**File S2 : Detailed methods**

Strain construction

CRISPR-Cas9 was used to edit the genomic sequence of the paralogs following (Ryan et al. 2016) in strains for which the SH3s were replaced by a stuffer DNA sequence (Dionne et al. 2021). The stuffer DNA sequence (GGCGGAAGTTCTGGAGGTGGTGGT) codes for a flexible linker (GGSSGGGG). The stuffer design was based on linkers used in structural protein studies and used to fuse proteins to minimize the effects on protein structure (Reddy Chichili et al. 2013; Li et al. 2016). PCR was used to create the donor DNA for the CRISPR-Cas9 transformations (pCAS-Stuffer3, gRNA = GGCGGAAGTTCTGGAGGTGG (Dionne et al. 2021)) using codon-optimized SH3 DNA sequences for expression in *S. cerevisiae* (Twist Bioscience) or purified yeast genomic DNA (phenol - chloroform protocol (Amberg 2005)) as DNA template. The oligonucleotides (Table S8, Eurofins) added 40-bp homology arms corresponding to the surrounding regions of the SH3 loci (Figure S6A). All PCRs to amplify DNA for genomic integration were done using the KAPA HiFi HotStart polymerase (Roche) with the following reaction mixes (except mentioned otherwise):

| **Reagent** | **Volume (µL)** |
|---|---|
| Kapa HF buffer 5x | 5 |
| dNTPs 10mM | 0.75 |
| Forward oligo 10µM | 0.75 |
| Reverse oligo 10µM | 0.75 |
| Kapa polymerase | 0.5 |
| Template DNA (15 ng/µL) | 0.75 |
| PCR grade water | 16.5 |
| Total | 25 |

with the following PCR cycle:

| | | |
|---|---|---|
| 98°C | 5 min | x1 |
| 98°C | 20 s | x30 |
| 60°C | 30 s | |
| 72°C | variable | |
| 72°C | 6 min | x1 |

Growth media YPD+NAT+G418 (1% yeast extract, 2% tryptone, 2% glucose, and 2% agar (for solid medium)) was used to select cells transformed with pCAS. Colonies were picked randomly to be grown in YPD without antibiotics to allow for plasmid loss. The genomic insertions of the DNA fragments were confirmed by PCR and the amplicons were sent to

sequencing (Centre hospitalier de l'Université Laval sequencing platform) to confirm the correct modifications. All confirmation PCRs were done using regular TAQ DNA polymerase (Bioshop Canada) on purified DNA from single colonies (Lõoke et al. 2011) using the following reaction mix:

| **Reagent** | **Volume (µL)** |
|---|---|
| TAQ polymerase buffer 10X | 2 |
| dNTPs 10mM | 0.4 |
| Forward oligo 10µM | 0.4 |
| Reverse oligo 10µM | 0.4 |
| $MgCl_2$ 25mM | 1.2 |
| DNA (Quick DNA extraction) | 2 |
| PCR grade water | 13.5 |
| Taq DNA polymerase 5 U/µL | 0.1 |
| Total | 20 |

with the following PCR cycle:

| | | |
|---|---|---|
| 95°C | 5 min | x1 |
| 95°C | 30 s | x35 |
| 54°C | 30 s | |
| 72°C | 1 min | |
| 72°C | 2 min | x1 |

The motifΔ (replacement of the motif with a linker) prey strains were constructed using a similar strategy. The predicted binding motif was replaced by the stuffer DNA sequence in the prey strain backgrounds (DHFR F[3] fusion). The NATMX4 cassette was amplified in reverse orientation by PCR while adding 40pb-homology arms surrounding the loci. Genes coding for the preys were replaced with the NATMX4 cassette by homologous recombination. The Q5 High-Fidelity Polymerase (reaction mix below, New Englands Biolabs) was used for the amplification of the DNA fragments and for the fusion of the motifΔ prey DNA sequences. The integrated NATMX4 cassette was targeted by a CRISPR-Cas9 transformation (pCAS-NAT, gRNA : TTCGTGGTCGTCTCGTACTC) to insert the motifΔ preys DNA sequences at the native prey loci (Figure S6B).

| Reagent | Volume (µL) |
|---|---|
| 5X Q5 Reaction Buffer | 5 |
| dNTPs 10mM | 0.5 |
| Forward oligo 10µM | 1.25 |
| Reverse oligo 10µM | 1.25 |
| Template DNA | variable |
| Q5 High-Fidelity DNA Polymerase | 0.25 |
| PCR grade water | to 25 |
| Total | 25 |

with the following PCR cycle:

| | | |
|---|---|---|
| 98°C | 30 sec | x1 |
| 98°C | 10 s | x5 |
| 66°C | 15 s | |
| 72°C | 2 min | |
| 94°C | 15 s | x30 |
| 70°C | 15 s | |
| 72°C | 2 min | |
| 72°C | 5 min | x1 |

The strain backgrounds for expressing the free SH3s were constructed based on the design of (Aranda-Díaz et al. 2017) creating the strain PL0001 (Table S12). This strain was again modified to express a genetic construct designated as a LandingPad (DHFR F[1,2] C-terminal tag : strain AKD0678; DHFR F[1,2] N-terminal tag: strain AKD0707) at the *GAL1* locus replacing the coding sequence only (Figure S6C). The insertions of the SH3 sequences in the LandingPad were done as described above (Figure S6A). All the strains constructed were confirmed by PCR and Sanger sequencing.

Protein-fragment Complementation Assays and analyses

A pin tool robotic platform (BM5-SC1, S&P Robotics Inc.) was used to perform the PCA screens as described in (Rochette et al. 2015). From 96-well plates, Matα DHFR F[3] tagged prey strains were individually arrayed in random positions (384 format) on YPD+HYG solid medium in OmniTray Single-Well Plates. The arrays were condensed into 1,536 format on YPD+HYG medium. To remove border effects on the growth of the colonies, a double border on each edge of the plates was added and used as a control. The control interaction was

between the bait Lsm8 and the prey Cdc39 (interaction of medium strength from (Tarassov et al. 2008)). Each prey was present in four or five replicates on the array. Mat**a** DHFR F[1,2] baits were replicated in 1,536 format from a lawn grown on YPD+NAT solid medium. Each bait was crossed twice with the prey array, resulting in a total of eight to ten replicates per PPI. Mating was performed on YPD solid medium without selection for 48h at 30°C. Next, two rounds of selection on YPD+HYG+NAT were used to select for diploids, each time for 48h at 30°C. As a quality control, pictures of the second diploid selection round were taken. All images were acquired with a EOS Rebel T5i camera (Canon). The diploids were finally replicated for two rounds on solid PCA selection media (0.67% yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, 2.5% noble agar, drop-out without adenine, methionine, and lysine). Each time, cells were grown for four days at 30°C in a spImager custom robotic platform (S&P Robotics Inc.). Final images from the second selection round were used for image analysis as in previous studies using this method (Dionne et al. 2021).

A second DHFR PCA experiment was performed using the motifΔ prey strains. The same protocol was followed except for the array design. The extantSH3, optSH3, AncC and SH3-depleted variants in both paralogs (n = 8) were used to create a randomized array in 384 format. The WT prey (n = 9) and motifΔ prey (n = 9) strains were also randomized in a 384 format. The baits and preys were condensed in 1536 format and independently crossed on four plates. The bait and prey arrays were designed to obtain six replicates for each PPI per plate (1536 format). After diploid selection, two replicates were performed by crossing on PCA medium resulting in 48 biological replicates for each PPI. However, two of the plates were lost due to incubation issues leaving 36 biological replicates per PPI.

A third DHFR PCA experiment was performed using strains AKD0678, AKD0707, p*GAL1*-SH3-DHFR F[1,2] (n = 10) and p*GAL1*-DHFR F[1,2]-SH3 (n = 10) as baits. The same protocol as described above was used, except for the array design. Using the second PCA experiment, 14 preys were chosen for this screen (confirmed proline motif, seven WT and seven motifΔ preys). The mating between the baits and the preys was done in liquid YPD then rearrayed in randomized 384 format. Arraying to 1536 format was done after the diploid selection step. There were four replicates for each PPI per plate. Successive printing scaled the number of plates to eight to pin on various inducer concentrations in the next step. The PCA medium used for this experiment was supplemented with β-estradiol (0 nM, 10 nM, 20 nM, 30nM, 40 nM, 60 nM, 80 nM, 100 nM) (Sigma-Aldrich) diluted in ethanol to activate transcription of the genetic constructs at increasinglevels. Each of the diploid plates was used to print two replicates on the same PCA condition resulting in eight replicates per PPI in each condition.

All pictures were transformed in reverse grayscale so that yeast colonies appear as dark grey (preferred by the image analysis software), then cropped to remove the edge of the omnitrays (ImageMagick Studio LLC). Pyphe (Kamrad et al. 2020), a python toolbox for phenotypic analysis of microbial growth, was used to quantify the colonies' area ('pyphe-quantify batch --grid auto_1536 --t 1 --d 3 --s 0.05') for each plate. Positions that showed no growth on the second diploid selection or on the prey array were considered missing data for the downstream analyses. The colony area values were log2 transformed. Then, the background and aberrant data were assessed and removed ($3 < \log_2(\text{area}) < 13.13$, Figure S2). A normalization using the maximum and minimum logarithm values on

each plate ($\log_2$(colony area) - $\log_2$(min))/($\log_2$(max)-$\log_2$(min)) transformed all logarithm colony areas on a scale between 0 and 1 (PPI score). PPI scores were considered only if there were two or more replicates remaining after data filtering for each PPI.

The analysis of the free SH3 PCA experiment had additional steps. After the image processing using Pyphe, the positions (10/1232) that were growing at 0 nM β-estradiol were removed from the dataset at each concentration because the genetic construct should not enable growth in a β-estradiol depleted media. Also, for the 40 nM and 80 nM β-estradiol conditions, the second technical replicate showed diminished growth (Figure S5), so we removed this data from the analysis. All the PPIs were tested in a set of concentrations induced, leading to increasing expression levels of the bait. Therefore, we selected a β-estradiol concentration for each prey that allowed the maximal range of sensitivity of the assay (Figure S5). We did this by computing the median PPI score difference (Δmedian PPI score) over three successive β-estradiol concentrations. For each PPI, we kept the middle of the three β-estradiol concentration value associated with the highest Δmedian PPI score between the maximum and minimum values. The β-estradiol concentrations conserved for each PPI were used to compute the median β-estradiol concentration (rounded up) grouped by prey.

A subset of 71 PPIs detected by DHFR PCA on solid medium were validated using liquid DHFR PCA growth measurements. The bait and prey strains were grown respectively in liquid YPD+NAT and in liquid YPD+HYG overnight at 30°C. The mating of the bait and prey strains was done in liquid YPD without selection (four replicates per PPI), then spotted on solid YPD+NAT+HYG (48h at 30°C). Diploids were grown in liquid SC pH 6 HYG+NAT (0.174 % yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, 1% succinic acid, 0.6% NaOH, 0.1% MSG), then diluted to an OD of 0.1 in liquid PCA selection medium using transparent polystyrene 96-well plates (Greiner Bio-One). Growth curves were generated by measuring the OD over four days of incubation at 30°C in an Infinite M Nano plate reader without shaking (Tecan). The initial OD value for each curve was subtracted from all the following measurements to correct for variation in initial cell inoculate density. The area under the logistic curve (AUC) was computed with the package R growthcurver (Sprouffske and Wagner 2016) for the first 65 hours of growth.

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