). At t=-30 minutes, galactose was added to 2% to induce expression of the degron tag’s cognate ubiquitin ligase. At t=0, cultures were spun down and resuspended in SC+2%raffinose +2% galactose +1mM BCS, and the culture was shifted to 36°C. t=2h, and, t=8h were collected for ribosome profiling, and equal A260 units of clarified yeast lysate were used for western blotting.

(B) Polysome profiles of SC-Ura media timepoints for replicate 1 collected for ribosome profiling.

(C) Violin plots of TL lengths from (Pelechano et al., 2013) for all mRNAs, as well as those containing oligo(U) in their TLs. The matched-length control set was chosen by fitting a Gaussian to the mean and standard deviation of the 7xU TL set, and using the
resulting probability distribution to pick TLs without oligo(U), without replacement, until an equal-sized gene set was achieved.

(D) Violin plots of log2 fold difference in ribosome density for each gene between the WT strain and the 4G degron strain. Red horizontal lines indicate the mean for each gene set, boxes show lower and upper quartile ranges, and whiskers indicate 95% intervals. P values are for comparison of the indicated gene set with the set of all genes, or against the 7xU gene sets for the length-matched controls, as computed by Mann-Whitney U test.

**Figure S4: mRNAs with long transcript leaders are particularly dependent on eIF4G.**
Violin plots of fold difference in TE upon factor depletion, with genes binned by TL lengths measured in (Pelechano et al., 2013). Gene bins are matched between all panels.

(A) Ribo-seq TE changes for eIF4G depletion from this study.

(B) Previously published ribo-seq TE changes for eIF4A or eIF4B depletion (Sen et al., 2016; 2015).

(C) Previously published polysome microarray TE changes for eIF4G depletion (Park et al., 2011).

**Figure S5: Additional in vitro testing of TLs with oligo(U)**
NanoLuc activity from in vitro translation extracts programmed with capped and polyadenylated NanoLuc mRNA bearing the indicated TL. Each point represents an independent experiment, and is the mean of 3 technical replicates. Horizontal lines indicate the mean of the three independent replicates. All differences between WT and mutant TLs are statistically significant (p<0.05 by two-tailed student’s t-test). For TL construct diagrams, TLs are drawn to as lines, U≥7 are indicated in vermillion, and
unfilled rectangles represent the N-terminal portion of the native coding sequence for the indicated gene.

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Figure S1
Figure S2

Panel A: Scatter plots showing the correlation between log_{2}\(\text{eIF4E RIP-seq enrichment}\) and log_{2}\(\text{eIF4G1 RIP-seq enrichment}\) for control samples. The correlation coefficients are r=0.741 (5563) and r=0.721 (5584).

Panel B: Box plots illustrating the distribution of log_{2} TL length across different conditions. The box plots are labeled with conditions: all (2556), 7U (193), less structure PARS 2.0-0.1 (711), more structure PARS 0.1-3.5 (122).

Panel C: Cumulative gene fraction plots for CAF20 and EAP1 log_{2} IP enrichment across different conditions: all, 7U, 7U BLS>1, control, etc. The significance levels are indicated for each condition.

Panel D: Similar cumulative gene fraction plots for eIF4E and PABP1 log_{2} IP enrichment.
Figure S3

A

|     | 2 hrs | 8 hrs | 2 hrs |
|-----|-------|-------|-------|
| WT  |       |       |       |
| 4G-ts |       |       |       |
| WT 1:2 |     |       |       |
| WT 1:4 |     |       |       |

replicate 1

replicate 2

B

Polysome/80S

WT, t=2h, replicate 1 1.13
WT, t=8h, replicate 2 0.52
4G-ts, t=2h, replicate 1 0.15
4G-ts, t=8h, replicate 2 0.14

C

all genes (n=5199)
mean = 47.0 nt

7xU (n=380)
mean = 213.9 nt

countrol set (n=380)
mean = 217.1 nt

TL length

D

log2(4G-ts/W T) TE change

2h depletion replicate 1
8h depletion replicate 1
2h depletion replicate 2
8h depletion replicate 2
Figure S4

(A) eIF4A depletion ribo-seq (tlf1-ts, 37°C, Sen et al. 2015)

(B) eIF4B depletion ribo-seq (tlf3-ts, 37°C, Sen et al. 2016)

(C) eIF4G depletion polysome microarray (Park et al. 2011)
**Figure S5**

- **WT Lysates**

Luciferase Units (x10^6)

| Gene Name  | Mutation | nLuc | Luciferase Units |
|------------|----------|------|-----------------|
| YMR158W    | -56      |      |                 |
| YPL172C    | -103     |      |                 |
| YOR064C    | -46      |      |                 |
| YIL083C    | -105     |      |                 |

125 nt
| Oligo Name | Use | Sequence (5’ to 3’) |
|------------|-----|---------------------|
| oBZ49      | T7 antisense primer for transcription | TAATACGACTCACTATA |
| oBZ52      | 20nt random library | CTTTGGCACCCGAGAATTCCANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
| 78 | NanoLuc TL reporter | CTATAGCGGGGTCTTTATATTTTAGGCTATAGTATCCTAACCAGCTATCTTATTTTTCTCTTTTAGAGTATCTTTCCTGAACCGATggtcttcacactcgaagattcggtg |
| --- | --- | --- |
| YORO64C--46_WT | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YORO64C--46_mut_28-35 | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YIL083C--105_WT | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YIL083C--105_mut_83-90 | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YJL084C--113_WT | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YJL084C--113_mut_44-51_65-77 | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YHR019C--42_WT | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YHR019C--42_mut_30-37 | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YHR074W--66_WT | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| YHR074W--66_mut_44-51 | GeneBlock for Gibson assembly of NanoLuc TL reporter | CTATGACATGATTACGCGCCAAGCTCATCCTAaggttaataacgactcataaggGCGCTAGCACACTCCGCTTTTTACAAGGAAGTTATTTTCGCTACAGAATGAACTCACAAGGGTAACATGCCCAAGGAAAACCGGATTCCGATATCATCAGTTCTTCTCTTAGACACTTTTGGATCTATTACCATCGATggtcttcacactcgaagattcggtg |
| GeneBlock for Gibson assembly of NanoLuc TL reporter | DNA oligos used in this study |
|-----------------------------------------------------|-------------------------------|
| YHR074W_-38_WT                                      | ACT TTA GCT ACA TGC AAC TTG AAT CAA TGG GCC CTA GAT TTT G CCatggttcacactggaagatttcgtg |
| YHR074W_-38_mut_16-23                                | CTATGACCATGATTACGCAAATGCTTATCTCTaagcttttataagactcactactagggAAATACCTGGCATTTTTTTTTGACTCCCTGCCCCAGTAATGCA CAT CTG ACT TTA GCT ACA TGC AAC TTG AAT CAA TGG GCC CTA GAT TTT GAA GGT AAT AGA GAC CGT ATC CTA CAG TCCatggttcacactggaagatttcgtg |

**Table S2**: DNA oligos used in this study
| Plasmid Name | Description | Source |
|-------------|-------------|--------|
| pWG682      | NanoLuc backbone plasmid with encoded polyA tail, for cloning in TL sequences. | Thomspon *et al.* eLIFE, 2016 |
| pWG1004     | T7, YMR158W -56 WT | This study |
| pWG1005     | T7, YMR158W -56 mutant | This study |
| pWG1006     | T7, YPL172C -103 WT | This study |
| pWG1007     | T7, YPL172C -103 mutant | This study |
| pWG1008     | T7, YOR064C -46 WT | This study |
| pWG1009     | T7, YOR064C -46 mutant | This study |
| pWG1010     | T7, YIL083C -105 WT | This study |
| pWG1011     | T7, YIL083C -105 mutant | This study |
| pWG1021     | T7, YJLO84C -113 WT | This study |
| pWG1022     | T7, YJLO84C -113 mutant | This study |
| pWG1023     | T7, YHR019C -42 WT | This study |
| pWG1024     | T7, YHR019C -42 mutant | This study |
| pWG1025     | T7, YHR074W -66 WT | This study |
| pWG1026     | T7, YHR074W -66 mutant | This study |
| pWG1027     | T7, YHR074W -38 WT | This study |
| pWG1028     | T7, YHR074W -38 mutant | This study |

Table S3: plasmids used in this study