Histone chaperone paralogs have redundant, co-operative and divergent functions in yeast

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Running title: Genetic dissection of paralog functions
Abstract

Gene duplications increase organismal robustness by providing freedom for gene divergence or by increasing gene dosage. The yeast histone chaperones Fpr3 and Fpr4 are paralogs that can assemble nucleosomes in vitro, however the genomic locations they target and their functional relationship is poorly understood. We refined the yeast synthetic genetic array (SGA) approach to enable the functional dissection of gene paralogs. Applying this method to Fpr3 and Fpr4 uncovered redundant, co-operative and divergent functions. While Fpr3 is uniquely involved in chromosome segregation, Fpr3 and Fpr4 co-operate to regulate genes involved in polyphosphate metabolism and ribosome biogenesis. We find that the TRAMP5 RNA exosome is critical for fitness in Δfpr3Δfpr4 yeast and leverage this information to identify an important role for Fpr4 at the 5’ ends of protein coding genes. Additionally, Fpr4 and TRAMP5 negatively regulate RNAs from the non-transcribed spacers of ribosomal DNA. Yeast lacking Fpr3 and Fpr4 exhibit a genome instability phenotype at rDNA which implies that these histone chaperones regulate chromatin structure and DNA access at this location. Taken together we provide genetic and transcriptomic evidence that Fpr3 and Fpr4 operate separately, co-operatively and redundantly to regulate a variety of chromatin environments.

Keywords: chromatin/ paralog/ histone chaperone/ genetic interactions/ nucleolus
**Introduction**

Gene duplication events play an important role both in driving protein evolution and in providing a mechanism for ensuring the robustness of biological systems. Since the earliest observations of duplications on chromosomes (Darlington and Moffett 1930; Bridges 1936) and redundant genes (Kataoka et al. 1984; Basson et al. 1986), models implicating gene duplication events as complex drivers of evolution have been proposed (Ohno 1970; Hughes 1994; Force et al. 1999; Francino 2005; Innan and Kondrashov 2010). Evolutionary forces can favor the retention of redundant genes for dosage reasons, for example, identical copies of histone and ribosomal genes are present in most eukaryotes. Alternately, duplicated genes provide an opportunity for functional divergence of gene pairs, or paralogs, over time.

The **FPR3** and **FPR4** genes encode two *S. cerevisiae* paralogs (Benton et al. 1994; Shan et al. 1994; Manning-Krieg et al. 1994; Dolinski et al. 1997) derived from a distant ancestral gene (Wolfe and Shields 1997; Kellis et al. 2004; Pemberton 2006). They code for highly similar proteins (58% identical and 72% similar in amino acid residues) with acidic N-terminal nucleoplasmin-like histone chaperone and C-terminal FK506-binding (FKBP) peptidyl-prolyl isomerase domains (Kuzuhara and Horikoshi 2004; Xiao et al. 2006; Park et al. 2014) (Figure 1A). Both proteins localize to the nucleus and are enriched in the nucleolus (Benton et al. 1994; Shan et al. 1994; Manning-Krieg et al. 1994; Huh et al. 2003). Notably, Fpr3 and Fpr4 interact with each other and share some common physical interactors (Krogan et al. 2006), including histones (Shan et al. 1994; Xiao et al. 2006; Nelson et al. 2006), and the Nop54 ribosome biogenesis factor (Sydorskyy et al. 2005). Additionally, both Fpr3 and Fpr4 are multi-copy suppressors of temperature sensitivity and mating defects resulting from the absence of the Tom1 E3 ubiquitin ligase (Utsugi et al. 1999; Davey et al. 2000) and both Fpr3 and Fpr4 are required for the degradation of the centromeric histone H3 variant Cse4 (Ohkuni et al. 2014). Therefore, there is good evidence that Fpr3 and Fpr4 co-operate.

There is also evidence that these paralogs have separate functions. Fpr3 has been identified as a regulator of chromosome dynamics at mitotic and meiotic centromeres. During meiosis, Fpr3 enhances recombination checkpoint delay (Hochwagen et al. 2005) and prevents meiotic chromosome synopsis initiation at centromeres (Macqueen and Roeder 2009). To our knowledge, no reports describe similar data for Fpr4. Thus Fpr3 may have functionally diverged. By contrast, Fpr4 can silence expression of a reporter at ribosomal DNA (rDNA) (Kuzuhara and Horikoshi 2004) but the degree to which Fpr3 regulates rDNA has not been described. Additionally, Fpr4 is involved in transcription induction kinetics through the isomerization of prolines on the amino tails of histones H3 and H4 (Nelson et al. 2006). Finally, microarray gene expression analysis of Δ*fpr3* and Δ*fpr4* yeast identified small changes
in partially overlapping sets of mRNAs (up to 4 fold changes in 385 and 161 genes respectively) (Park et al. 2014).

Loss-of-function phenotypes and genetic interactions usually provide insight into gene function. For example, the ASF1 and RTT106 genes, encoding histone chaperones, display clear chromatin-related genetic interactions in synthetic genetic array (SGA) screens (Costanzo et al. 2010, 2016). We noted that the genetic interactomes of FPR3 and FPR4 contained few chromatin-related hits (Collins et al. 2007; Costanzo et al. 2010, 2016; Stirling et al. 2011; Milliman et al. 2012) and hypothesized that the high similarity of these paralogs could render them semi-redundant, masking their genetic interactions.

Here, through a set of comprehensive genetic interaction screens designed for paralogs and a series of RNA-seq transcriptome surveys, we demonstrate that Fpr3 and Fpr4 operate separately, co-operatively, and redundantly. Unique genetic interaction profiles and differentially expressed genes demonstrate that these histone chaperones are not equivalent; for example, Fpr3 appears uniquely involved in chromosome segregation. By contrast, shared genetic interactions of FPR3 and FPR4 with the SWI/SNF and ADA chromatin regulators predicted that Fpr3 and Fpr4 co-operate to regulate genes. The identification of polyphosphate metabolism and ribosome biogenesis genes as Fpr3/4 targets confirms this prediction. Finally, we find that the TRAMP5 RNA exosome becomes critical for fitness in ∆fpr3∆fpr4 yeast and leverage this information to perform a sensitized survey for Fpr4 regulated genomic loci. This strategy identified an important role for Fpr4 at the 5' ends of protein coding genes as well as at the non-transcribed spacer regions of rDNA. Finally, we show that yeast lacking Fpr3 and Fpr4 exhibit a genome instability phenotype at rDNA implying that these histone chaperones regulate chromatin structure at these regions. Taken together we provide genetic and transcriptomic evidence that Fpr3 and Fpr4 operate separately, co-operatively and redundantly to regulate a variety of chromatin environments.

Materials & Methods

Yeast strains and plasmids

Yeast strains used in this study are described in Appendix file 5. Strains in the MAT a non-essential yeast deletion collection (DMA) used for the SGA analysis are all isogenic to BY4741 and were purchased from Thermofisher Dharmacon. The plasmid rescued double genomic deletion ∆fpr3∆fpr4 SGA query strain (YNS 35) was created in a Y7092 genetic background as follows. The endogenous FPR4 locus on a Y7092 WT strain was replaced with a nourseothricin resistance (MX4-NATR) PCR product deletion module. The resulting single gene ∆fpr4 deletion mutant was subsequently
transformed with prs316  FPR4: a single copy URA3 marked  shuttle vector carrying an untagged full length copy of the FPR4 open reading frame with endogenous promoter and terminator (originally described in (Nelson et al. 2006)). The endogenous FPR3 locus on this plasmid rescued Δfpr4 deletion mutant was subsequently replaced with a LEU2 PCR product deletion module.

Triple deletion mutants: Δrrp6Δfpr3Δfpr4 and Δtrf5Δfpr3Δfpr4 and their corresponding mixed population total haploid meiotic progeny controls used in the validating growth curves were generated from the SGA cross (see below).

Single gene deletion mutants of Δfpr3, Δfpr4, and Δsir2 used for the RNA sequencing are all isogenic to BY4741 and were either purchased from open biosystems, or taken from the yeast deletion collection (purchased from Thermofisher Dharmacon). The isogenic double deletion Δfpr3Δfpr4 mutant was constructed from the open biosystems Δfpr3 single gene deletion mutant by replacing the endogenous FPR4 locus with a nourseothricin resistance (MX4-NATR) PCR product deletion module. The FPR4(Δfpr3Δtrf5) and Δfpr3Δfpr4Δtrf5 isogenic strains and their corresponding total haploid mixed population controls were generated from the SGA cross (see below).

The Δfpr3 and Δfpr4 deletion mutant strains used in the rDNA reporter spotting assays were generated from a cross of the MAT α UCC1188 (VanLeeuwen et al. 2002) with a MATa BY4741 deletion mutant see Appendix table 5 for details. The UCC1188 background Δfpr3Δfpr4 double deletion mutant, UCC1188 background Δsir2 deletion mutant, and HMLα reporter expression mutants were generated by lithium acetate transformation of either UCC1188 or UCC7266 (VanLeeuwen et al. 2002) with PCR product deletion modules. The Δfpr3Δfpr4 and Δsir2 deletion mutant strains used in the propagation assays were generated from a transformation of UCC1188 with PCR product deletion modules.

Synthetic Genetic Array (SGA) Analysis

SGA analysis was performed using a Singer Instruments ROTOR microbial arraying robot as previously described (Tong and Boone 2006) with the following modifications. The MAT a/a diploid zygotes resulting from the query strain DMA cross were pinned onto diploid selective YPD + G418/clonNAT plates a total of two times for greater selection against any residual haploids. Sporulation was carried out at room temperature for 14 days. Spores were pinned onto Mat a selective germination media for two rounds of selection as previously described (Tong and Boone 2006).

The resulting MAT a progeny were subsequently replica plated onto four kinds of selective media: control media selective for the total haploid meiotic progeny population (SD media lacking histidine, arginine, lysine and containing canavanine and thialysine both at a final concentration of 50mg/l, and
G418 at a final concentration of 200mg/L), media selective for ΔxxxΔfpr3 haploid meiotic progeny (SD media lacking histidine, arginine, lysine, leucine, uracil, and containing canavanine and thialysine both at a final concentration of 50mg/l, G418 and clonNAT both at a final concentration of 200mg/L), media selective for ΔxxxΔfpr4 haploid meiotic progeny (SD media lacking histidine, arginine, lysine, and containing canavanine and thialysine both at a final concentration of 50mg/l, G418 and clonNAT both at a final concentration of 200mg/L, and 5-fluoroorotic acid at a final concentration of 1000mg/L), and finally media selective for ΔxxxΔfpr3Δfpr4 haploid meiotic progeny (SD media lacking histidine, arginine, lysine, leucine, and containing canavanine and thialysine both at a final concentration of 50mg/l, G418 and clonNAT both at a final concentration of 200mg/L, and 5-fluoroorotic acid at a final concentration of 1000mg/L). Plates were incubated at 30°C for 24 hours and were then expanded into triplicate and incubated for an additional 24 hours at 30°C.

Images of each plate were scanned and subsequently processed using the Balony image analysis software package as previously described (Young and Loewen 2013). In brief, pixel area occupied by each colony was measured to determine colony size. Progeny fitness was then scored as follows. The ratio of each double (ΔxxxΔfpr3, ΔxxxΔfpr4) and triple (ΔxxxΔfpr3Δfpr4) mutant colony size relative to its corresponding total haploid meiotic progeny control colony was determined. Ratio cut-off thresholds were estimated automatically by the software by extrapolating the central linear portion of the ratio distributions and finding the y-intercepts at either ends of the x-axis. Genetic interactions were identified using the automatically estimated upper and lower cut-off thresholds and default Balony hit parameters (i.e. reproducibility in 3/3 sets and p-values < 0.05) (a complete list of all genetic interactions generated from each dataset is presented in Appendix file 1).

**SGA Data Processing**

Unique, common, and masked synthetic sick/lethal interactors were identified as follows. First, duplicate genes in the lists of hits from each dataset were removed. The three lists of hits were then compared to each other. The Δfpr3 and Δfpr4 screens were compared to identify unique and common interactors. Genes uniquely present in the Δfpr3Δfpr4 double mutant screens were defined as masked interactors. Unique, common, and masked suppressor interactors were identified the same way.

The lists of unique, common, and masked synthetic sick/lethal and suppressor genetic interactors were subsequently analyzed using the web based FunSpec bioinformatics tool (http://funspec.med.utoronto.ca/, Dec 2017). The analysis was performed using a p-value cut-off score of 0.01, and without Bonferroni-correction. A full list of the ontologies uncovered and their corresponding p-values are presented in Appendix file 2. Networks illustrating the unique and common
complex related genetic interactions were drawn using the Cytoscape software platform (http://www.cytoscape.org/).

**Growth Curves**

Growth curves to validate the synthetic sickness phenotypes were carried out as follows. Colonies generated from the SGA assay corresponding to each triple mutant of interest and its respective control colony were isolated and validated for correct genotype by PCR. Confirmed strain isolates were then resuspended in fresh YPD media, normalized to an OD<sub>600</sub> of 0.2 and distributed into triplicate wells of a 24 well cell culture plate. Plates were subsequently grown for 16h at 30ºC in a shaking plate reader. Readings of OD<sub>600</sub> were taken every 30 minutes.

**RNA-Seq Library Preparation and Sequencing**

Single colony isolates of each strain were grown to mid log phase in 50ml of liquid yeast extract-peptone-dextrose (YPD) media. Samples were then pelleted and washed once with sterile water before being flash frozen in liquid nitrogen and stored for 16 hours at -80ºC. Samples were thawed on ice, and RNA was extracted using a phenol freeze based approach as previously described (Schmitt et al. 1990). The extracted RNA was subsequently treated with RNase- free DNase I (Thermo Fisher Scientific).

RNA samples were processed and sequenced at the BC Cancer agency Michael Smith Genome Sciences Centre following standard operating protocols. Briefly, total RNA samples were ribo-depleted using the Ribo-Zero Gold rRNA Removal Kit (Yeast) (Illumina) and analyzed on an Agilent 2100 Bioanalyzer using Agilent 6000 RNA Nano Kit (Agilent Technologies, Santa Clara, California). cDNA was generated using the Superscript Double-Stranded cDNA Synthesis kit (ThermoFisher) and 100bp paired-end libraries were prepared using the Paired-End Sample Prep Kit (Illumina, San Diego, California).

**Processing of Sequencing Data**

Sequenced paired-end reads were aligned to the sacCer3 reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_000146045.2/) using the BWA aligner (Li and Durbin 2010) (version 0.6.1-r104-tpx). We observed that out of 5110 *Saccharomyces cerevisiae* genes annotated in Ensembl v90 only 267 are spliced with and most of spliced genes (251) having one intron. Therefore, we considered genomic alignment of RNA-seq reads as a good approximation for the yeast
transcriptome analysis. For every library total of ~1.5-2M reads were sequenced, of which ~75-95% of reads were aligned.

To quantify gene expression, we filtered reads that aligned to multiple locations (and therefore can’t be placed unambiguously) by applying a BWA mapping quality threshold of 5. We further collapsed fragments that were duplicated (only counting a single copy of a read pair if a number of pairs with the same coordinates was sequenced) as well removed chastity failed reads and considered only reads that were properly paired. Post-processing was performed using the ‘pysam’ application for python (https://github.com/pysam-developers/pysam). The alignment statistics were calculated using the ‘sambamba’ tool v 0.5.5 (Tarasov et al. 2015).

We considered cDNA fragment lengths distributions as well as genome-wide distributions of read coverage (data not shown) in order to ensure that these characteristics are similar for the pairs of data sets in the differential gene expression (DE) analysis. Genome wide pair-ended fragment coverage profiles for both strands were generated as well as read counts for every gene for further DE analysis.

The reads-per-kilobase-per-million (RPKM) values were calculated for every gene, and DE analysis was performed using the DEfine algorithm (M.Bilenky et al., unpublished). First, the chi2 p-value was estimated for every gene under the null hypothesis that the gene is not differentially expressed between two data sets. The Benjamini-Hochberg FDR-control procedure was applied (FDR=0.05) to find a p-value threshold. To further reduce noise, we only considered genes with the fold-change (FC) between RPKM values FC>1.5, as well required minimal number of aligned reads >5 per gene. Only reads aligned to the proper strand were considered in the DE analysis.

In addition to the standard DE analysis, where gene expression quantification was done by counting reads falling into the gene boundaries, we considered a model independent approach by calculating read counts in every 175bp long bin genome wide (for both strands), and performed DE analysis between bins (with the same approach we used for genes, see above). After defining the DE bins, we overlapped their locations with gene coordinates to determine DE genes. This second approach also provided a list of potential DE expressed intergenic regions. A full list of the DE genes is presented in Appendix file 3.

Quantitative real time PCR (qRT-PCR) validation of DE transcripts

Total RNA was prepared from single colony isolates of each strain grown to mid log phase in 50ml of liquid yeast extract- peptone- dextrose (YPD) media using a phenol freeze based approach as previously described (Schmitt et al. 1990). The extracted RNA was subsequently treated with RNase-free DNase I (Thermo Fisher Scientific) and cDNA was prepared using a High- capacity cDNA reverse
transcription kit (Applied Biosystems). Quantitative real time PCR was performed using the Maxima
SYBR Green qPCR Master Mix (Thermo Scientific) and the forward and reverse primers listed in
Appendix file 6. Experimental gene Ct values were normalized to the mean Ct values of two
housekeeping gene normalizers; *TCM1* and *GPD1*.

**Ontology analysis of DE genes**

Ontologies associated with differentially expressed genes or genetic interactions were identified
using the web based FunSpec bioinformatics tool (http://funspec.med.utoronto.ca/, Dec 2018). The
analysis was performed on genes displaying a fold change or 1.3 and up using a p-value cut-off score
of 0.001, and with Bonferroni-correction. A full list of the ontologies uncovered and their
corresponding p values is presented in Appendix file 4.

**Averaged gene read maps**

Universal gene coverage profiles were generated as follows; we first created cDNA fragment
coverage profiles genome wide for both strands using all aligned read-pairs. Next, we selected profiles
for individual genes and scaled them to 100 units and normalized by the total gene coverage. After that
we agglomerated all scaled and normalized gene coverage profiles together. When doing this, the
profiles for genes on the negative strand were inverted (in other words we always agglomerate profiles
from 5’ to 3’ of gene).

**rDNA Reporter Propagation Assays**

The URA+ status of each reporter containing strain was first confirmed by growth on SD media
lacking uracil. Saturated overnights were then prepared from single colony isolates of each confirmed
strain in liquid YPD media. Cultures were prepared from the overnights in 50ml YPD media and grown
at 30°C to mid log phase. Cells were subsequently collected, washed once, resuspended in sterile
deionized water, and normalized to an OD$_{600}$=0.5. Normalized cell suspensions were subsequently
diluted 10-fold and 250µl of each dilution was plated on 25ml SD 5-FoA plates. Plates were incubated
at 30°C for 16 hours. A total of 96 well-isolated colonies were randomly picked from each 5-FoA plate
using the Genetix QPix-2 colony picking robot and deposited onto non-selective solid YPD plates.
Plates were incubated for 5 days at 30°C. All 96 colonies on each YPD plate were then replica plated
onto SD complete control media and SD media lacking uracil and incubated for 5 days at 30°C before
being imaged.
Data Availability Statement
Supplemental files available at FigShare. Appendix 1 contains lists of all genetic interactions detected in this study. Appendix 2 contains gene ontology analysis of genetic interactions. Appendix 3 contains lists of all differentially expressed genes detected in this study. Appendix 4 contains gene ontology analysis of differentially expressed genes. RNA-seq data are deposited in the Gene Expression Omnibus Repository (GSE134075). All yeast strains and primers used in this study are listed in Appendices 5 and 6, respectively.

Results
Genetic interactions reveal separate, co-operative, and redundant functions of FPR3 and FPR4

Since Δfpr3 and Δfpr4 yeast are viable, but double Δfpr3Δfpr4 mutants display a synthetic sick phenotype (Dolinski et al. 1997; Costanzo et al. 2010) we reasoned that partial redundancy may be masking genetic interactions. To address this and determine the biological processes sensitive to these histone chaperones we performed a modified synthetic genetic array (SGA) screen designed to dissect functional redundancy of gene paralogs (Figure 1B, see Materials and Methods). To this end we crossed a dual-query Δfpr3Δfpr4 double mutant strain to the 4784-strain non-essential yeast deletion mutant array (DMA), so that the fitness of all double (Δfpr3Δxxx and Δfpr4Δxxx) and triple (Δfpr3Δfpr4Δxxx) mutant meiotic progeny could be measured in parallel. The query strain also harbored an episomal URA3 plasmid with a functional FPR4 gene to avoid the slow growth phenotype of Δfpr3Δfpr4 dual deletion yeast, and its vulnerability to suppressor mutations. This plasmid was maintained until the final step of the screen when counter-selection with 5′FOA created the fpr4 null status. Using standard selection methods, the spores of this single cross were manipulated to generate three separate SGA screens that identified all genetic interactions with Δfpr3, with Δfpr4 and genes whose disruption impacted the fitness of yeast lacking both Δfpr3Δfpr4.

We identified 456 and 138 genetic interactors that were unique to either FPR3 or FPR4, respectively, revealing that these paralogs are not equivalent (Figure 1C top). An additional 78 genes interacted with both FPR3 and FPR4, implying that there are specific contexts of paralog co-operativity; that is, situations where both histone chaperones are required for function. We also uncovered 75 masked interactors, defined as genes whose deletion only impacts the fitness Δfpr3Δfpr4 yeast (Figure 1C bottom). These genes highlight processes where paralog function is redundant. The
complete list of these genes and a Gene Ontology (GO) analysis are provided in Appendix files 1 and 2, respectively.

FPR3 genetic interactors fall into a diverse collection of protein complex ontologies including members of the large and small mitochondrial ribosomal subunits (p<10^{-14} and p=7.49x10^{-7} respectively), the mitochondrial pyruvate dehydrogenase complex (p=1.16x10^{-3}), the cytochrome bc1 complex (p=3.11x10^{-3}) and components of the ESCRT II endosomal sorting complex (p=3.06x10^{-4}) (Figure 1D). We also identified all three components of the Ctk1 kinase complex (p=3.06x10^{-4}), and four components of the Swr1 chromatin remodeler (p=9.00x10^{-3}) supporting at least some potential chromatin centric roles of Fpr3. Most notably, we uncovered complexes involved in chromosome segregation such as the astral microtubule (p=6.48x10^{-6}), kinetochore (p=1.14x10^{-4}), and the Mrcl/Csm3/Tof1 complex (p=3.06x10^{-4}) as genetic interactors unique to Fpr3, and not Fpr4. These systems-level data support reports which indicate that Fpr3, but not Fpr4, regulates mitotic and meiotic chromosome dynamics, including those associated with centromeres (Hochwagen et al. 2005; Macqueen and Roeder 2009; Ohkuni et al. 2014). Although we identified 138 FPR4 specific genetic interactions, they fall into limited ontologically related protein complex categories. Several genes coding for components of the pre-autophagosome and associated with the process of mitochondrion degradation (p=2.89x10^{-3}) were the exception, but the relationship between Fpr4 and this process is not clear. Taken together, the number and nature of negative genetic interactions from single query screens suggests that Fpr4 cannot fulfil many of Fpr3’s biological functions, particularly those in chromosome dynamics, and mitochondrial ribosome biology. However, Fpr3 might be competent to substitute for Fpr4 (see below).

Shared genetic interactions would be expected if both paralogs were required for the efficient execution of a biological process. Among genetic interactors common to both FPR3 and FPR4 are genes coding for the ESCRT III complex (p=6.05x10^{-6}) which functions in endosomal sorting, the Ada2/Gen5/Ada3 histone acetyltransferase (p=1.50x10^{-5}) and the ATP-dependent SWI/SNF chromatin remodeler (Figure 1D). Shared genetic interactions with the SWI/SNF remodeler were confirmed using spotting assays (data not shown). The proposed co-operation of Fpr3 and Fpr4 is supported by the fact these proteins co-purify (Krogan et al. 2006) and, like nucleoplasmin, have the intrinsic propensity to form oligomers (Dutta et al. 2001; Edlich-Muth et al. 2015; Koztowska et al. 2017). Thus, these shared genetic interactions with known chromatin regulatory complexes support published protein complex data and indicate that Fpr3 and Fpr4 likely co-operate in some contexts.

75 masked genetic interactions are only detectible in double Δfpr3Δfpr4 mutants (Figure 1C bottom). These genes are essential only when both paralogs are absent, and thus highlight processes in
which Fpr3 and Fpr4 are redundant. Most notably these interactors include TRF5 and AIR1 (Figure 2A), two non-essential components of the TRAMP5 nuclear RNA exosome, an RNA surveillance factor that recognizes, polyadenylates and degrades aberrant RNA transcripts (Figure 2B) (LaCava et al. 2005; Houseley and Tollervey 2008; San Paolo et al. 2009; Wery et al. 2009). An additional non-essential subunit of the nuclear RNA exosome (RRP6) was at the threshold of significance using default Balony settings (Figure 2A). We independently confirmed synthetic sickness of Δfpr3Δfpr4 with Δtrf5 and Δrrp6 using growth curves (Figure 2C). Negative genetic interactions with three non-essential components of the TRAMP5 exosome strongly suggests that Fpr3 and Fpr4 have redundant biological functions likely involving the negative regulation of RNAs.

### Suppressor genetic interactions of FPR3 and FPR4

The SWI/SNF and ADA complexes are particularly important for the fitness of Δfpr3 and Δfpr4 yeast (Figure 1D). In support of a chromatin defect underlying these phenotypes, we found that several genetic suppressors (Figure 3), which alleviate the slow growth phenotype of Δfpr3Δfpr4 yeast, are themselves chromatin modifiers. These include: Hos2, Hda1 and Hos3, three NAD+ independent histone deacetylases (p= 6.33x10⁻⁵); Hir1, Hpc2, and Hir3, three of the four components of the HIR replication-independent nucleosome assembly complex (p=1.29x10⁻⁵); and Swd3 and Sdc1, two of the eight components of the Set1/COMPASS histone H3K4 methylase complex, (p= 5.87x10⁻³). We note that the Swd2 subunit of COMPASS is encoded by an essential gene and the Δset1 knockout is not present in our deletion strain collection. It is particularly notable that we find histone deacetylases enriched among suppressor interactions and histone acetyltransferases among synthetic sick and lethal interactions. The presence of both aggravating and alleviating chromatin-related genetic interactions in our modified SGA screen is consistent with a chromatin-centric mode of action for Fpr3 and Fpr4.

### Fpr3 and Fpr4 regulate partially overlapping sets of genes

The genetic interactions of Fpr3 and Fpr4 with known chromatin modifiers suggest that they regulate transcription. Indeed a microarray study determined these histone chaperones regulate the expression of a broad set of functionally diverse protein coding genes (Park et al. 2014). Because these experiments did not include an analysis of Δfpr3Δfpr4 double mutants and were restricted to protein coding regions of the genome we sought to obtain a more complete view of the impacts of Fpr3 and Fpr4 on the transcriptome. To this end we performed a singlicate RNA-seq survey screen of the ribo-minus fraction of RNAs from WT, Δfpr3, Δfpr4 and Δfpr3Δfpr4 yeast (Figure 4A, B). To verify this survey approach, we included a Δsir2 strain as a control, which in our analysis displayed 854
differentially expressed genes (Figure 4A) using a lenient cut-off of 1.3 fold (a similar threshold to that of Park et al). The number and nature of Sir2-regulated genes we identified is in good agreement with previous reports of Sir2 regulated genes and binding sites (Li et al. 2013; Ellahi et al. 2015). A complete list of differential expressed genes from these experiments can be found in Appendix file 3.

Single deletion mutants of \(\Delta fpr3\) and \(\Delta fpr4\) had 529 and 549 differentially expressed genes, respectively (Figure 4A, Appendix file 3). Two general observations are consistent with previous microarray analyses (Park et al. 2014). First, roughly one third of Fpr3 regulated transcripts are also regulated by Fpr4, and vice versa, confirming that on these genes, transcriptional regulation requires co-operation between paralogs (Figure 4B). Second, the effect of these histone chaperones on gene expression can be positive or negative, but the impact of Fpr3 and Fpr4 is always in the same direction. Since approximately 2/3 of differentially expressed genes were down-regulated (Figure 4A, B blue), these histone chaperones likely predominantly promote gene expression.

Do determine if Fpr3 and Fpr4 have distinct impacts on the transcriptome, we subjected the gene lists represented by sectors in Figure 4B to GO analysis. While the singlicate nature of our comparative RNA-seq approach means the interpretation of DE genes should be taken with caution, it is noteworthy that genes uniquely regulated by Fpr3 and Fpr4 appear to fall into functionally distinct categories (Appendix file 4). The 337 Fpr3 regulated genes retrieved the terms transferase activity (\(p=2.11 \times 10^{-7}\)) and the generic term of metabolic process (\(p=1.99 \times 10^{-5}\)), while Fpr4 regulated genes are enriched in RNA binding (\(p=7.69 \times 10^{-6}\)), nucleotide binding (\(p=1.91 \times 10^{-5}\)) functions, and ribosome biogenesis (\(p=1.07 \times 10^{-11}\)) and rRNA processing (\(p=8.51 \times 10^{-9}\)) processes. 338 genes were uniquely misregulated in \(\Delta fpr3\Delta fpr4\) double mutants but these genes generally fall into previously described Fpr3 and Fpr4 categories including transferase activity (\(p=4.45 \times 10^{-5}\)) and rRNA binding (\(p=4.99 \times 10^{-4}\)). Taken together these results indicate that Fpr3 and Fpr4 have non-overlapping impacts on a fraction of the transcriptome, but may be functionally redundant on some genes.

We identified 127 genes (62 up, 65 down) that are differentially expressed in all three RNA-seq libraries (\(\Delta fpr3\), \(\Delta fpr4\) and \(\Delta fpr3\Delta fpr4\)). Genes downregulated in all three experiments are enriched in factors involved in iron siderophore transport (\(p=4.33 \times 10^{-9}\)). We found that the 62 genes upregulated are highly-enriched in ribosomal protein genes (\(p=5.07 \times 10^{-8}\)), factors involved in phosphate transport (\(p=1.24 \times 10^{-6}\)) and polyphosphate metabolism (\(p=4.20 \times 10^{-7}\)). In fact, the most differentially expressed genes in our survey (up to 60-fold upregulated) are phosphate metabolic genes such as \(PHO5\) and \(PHO11/12\), encoding acid phosphatases; and \(PHO89, PHO84\) and \(PIC2\), encoding phosphate transporters. Since previous studies had not identified the \(PHO\) genes as Fpr3/4-regulated, we verified our RNA-seq observations using independent biological replicates and quantitative RT-PCR of two
PHO genes (Figure 4C), as well as one down-regulated siderophore transporter, SIT1. The identification of polyphosphate metabolism and ribosomal protein genes as Fpr3/4 targets is noteworthy given a recent report that identified Fpr3 and Fpr4 as major direct targets of protein polyphosphorylation and established conserved links between the polyphosphorylation and ribosome biogenesis network in yeast and human cells (Bentley-DeSousa et al., 2018).

In summary, our RNA-seq experiments demonstrate that Fpr3 and Fpr4 have non-overlapping impacts on the transcriptome. Most significantly we find that both paralogs are required for repression of genes involved in phosphate uptake and polyphosphate metabolism, as well as ribosomal protein genes.

The TRAMP5 RNA exosome masks the impacts of Fpr4 on transcription

Deletion of TRF5, encoding the defining component of the TRAMP5 nuclear RNA exosome, induces severe sickness in Δfpr3Δfpr4 yeast (Figure 2). We therefore wondered whether TRAMP5 might be required for the degradation of transcripts negatively regulated by these paralogs. To test this idea, we focused on Fpr4 regulated genes by sequencing the ribo-minus transcriptomes of two strains from our SGA screen: Δtrf5 haploids with a functional Fpr4 (Δfpr3Δtrf5), and isogenic haploids from the same spores that lack both Fpr3/4 proteins (Δfpr3Δfpr4Δtrf5). This provides a sensitized approach to reveal Fpr4-regulated RNAs because functional compensation by Fpr3 is not possible and potential degradation of upregulated RNAs by TRAMP5 is eliminated. This comparison (Δfpr3Δtrf5 vs Δtrf5Δfpr3Δtrf5) uncovered a total of 1321 differentially expressed genes (967 upregulated and 354 downregulated) (Figure 4D). A summary of GO analysis of upregulated genes is provided in Figure 4E. Genes encoding protein components of the cytosolic ribosome (p=3.21x10^{-12}) and genes associated with rRNA processing (p=1.14x10^{-8}) are highly enriched as Fpr4 targets. Also enriched were genes coding for constituents of the fungal-type cell wall (p=1.87x10^{-4}) and the electron transport chain (p=6.12x10^{-8}) (Figure 4E). These results partially explain the underestimation of genes negatively Δfpr4 transcriptomes (Figure 4A). That is, the TRAMP5 RNA exosome may buffer changes in the levels of some Fpr4 regulated RNAs.

A signature of incomplete elongation is present in Δfpr4 yeast

Further interrogation of our transcriptome data reveals additional evidence for Fpr4 in the regulation of transcription: we noticed that a significant proportion (~40%) of differentially expressed genes in Δfpr3Δfpr4Δtrf5 yeast displayed an accumulation of reads towards the 5’ end of the annotated transcript. Subsequent bioinformatic analysis of the total transcriptomes of Δfpr3Δfpr4Δtrf5 and
Δfpr3Δtrf5 mutants revealed that this asymmetry (or 5′-bias) is widespread, and detectable in genes irrespective of their net change in transcription (Figure 5A). RNA-seq reads on two example genes illustrating this asymmetry signature are presented in Figure 5B; SSF1 codes for a constituent of the 66S pre-ribosome and is required for large ribosomal subunit maturation, while UTP9 codes for a component required for proper endonucleolytic cleavage of 35S rRNA. The paired-end tag coverage on both of these genes, but not the ACT1 gene (Figure 5C), displays the characteristic 5′-asymmetry in Δfpr3Δfpr4Δtrf5 yeast. We verified these observations using independent biological replicates and quantitative RT-PCR using 5′ and 3′ amplicons of UTP9 and SSF1, which were normalized to the unchanged GPD1 gene (Figure 5D). This transcriptome signature demonstrates three novel findings: first, Fpr4 negatively regulates transcription from many genes even though total reads per gene may not change. Second, Fpr4 action is critical at a stage after initiation, likely transcriptional elongation. Third, because this signature of accumulated 5′ reads on genes is only readily detectable in the absence of Trf5, the TRAMP5 RNA exosome can mask subtle transcriptional defects (Figure 4D). While we cannot rule out potential impacts of Fpr3 and Fpr4 on the stability of RNAs, given the role histone chaperones play in nucleosome dynamics, we favor a model that explains this bias as a consequence of altered passage of polymerase through genes. Additional experiments probing transcriptional processivity in Δfpr3 and Δfpr4 mutant yeast are needed to resolve the mechanism(s) by which these histone chaperones facilitate the full transcription of genes.

Fpr4 inhibits transcription from the non-transcribed spacers of ribosomal DNA

The ribosomal DNA locus in yeast consists of a series of 150-200 tandem repeats of a 9.1kb unit containing the 35S and the 5S rRNAs each separated by two non-transcribed spacer sequences (NTS1 and NTS2) (Johnston et al. 1997). Given the nucleolar enrichment of Fpr3 and Fpr4, and the ability of Fpr4 to repress reporter expression from rDNA (Kuzuhara and Horikoshi 2004), we asked if yeast lacking Fpr3 and Fpr4 display transcriptional defects at rDNA. While our RNA-seq analysis was performed on ribo-minus RNA, reads from within the rRNA are readily detected (presumably from incomplete rRNA depletion) and indicate no change in rRNAs in Δfpr3, Δfpr4, or Δfpr3Δfpr4 strains (Figure 6A), which we have also observed in Northern and qRT-PCR analyses (data not shown). Surprisingly, we did not observe evidence for the reported loss of NTS silencing in Δfpr4 (or Δfpr3 or Δfpr3Δfpr4) yeast (Kuzuhara and Horikoshi 2004) (Figure 6A). Given that TRAMP5 buffers the loss of Fpr4 (Figure 4D), we asked if Trf5 might be degrading NTS RNAs in Δfpr4 yeast. Consistent with this idea, we observe transcripts templated from both strands of NTS1 and NTS2 in Δfpr3Δfpr4Δtrf5, but not Δfpr3Δtrf5 strains. Taken together, these results support a model where Fpr4 establishes a
transcriptionally silent chromatin state at rDNA. In the absence of this chromatin structure, pervasive transcription can occur from both strands of NTS1 and NTS2. These RNAs are presumably degraded by TRAMP5.

**Fpr3/Fpr4 are required for genomic stability at ribosomal DNA**

Ribosomal RNAs comprise approximately 80% of the total RNA in yeast; accordingly, active rDNA repeats are the most heavily transcribed, and nucleosome-free, genes in the cell (Warner 1999; Vogelauer et al. 2000; Nomura et al. 2004). Reciprocally, the adjacent NTS spacers and inactive rDNA repeats are chromatinized and potently silenced. This arrangement is thought to generate a chromatin template that is refractory to recombination between rDNA repeats and the deleterious loss of rDNAs from chromosome XII, which is a major driver of yeast replicative aging (Sinclair and Guarente 1997). For this reason, failure to generate heterochromatin environments at rDNA, as occurs in Δsir2 histone deacetylase mutants, decreases genomic stability at this locus (Gottlieb and Esposito 1989; Kobayashi et al. 2004).

We reasoned that if Fpr3 or Fpr4 were silencing the NTS regions via a mechanism that involves chromatin structure, that yeast lacking these enzymes should also exhibit genomic instability at this locus. To test this hypothesis, we introduced Δfpr3Δfpr4 and Δsir2 deletions into a strain with a reporter gene (URA3) integrated at NTS1 (VanLeeuwen et al. 2002; VanLeeuwen and Gottschling 2002). First, URA+ status of each strain was ensured by propagation in media lacking uracil. Next, cells were grown in non-selective media (YPD) for two days to permit reporter silencing or loss. Phenotypically ura- cells, were isolated on 5′FOA and ~96 colonies picked using a colony picking robot. These ura- cells could arise in two ways: epigenetic silencing of URA3 at NTS1, or from URA3 gene loss via recombination (Figure 7A). To discriminate between these events, we replica plated these individual isolates to media lacking uracil, where growth indicates that the URA3 phenotype was a consequence of epigenetic silencing. Reciprocally, isolates that failed to grow would represent reporter loss events (Figure 7A). These propagation assays revealed that normally, the rate of epigenetic switching of URA3 is much higher than reporter loss: 82% of ura- isolates still have a URA3 gene at the end of our propagation assay as exemplified growth in the absence of uracil (Figure 7B and C), and by PCR of genomic DNA (not shown). As expected, Δsir2 yeast are unable to establish silent chromatin at NTS1, and can only grow on 5′FOA via loss of the reporter. Finally, we observe that Δfpr3Δfpr4 yeast are compromised in their ability to silence URA3 epigenetically: only 30% of 5′FOA resistant colonies retain the URA3 gene. Thus, in Δfpr3Δfpr4 yeast recombination and URA3 reporter gene loss are more frequent than epigenetic silencing. This observation supports a model where
Fpr3/Fpr4 build chromatin structures at the NTS regions of rDNA locus. These structures are critical to maintaining genome stability at rDNA.

**Discussion**

Gene duplication events play a critical role in protein and organism evolution. However, the high similarity of duplicated genes can lead to complete or partial compensation when one paralog is deleted, as is in the case in conventional genetic interaction analysis. Here we present a dual-query SGA screening approach where one genetic cross can report the separate, shared and masked genetic interactions of gene paralogs. Using this approach on two nucleoplasmin-like histone chaperones revealed that they perform separate, cooperative, and redundant chromatin-related functions. Given that approximately 13% of yeast protein coding genes are duplicates (Wolfe and Shields 1997), this approach may have applications in the analysis of other paralogs.

The genetic interactions annotated here support a unique function for Fpr3 in orchestrating centromeric chromatin dynamics during chromosome segregation. This is fully consistent with existing literature (Hochwagen et al. 2005; Krogan et al. 2006; Macqueen and Roeder 2009; Ghosh and Cannon 2013; Ohkuni et al. 2014). Our comparative analysis provides additional systems-level evidence that this role is not shared with Fpr4 indicting that Fpr3, potentially as homo-oligomers, may regulate chromatin in a way that impacts chromosome segregation (Hochwagen et al. 2005; Macqueen and Roeder 2009). Furthermore, the fact that Δfpr3Δfpr4 double mutants display fewer genetic interactions than single gene Δfpr3 mutants (Appendix file 1) indicates that Fpr4 may be toxic in the absence of Fpr3 (Ohkuni et al. 2014). This model predicts that in the absence of Fpr3 the partial engagement or modification of chromatin by Fpr4 is deleterious.

Several members of the ADA and SWI/SNF chromatin regulatory complexes exhibit negative genetic interactions with both Fpr3 and Fpr4. These results could be explained by reduced dosage of a histone chaperone activity. Alternately, these genetic interactions are consistent with a model where Fpr3 and Fpr4 act together to chaperone nucleosomes, facilitating chromatin dynamics as SWI/SNF does. Whether this means that the paralogs operate together in a sequence of events, such as the removal and subsequent redeposition of nucleosomes during transcription or, in concert as a hetero-oligomeric complex, is not yet clear. The fact that Fpr3 and Fpr4 co-purify (Krogan et al. 2006) supports the latter model, but does not exclude the former.

The repression of several phosphate and polyphosphate metabolism genes in rich media requires both Fpr3 and Fpr4. It is therefore intriguing that both Fpr3 and Fpr4 were recently identified as two of the most heavily polyphosphorylated proteins in the yeast proteome, along with several proteins in an
evolutionarily conserved network of ribosome biogenesis factors (Neef and Kladde 2003; Bentley-DeSousa et al. 2018). The precise sites of Fpr3/4 polyphosphorylation and the impact of this post-translational modification on Fpr3/4 function is not yet clear. Fpr3/4 also impact the steady-state levels of mRNAs encoding ribosomal protein genes and rRNA processing machinery. Thus, Fpr3/4 may function as master regulators of ribosome biogenesis by coordinating both ribosomal protein abundance and rRNA processing. Given that many ribosomal and rRNA processing protein genes are driven by common regulators, Fpr3/4 may recognize common DNA sequences or transcription factors to accomplish this function (Fermi et al. 2016). As already stated, the links between polyphosphorylation of Fpr3/4 and the ribosome biogenesis network also require further investigation. It appears that at least some elements of this regulatory system may be conserved in the human nuclear FKBP25 protein (Gudavicius et al. 2014; Dilworth et al. 2017) and the acidic-tract containing nucleolin protein (Bentley-DeSousa et al. 2018).

The yeast TRAMP5 complex recognizes and polyadenylates aberrant RNA transcripts to target them for degradation by the Rrp6 ribonuclease (Karyn Schmidt and J. Scott Butler 2013). TRAMP5 targets include both ribosomal protein coding mRNAs and cryptic unstable transcripts generated from intragenic sites on the genome including those within the ribosomal DNA locus (LaCava et al. 2005; Reis and Campbell 2007; San Paolo et al. 2009; Wery et al. 2009). Here we found that deletion of Δtrf5 enabled the detection of a previously invisible transcriptome signature Δfpr4 yeast where there is a bias in RNA-seq reads towards the 5’ end of genes. This is consistent with Fpr4 promoting the transcriptional elongation process. It is noteworthy that these reads appear to cover the first 1-3 nucleosomes of genes because Fpr4 is capable of both histone and nucleosome binding (Leung et al. 2017), and was previously shown to be important for the kinetics of transcriptional induction (Nelson et al. 2006). Thus, the nucleosomes near the transcriptional start site are candidates targets of Fpr4. This regulation could involve either the installation of nucleosomes within promoters to inhibit transcriptional initiation or nucleosome/histone eviction from sequences downstream of the promoter in order to remove nucleosome blocks to the polymerase. The cryo-EM structures of nucleoplasmin pentamers engaging intact histone octamers provides further support for these models (Franco et al. 2019). We recently showed that Fpr4’s nucleoplasmin-like acidic regions bind to free histones while its basic surfaces permit nucleosome binding (Leung et al. 2017). Precisely how these activities and Fpr4’s peptidyl-prolyl isomerase activity towards the histone H3 tail (Nelson et al. 2006) (Monneau et al. 2013) co-operate to regulate chromatin dynamics is still unclear. However, the genetic and transcriptional read-outs identified here provide complementary assays for dissecting the importance of each of these features.
In addition to regulating the transcription of protein coding genes Fpr4 restricts transcription from the non-transcribed spacers (NTS) sequences of ribosomal DNA. This is consistent with both nucleolar enrichment and data indicating that Fpr4 inhibits transcription of exogenous reporters at rDNA in yeast (Kuzuhara and Horikoshi 2004) and orthologues operate similarly in plants (Li and Luan 2010). In yeast the NTS loci contain important DNA sequence features including as two terminators for the RNA PolI transcribed RDN35 repeat, a replication fork barrier site, and an autonomous replication site. Two separate observations suggest that Fpr4 builds chromatin at rDNA in order to insulate DNA at these spacers. First, using a strain sensitized to reveal Fpr4 regulated RNAs accumulates large amounts of NTS transcripts, and these RNAs are templated by both DNA strands. Second, consistent with a chromatin structural defect underpinning this phenomenon, the rDNA locus in Δfpr3Δfpr4 yeast is also hyper-recombinogenic (Figure 7). Thus, these histone chaperones of particular importance at the 100-200 rRNA repeats where they mediate the stability and silencing of spacers between the most heavily transcribed sequences in the cell. How these chaperones regulate chromatin structure at this locus, and how the structure differs from other targets in the nuclear genome, remain open questions that can now be addressed in future studies.

**Competing Interests**

The authors declare they have no conflict of interest.

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Figure Legends

Figure 1 – Fpr3 and Fpr4 have separate, co-operative and redundant functions.

A. Domain architectures of Fpr3 and Fpr4. Both proteins have an N-terminal nucleoplasmin-like domain with characteristic patches of acidic and basic residues, and a C-terminal FK506-binding (FKBP) peptidyl prolyl isomerase domain.
B. Schematic illustrating modified paralog-SGA workflow. Spores from a single cross of the double deletion Δfpr3Δfpr4 query to the 4784- strain DMA are manipulated to generate three separate sets of meiotic progeny for interactome analysis. The query strain also harbored an episomal URA3 plasmid with a functional FPR4 gene to avoid the slow growth phenotype of Δfpr3Δfpr4 dual deletion, and its vulnerability to suppressor mutations. This plasmid was selected for (for FPR3 interactors) or against (for FPR4 interactors) in the last step of the screen.

C. Venn diagram illustrating shared and unique negative genetic interactions from Δfpr3 and Δfpr4 paralog-SGA screens. The number of negative genetic interactions only detectable in double deletion Δfpr3Δfpr4 mutants is represented below.

D. Network illustrating complex related ontologies enriched among unique and shared negative genetic interactors of FPR3 and FPR4. *= SNF2, SNF5 and SNF6 were identified as hits in the FPR4 screen only, but displayed a synthetically sick phenotype with both Δfpr3 and Δfpr4 mutations in confirmatory spotting assays (not shown).

Figure 2 – The TRAMP5 nuclear RNA exosome is a masked genetic interactor of FPR3 and FPR4.

A. Fitness ratios of the indicated single, double, and triple mutants generated from paralog-SGA screens. The mean colony size ratios of Δfpr3Δxxx, Δfpr4Δxxx, and Δfpr3Δfpr4Δxxx mutants relative to colony sizes of Δxxx total haploid meiotic progeny are plotted as histograms. Ratios significantly below the default cut-off threshold (dotted green line) are indicated with green bars. A Balony software generated image of the mean colony size of each mutant, normalized to the plate median colony size, is illustrated along x-axis. GLO1 is a negative control that does not display a genetic interaction in Δfpr3Δ, Δfpr4, or Δfpr3Δfpr4 screens.

B. A schematic of the TRAMP5 complex (top right) interacting with the nuclear RNA exosome (bottom left). Genetic interactors identified in panel A are colored red. Pink text indicates essential components. Illustration is adapted from (Wolin et al. 2012).

C. Growth curves for select triple deletion mutants and corresponding total haploid meiotic progeny control populations confirm the slow growth of Δfpr3Δfpr4Δtrf5 and Δfpr3Δfpr4Δrrp6 mutants.

Figure 3 -Suppressor genetic interactions support chromatin-centric functions for Fpr3 and Fpr4.
A. Venn diagram illustrating shared and unique suppressor interactors from $\Delta fpr3$ and $\Delta fpr4$ paralog-SGA screens. The number of suppressor genetic interactions only detectable in double deletion $\Delta fpr3 \Delta fpr4$ mutants is represented below.

B. Plot of fitness ratios for all $\Delta fpr3 \Delta fpr4 \Delta xxx$ triple mutants relative to $\Delta xxx$ total haploid meiotic progeny controls. Green dots indicate negative genetic interactions, red dots indicate all suppressor genetic interactions. Threshold cut-offs are indicated by red and green dashed horizontal lines. The location of significant hits coding for components of chromatin modifiers are labeled and accompanied with schematic illustrations of their complex components. Components coded for by para-log SGA hits are colored. Red text denotes essential complex components.

**Figure 4 - Fpr3 and Fpr4 have partially overlapping impacts on the transcriptome.**

A. Numbers of differentially expressed genes in $\Delta fpr3$, $\Delta fpr4$, $\Delta fpr3 \Delta fpr4$ and $\Delta str2$ mutants.

B. Venn diagrams depict the partial overlap in up- and downregulated genes in $\Delta fpr3$, $\Delta fpr4$, and $\Delta fpr3 \Delta fpr4$ mutants. Genes at the centres of the diagrams (*) are differentially expressed in all three RNA-seq data sets and are enriched in the indicated Gene Ontology terms.

C. Confirmation of select differentially expressed genes (PHO5, PHO84, and SIT1) by quantitative RT-PCR of RNA isolated from independent biological replicates. Fold changes in gene expression are shown relative to WT.

D. Comparing the transcriptome of $\Delta fpr3 \Delta fpr4 \Delta trf5$ triple deletion mutants to $\Delta fpr3 \Delta trf5$ double mutants reveals an increase number Fpr4-repressed RNAs (red dots).

E. Gene ontology enrichment analysis for upregulated transcripts in $\Delta fpr3 \Delta fpr4 \Delta trf5$ triple deletion mutants. Enriched genes were classified by molecular function, biological process, cellular component, and MIPS functional database classification by FunSpec (http://funspec.med.utoronto.ca/).

**Figure 5 – A signature of incomplete elongation is present in $\Delta fpr4$ yeast.**

A. Plots of RNA-seq read density as a function of position on a scaled average gene. Upregulated, downregulated and unchanged transcripts generated form $\Delta fpr3 \Delta trf5$ double mutants are (left) and $\Delta fpr3 \Delta fpr4 \Delta trf5$ triple mutants (right) are shown.

B. RNA-seq read density plots on two genes showing a signature of incomplete elongation: SSF1 (left), UTP9 (right).

C. RNA-seq read density plots on ACT1, a gene without a signature of incomplete elongation IDP1.
D. Quantitative RT-PCR validation of RNA read densities on UTP9, SSF1, and ACT1. 5’ and 3’
amplicons were normalized to the unchanged GPD1 gene. RNAs were extracted from independent
biological replicates (from those subjected to RNA-seq).

**Figure 6 - Fpr4 is required to silence the non-transcribed spacers (NTS) of rDNA.**
A. Plots of RNA-seq read density across the rDNA locus on chromosome XII in WT, Δfpr3, Δfpr4 and
Δfpr3Δfpr4 mutants. The lack of reads mapping to NTS2-1, NTS1-1 (centre) and NTS2-2 (right)
suggests transcriptional silence in maintained in all strains.
B. Plots of RNA-seq read density across the rDNA locus on chromosome XII (top), and across NTS2-2
(bottom) in WT, Δfpr3Δtrf5 and Δfpr3Δfpr4Δtrf5 mutants. The reads mapping to NTS2-1, NTS1-1
(centre) and NTS2-2 (right) in Δfpr3Δfpr4Δtrf5 reveals that Fpr4 is required to transcriptionally silence
the NTSs.

**Figure 7 – Fpr3 and Fpr4 are required for genomic stability at the rDNA locus.**
A. Diagrams illustrating the propagation experiment carried out to assess frequency of reporter loss.
Top: The rDNA(NTS1)::URA3 gene stochastically switches between an active euchromatin state (dark
blue cells) and a silenced heterochromatin-like state (light blue cells). Bottom: Individuals that lose the
reporter due to instability can be distinguished from cells with a stochastically silenced reporter with
the indicated workflow.
B. Images of the 96 individuals selected for after propagation on SD-complete control media and on
SD-URA experimental media. Those growing on the experimental media represent the fraction of the
population in which the reporter was epigenetically silenced. Those that fail to grow indicate
permanent loss of the reporter.

C. Percentage of total colonies recovered after strain propagation that have retained or lost the ability
to grow on SD-complete media.
**Figure 1, Savic et al**

**A**

- Red: Acidic Region
- Blue: Basic Region
- Purple: Peptidyl Proline Isomerase

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Nucleoplasmin-like histone chaperone
FKBP

**B**

- query strain
- meiotic progeny
- SGA Workflow
- 4784-strain DMA

**C**

Δfpr3
456
Δfpr4
78
Δfpr3Δfpr4
138
Δfpr3
75

**D**

- Mitochondrial pyruvate dehydrogenase complex
- Cytochrome bc1 complex
- Astral microtubule
- Dynactin complex
- Pre-autophagosomal structure
- Ada2/Gcn5/Ada3 transcription activator complex
- SWI/SNF Complex
- Ctk1p complex
- Swr1 complex
- ESCRT III complex
- ESCRT II complex
Figure 2, Savic et al

A

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AIR1

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<td>0.6</td>
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<td>(exp/ctrl)</td>
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<td>Time (h)</td>
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<td>0.2</td>
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B

The TRAMP5 complex

C

The Nuclear RNA Exosome
**Figure 3, Savic et al**

A

Δfpr3 Δfpr4

<table>
<thead>
<tr>
<th>218</th>
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<tbody>
<tr>
<td>191</td>
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Δfpr3Δfpr4

Red text= essential gene, 
**= mutant not present in DMA, 
* gene is a masked interactor only

**The SWI/SNF Complex**

Δfpr3 Δfpr4

**The ADA Complex**

**B**

NAD+ independent histone deacetylases

HIR Complex

Set1/ COMPASS Complex

HOS2  HIR1  HDA1  HOS3  HPC2  SWD3  HIR3  SDC1

Ratio of Fitness (Δfpr3/Δfpr4/Δxxx)

Deletion Mutant
Figure 4, Savic et al

A

- UP genes
- DOWN genes

<table>
<thead>
<tr>
<th>Δfpr3</th>
<th>Δfpr4</th>
<th>Δfpr3Δfpr4</th>
<th>Δfpr3Δfpr4</th>
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<tbody>
<tr>
<td>529</td>
<td>549</td>
<td>683</td>
<td>827</td>
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</tbody>
</table>

B

UP Genes

Δfpr3

- 89
- 13
- 31
- 145

Δfpr4

- 62
- 74
- 36

Δfpr3Δfpr4

- 31
- 36

DOWN Genes

Δfpr3

- 160
- 52
- 153
- 193

Δfpr4

- 57
- 65
- 94

Δfpr3Δfpr4

- 38
- 58
- 100

C

Fold change relative to WT

WT Δfpr3 Δfpr4 Δfpr3Δfpr4

<p>| | | | |</p>
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<thead>
<tr>
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<td>Δfpr3</td>
<td>Δfpr4</td>
<td>Δfpr3Δfpr4</td>
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<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
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</tbody>
</table>

D

UP Genes

- SIT1PHO84PHO5

- structural constituent of ribosome [GO:0003735]
  - p=5.07 x 10^-8

- polyphosphate metabolic process [GO:0006797]
  - p=4.20 x 10^-7

DOWN Genes

- siderophore transport [GO:0015891]
  - p=4.31 x 10^-9

E

- rRNA processing
- electron transport
- ribosomal proteins
- cytosolic large ribosomal subunit
- ribosome
- nucleus
- nucleolus
- cell wall
- fungal-type cell wall
- extracellular region
- intracellular
- ribonucleoprotein complex
- cellular response to oxidative stress
- ribosome biogenesis
- rRNA processing
- structural constituent of ribosome

- MIPS Functional Classification
- Biological Process
- Cellular Component
- Molecular Function

- log (corr P-value)
Figure 5, Savic et al

A

Δfpr3Δtrf5

Δfpr3Δfpr4Δtrf5

Agglomerated mean gene coverage

Scaled gene length%

5' 3'

B

SSF1

UPF9

Chromosomal position (bp) [chrVII]

Normalized read counts

Δfpr3Δtrf5

Δfpr3Δfpr4Δtrf5

C

ACT1

Chromosomal position (bp) [chrVII]

Normalized read counts

Δfpr3Δtrf5

Δfpr3Δfpr4Δtrf5

D

Normalized read counts

Amplicon / Gpd1 Ratio relative to Δfpr3Δtrf5

Δfpr3Δtrf5

Δfpr3Δfpr4Δtrf5

p=3.2 x 10^-2

p=8.3 x 10^-5

p=0.18
**Figure 6, Savic et al**

A

![Diagram A](image)

B

![Diagram B](image)
Figure 7, Savic et al

A

![Diagram A]

- Reporter in euchromatin
- Reporter in heterochromatin
- Reporter lost from genome

- SD - Ura
- YPD
- 5-FOA
- SD - Ura

Uracil auxotrophy bottleneck
Non-selective media (permits silencing or loss)
Select phenotypically OFF individuals
Score silencing vs loss events

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Δfpr3Δfpr4</th>
<th>Δsir2</th>
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<tr>
<td>SD complete</td>
<td>SD - Ura</td>
<td>SD complete</td>
<td>SD complete</td>
</tr>
<tr>
<td>SD - Ura</td>
<td></td>
<td>SD - Ura</td>
<td></td>
</tr>
</tbody>
</table>

C

![Bar Chart]

Percentage URA3 silenced or lost

WT  Δfpr3Δfpr4  Δsir2

0%  10%  20%  30%  40%  50%  60%  70%  80%  90%  100%