

1 **Uncovering Natural Longevity Alleles from Intercrossed Pools of**
2 **Aging Fission Yeast Cells**

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1 **ABSTRACT**

2 Quantitative traits often show large variation caused by multiple genetic factors.
3 One such trait is the chronological lifespan of non-dividing yeast cells, serving as
4 a model for cellular aging. Screens for genetic factors involved in ageing typically
5 assay mutants of protein-coding genes. To identify natural genetic variants
6 contributing to cellular aging, we exploited two strains of the fission yeast,
7 *Schizosaccharomyces pombe*, that differ in chronological lifespan. We generated
8 segregant pools from these strains and subjected them to advanced intercrossing
9 over multiple generations to break up linkage groups. We chronologically aged
10 the intercrossed segregant pool, followed by genome sequencing at different
11 times to detect genetic variants that became reproducibly enriched as a function
12 of age. A region on Chromosome II showed strong positive selection during
13 ageing. Based on expected functions, two candidate variants from this region in
14 the long-lived strain were most promising to be causal: small insertions and
15 deletions in the 5'-untranslated regions of *ppk31* and *SPBC409.08*. Ppk31 is an
16 orthologue of Rim15, a conserved kinase controlling cell proliferation in
17 response to nutrients, while SPBC409.08 is a predicted spermine
18 transmembrane transporter. Both Rim15 and the spermine-precursor,
19 spermidine, are implicated in ageing as they are involved in autophagy-
20 dependent lifespan extension. Single and double allele replacement suggests that
21 both variants, alone or combined, have subtle effects on cellular longevity.
22 Furthermore, deletion mutants of both *ppk31* and *SPBC409.08* rescued growth
23 defects caused by spermidine. We propose that Ppk31 and SPBC409.08 may
24 function together to modulate lifespan, thus linking Rim15/Ppk31 with
25 spermidine metabolism.

1 INTRODUCTION

2 Both between and within species, even within individual organisms, the lifespan
3 of cells can vary enormously. However, from simple microorganisms to tissues of
4 multicellular eukaryotes, the genetics underlying this variation in natural
5 populations is poorly understood. There are two ways to measure a cell's
6 lifespan. One is to count the number of mitotic divisions it can undergo – termed
7 'replicative lifespan'. The 'chronological lifespan', on the other hand, is a measure
8 of a non-dividing cell's ability to remain viable over time. The relative
9 importance of replicative or chronological lifespan depends on the type of cell.
10 Post-mitotic cells no longer divide and are not limited by their replicative
11 lifespan. For example, during times of nutritional deprivation, many single-celled
12 organisms from bacteria to yeast stop dividing and begin to age chronologically
13 (Fabrizio and Longo 2003; Gonidakis and Longo 2013). Chronological lifespan
14 also applies to multicellular eukaryotes, for terminally differentiated post-
15 mitotic cells such as neurons (MacLean *et al.* 2001; Rando and Chang 2012;
16 Magrassi *et al.* 2013) or for reversibly quiescent stem cells (Rodgers and Rando
17 2012; Roche *et al.* 2017).

18 Chronological lifespan is affected by a multiplicity of genes (Gems and
19 Partridge 2013) and is thus a complex trait. Genome-wide approaches in
20 genetically tractable model organisms are therefore crucial for identifying the
21 different cellular processes involved. Work in budding yeast and, to a lesser
22 extent, fission yeast have helped reveal a number of well-annotated coding genes
23 which show large effects on chronological lifespan when deleted (Powers *et al.*
24 2006; Matecic *et al.* 2010; Fabrizio *et al.* 2010; Rallis *et al.* 2013, 2014; Garay *et*
25 *al.* 2014; Sideri *et al.* 2014) or overexpressed (Ohtsuka *et al.* 2013). Along with

1 studies in other organisms, this work has helped to uncover diverse protein-
2 coding genes acting on a range of cellular processes, which extend or shorten
3 chronological lifespan. Notably, the roles in ageing of many of these pathways
4 are conserved. For example, inhibition of the target of rapamycin complex 1
5 (TORC1) pathway extends chronological lifespan in yeast, and organismal
6 lifespan in worms, flies and mice (Fontana *et al.* 2010). However, as valuable as
7 these systematic, reverse genetic approaches are, they have some limitations.
8 First, they only consider coding regions, ignoring any role of non-coding RNAs or
9 regulatory regions. Second, gene deletion and overexpression are quite crude
10 genetic tools that fail to capture weak effects typical of natural genetic variations,
11 the combination of which quantitatively contributes to the genetic basis of
12 complex phenotypes. To better understand the complexity of chronological
13 lifespan, we need to identify the effects of natural genetic variations, however
14 subtle, throughout the genome.

15 Many species show substantial variation in lifespan. Studies in worms
16 (Ayyadevara *et al.* 2003), flies (Nuzhdin *et al.* 1997; Mackay 2002; De Luca *et al.*
17 2003; Highfill *et al.* 2016) and humans (Sebastiani *et al.* 2012; Deelen *et al.* 2014;
18 Erikson *et al.* 2016; Zeng *et al.* 2016) have harnessed the segregating genetic
19 variation in natural populations to identify loci involved in organismal ageing.
20 Furthermore, with diminishing sequencing costs, recent studies could detect
21 variants with subtle effects on lifespan in both coding and non-coding regions.
22 Natural genetic variation can also be used to understand particular aspects of
23 cellular ageing, such as the genetic basis of chronological lifespan. In budding
24 yeast, segregant mapping panels from F1 crosses have identified Quantitative
25 Trait Loci (QTL) involved in both replicative (Stumpferl *et al.* 2012) and

1 chronological ageing (Kwan *et al.* 2013). Due to the large sample sizes, pooled
2 experiments with yeast cells can provide greater power to detect multiple loci of
3 small effect in QTL mapping studies (Ehrenreich *et al.* 2010). Furthermore,
4 studies of other phenotypes have maximized the QTL resolution by applying
5 selection to large pools of segregants from Advanced Inter-crossed Lines (AILs),
6 where multiple generations of recombination break up linkage groups to
7 separate nearby variants and generate diverse variant combinations in the
8 segregant pool (Parts *et al.* 2011; Liti and Louis 2012).

9 Here, we use such an intercross QTL (iQTL) approach in the fission yeast,
10 *Schizosaccharomyces pombe*, to uncover genetic variants involved in
11 chronological lifespan. Several studies have reported aspects of the genetic and
12 phenotypic diversity of wild *S. pombe* strains, isolated from different geographic
13 regions (Brown *et al.* 2011; Teresa Avelar *et al.* 2013; Fawcett *et al.* 2014;
14 Jeffares *et al.* 2015, 2017). Cellular lifespan, however, has not been studied as a
15 natural phenotype in fission yeast. We generated an AIL using a long-lived
16 natural isolate of *S. pombe* and a laboratory strain as parents. By deeply
17 sequencing non-dividing, ageing pools of the resulting segregants over time, we
18 identify genetic variants that become increasingly over- or under-represented as
19 a function of age. We show that the long-lived parent's haplotype across a region
20 of Chromosome II repeatedly undergoes selection across replicates during
21 ageing. We analyze two candidate causal alleles in this region, and show that
22 variants at the two loci have very subtle effects on chronological lifespan. We
23 discuss the possibility that these neighboring genes, both of which have been
24 implicated in autophagy and lifespan, act in the same pathway.

25

1 **MATERIALS & METHODS**

2 *Lifespan Assays*

3 For all lifespan experiments with parental strains or pooled segregants, cells
4 were inoculated from plates into liquid yeast extract supplemented (YES)
5 medium, and the optical density of cultures was monitored during growth. To
6 most accurately reflect the point at which the majority of cells in the population
7 had stopped dividing, Day 0 measurements were taken when cultures stopped
8 increasing in optical density. Subsequent time points were then taken at the
9 same time of each day. Over time, the proportion of living cells in the culture was
10 estimated by reviving samples on YES agar, counting colony forming units, and
11 comparing this count to the number at Day 0 (Rallis *et al.* 2013). For each time
12 point, colony-forming units were measured in triplicate on three plates.

13

14 *Generation of Advanced Intercrossed Line*

15 To generate the AIL, the two parental strains, DY8531 and Y0036, were left to
16 mate on solid malt extract agar (MEA) medium for 3 days. Parental strains were
17 of opposite mating types, so no selection against self-crosses was required. The
18 cross was checked for zygotes using microscopy to ensure mating was efficient.
19 To kill any vegetative parental cells in the sample, leaving only spores, cell
20 samples were scraped off these plates, re-suspended in zymolyase, and
21 incubated for 30min at 32°C. They were then spun down, re-suspended in 40%
22 EtOH, and left at room temperature for 10min. Spores were inoculated into 50ml
23 rich liquid YES media and grown overnight. These cultures were spun down, and
24 500µl samples plated on MEA. Samples were left to mate for 3 days, followed by
25 repetition of the entire process for the next generation of intercrossing. This

1 intercrossing procedure was performed for 20 generations, and glycerol stocks
2 were made for each generation.

3

4 *Testing whether Re-Growth of Samples Skews Allele Frequencies*

5 For the selection experiment with pooled segregants, samples taken throughout
6 the timecourse contain both live and dead cells. When sequencing and analyzing
7 allele frequencies, samples could be re-grown first to avoid introducing noise
8 from dead cells (Ehrenreich *et al.* 2010; Matecic *et al.* 2010; Fabrizio *et al.* 2010).
9 However, genes involved in growth or stress response often feature antagonistic
10 pleiotropy. In our segregant pools, many alleles that increase in frequency as a
11 function of age may therefore decrease in frequency when samples are re-grown.
12 To see if re-growth treatment affected allele frequencies at loci involved in
13 longevity, we performed a pilot experiment using separate pools. First, we
14 measured the change in frequencies as cells aged, by comparing allele
15 frequencies at Day 0 in the pools (i.e. not regrown) with those at Day 6 in the
16 pools. Second, we measured the change in frequencies as the aged cells were
17 regrown, by comparing allele frequencies at Day 6 in the pool, with those same
18 samples after re-growth (Supp. Fig. 4A).

19 Two replicate pools of segregants were left to age and sampled at Day 0
20 and Day 6, with another Day 6 sub-sample being re-grown. DNA was then
21 extracted from these three samples, DNA libraries were prepared (see below),
22 and sequenced at low coverage (~10x) using the Illumina MiSeq platform. Reads
23 were aligned to the reference genome, and raw allele frequencies were obtained
24 (see below). To measure changes in allele frequency (AF) with age, we calculated
25 the difference between AF at the start and end of the ageing timecourse

1 (Lifespan Δ AF). To measure the change in AF with growth, we calculated the
2 difference between AF at the end of the ageing time course before and after the
3 sample was re-grown (Growth Δ AF). Using an arbitrary cut-off of 0.15, we found
4 that the alleles at a large proportion of loci changed frequency with
5 chronological age (20%), and with growth (16%). Interestingly, when the allele
6 frequency change with growth was plotted against the change with chronological
7 age for each locus (Supp. Fig 4B), we found a weak negative correlation
8 (Pearson's correlation=0.31, $p < 0.01$), suggesting the existence of a modest
9 number of loci whose alleles are antagonistic with respect to these two traits.
10 Again, using 0.15 cut-offs, this equated to around 6% of loci (Supp. Fig 4B, red
11 dots). Note that, due to the low sequencing coverage in this pilot experiment,
12 many loci did not have sufficient read depth in all samples to measure allele
13 frequency changes. Because re-growth biases allele frequencies at a subset of
14 loci, we decided to not re-grow samples from ageing pools prior to sequencing.

15

16 *Efficacy of Applying Age-Based Selection to Segregant Pools*

17 To determine the efficacy of our experimental design, we tested whether
18 sampling a non-dividing pool later in time does indeed select for more long-lived
19 segregants. We sampled two replicate pools through time, and used these
20 samples to seed new pools. By measuring the survival integral (area under
21 lifespan curve) for each re-grown population, a trend for later samples to
22 generate more long-lived cells could be measured (Supp. Fig. 2A). Pools were
23 sampled from the original ageing pools at Day 0 (early), Day 3 (middle) and Day
24 6 (late). Indeed, we found that sampling later in time leads to an increased cell-
25 survival integral (Supp. Fig 2B).

1 During the pilot studies, when re-growing samples from the pools, later
2 samples would have fewer live cells per volume. To prevent a bottleneck effect
3 when re-growing, an effort was made to keep the number of live cells constant in
4 all samples through time. We therefore estimated the proportion of dead cells in
5 the population at each time point using the phloxine-staining assay (Rallis *et al.*
6 2013): 100µl of cells were resuspended in 1x phloxine-B, and incubated for
7 15min at 32°C. Slides were then prepared, and visualised on a Zeiss Axioskop
8 microscope with rhodamine filter, using a 63x 1.4 NA oil immersion objective. In
9 total, 500 cells were counted, and the proportion of phloxine-stained cells was
10 recorded. The proportion of live to dead cells was then used to calculate the
11 sample size required to maintain the same effective number of live cells.

12

13 *DNA Extraction and Library Preparation*

14 DNA was extracted from samples using a standard phenol-chloroform method,
15 sheared to ~200bp using a Covaris sonicator (S series), and cleaned using Qiagen
16 PCR purification columns. Libraries were prepared with NEB Next Ultra DNA
17 library preparation kits, according to the manufacturer's protocol. The 48
18 samples for the main study, 8 repeats with 6 time points each, were pooled and
19 sequenced on the Illumina HiSeq platform (SickKids hospital, Toronto, Canada).

20

21 *Read Alignment and Raw Allele Frequency Estimation*

22 To estimate allele frequencies, we needed to identify segregating sites between
23 the parental strains. The BAM files of the Y0036 (Clément-Ziza *et al.* 2014) and
24 DY8531 (Hu *et al.* 2015) genome sequences were obtained from the European
25 Nucleotide Archive (<http://ebi.ac.uk/ena>) and NCBI SRA

1 (<http://www.ncbi.nlm.nih.gov/sra>). For the sake of continuity, all files were then
2 re-mapped with BWA-MEM (Li and Durbin 2009; Li 2013). PCR duplicates were
3 filtered using samtools (v0.1.18, Li *et al.* 2009), and bam files were InDel
4 realigned using GATK (McKenna *et al.* 2010). Variant sites were called using both
5 GATK's HaplotypeCaller (McKenna *et al.* 2010) and bcftools (Li *et al.* 2009).
6 These lists were then combined and filtered based on a number of criteria,
7 including: the read depth at that site; the alternate allele frequency (>99%); the
8 number of badly mapped or split reads at that site; the proximity to other SNPs
9 and InDels; and the repetitiveness of the region. Filtration made use of a number
10 of programmes including bcftools, vcftools (Danecek *et al.* 2011) and GATK.
11 Repetitive regions were annotated in PomBase (Wood *et al.* 2012; McDowall *et*
12 *al.* 2015). Our final list of polymorphic markers showed fairly even distribution
13 throughout the genome, except for a small region on Chromosome II which
14 showed a very high marker density (Supp. Fig. 9).

15 Prior to processing, sequencing results were briefly checked in FastQC
16 (Andrews 2010). Reads were then aligned to the reference genome (accessed
17 May 2015, Wood *et al.* 2002) using BWA-MEM (Li and Durbin 2009; Li 2013). To
18 prevent bias from PCR amplification during library preparation, PCR duplicates
19 were removed using samtools (v0.1.18, Li *et al.* 2009). Bam files were then InDel-
20 realigned using GATK (McKenna *et al.* 2010). Pileups were made at each variant
21 site using samtools to obtain the frequency of each allele at these sites. For the
22 initial pilot study, these raw allele frequencies were used directly. For the main
23 experiment, the filterHD algorithm (described in Fischer *et al.* 2014) was used to
24 get a more accurate estimation of the true underlying allele frequencies. Sliding
25 window averages were calculated using a custom Python script.

1

2 *Scoring of Allele Frequency Trajectories*

3 Scores were generated for each trajectory (a set of allele counts corresponding to
4 the time points) independently, in the following way. First, a null model was
5 learned as the best single allele frequency that explains the observed counts
6 within a trajectory, assuming a binomial distribution. This was contrasted to a
7 model where each time point got its own allele frequency (number of variant
8 alleles divided by the read depth), the observed counts were then scored using a
9 binomial model with these allele fractions. The score difference between these
10 two ways of scoring reports how much a given observed trajectory differed from
11 a best no change line. Before calling loci, we addressed a number of anomalously
12 high scores. Although intercrossing will have broken up a considerable amount
13 of genetic linkage between neighboring loci, a substantial number of segregants
14 in each population carrying large unbroken linkage blocks at any given region
15 were still expected. We therefore expected high-scoring loci near other high-
16 scoring loci whose alleles have ‘hitch-hiked’ with the causal allele(s). High-
17 scoring outliers with no neighboring support are therefore likely to be false
18 positives. To only accept scores with support from neighboring variants, any loci
19 whose scores were further than the population inter-quartile range from either
20 of its neighboring loci were filtered out.

21

22 *Generation of Allele Replacement Strains*

23 When combined with an oligonucleotide template for Homologous
24 Recombination (HR), CRISPR-Cas9 can be used to specifically target alleles for
25 replacement at the nucleotide level, in a single step (Ran *et al.* 2013). However,

1 this approach is only feasible if either the Protospacer Adjacent Motif (PAM), or
2 the region immediately upstream, are altered after HR (Paquet *et al.* 2016).
3 There is an unusual dearth of PAMs in the regions surrounding both genetic
4 variants targeted in this study, precluding the use of this method. We therefore
5 designed a two-step approach (similar in design to Paquet *et al.* 2016). We first
6 used a more distal PAM to precisely target the region containing the InDel for
7 deletion, and then targeted the deleted region for re-insertion of a template
8 containing the alternative allele (Supp. Fig. 5). Deletions of ~200bp were made
9 as previously described (Rodríguez-López *et al.* 2017), with the small addition of
10 an arbitrary modification in the HR template (Supp. Fig. 5A-C). The modification
11 was designed to neighbor a PAM site lying at the edge of the deletion. After
12 integration during the deletion step, this PAM site was then targeted by Cas9 for
13 the insertion step. Because the temporary modification integrated during the
14 deletion step was not present at the replacement locus, it could be removed
15 during HR-based insertion of the template, preventing any further cutting by
16 Cas9 and leaving the locus scar-free (Supp. Fig. 5D-E). A strain containing both
17 allele replacements was obtained by repeating this deletion-insertion approach
18 at the *SPBC409.08* locus in the *ppk31* allele replacement strain.

19 Step one deletion mutants were obtained at a low frequency and
20 confirmed by PCR (Supp. Fig. 6A). Step two insertion mutants were obtained at a
21 much higher frequency (Supp. Fig. 6B) and confirmed by Sanger sequencing. The
22 reason for the difference in efficiency of these two reactions remains unclear, but
23 may reflect either the chromatin structure becoming more accessible after the
24 initial deletion step or a reduced efficiency for DNA repair that results in
25 deletion. The latter possibility is appealing as the probability of a modification

1 being integrated is known to decrease with the distance from the cut site (Elliott
2 *et al.* 1998; Beumer *et al.* 2013), presumably because of the large resection
3 required before HDR.

4

5 *Spot Assays of Deletion Strains*

6 Generation of prototrophic deletion strains was previously described (Malecki
7 and Bähler 2016), and deletions were confirmed by PCR. Strains were woken up
8 on YES agar before being grown to saturation in polyamine-free minimal
9 medium (EMM). Optical densities were normalized to OD₆₀₀ 1, and five 5-fold
10 serial dilutions were made of each strain in a 96-well plate. These serial dilutions
11 were then arrayed onto EMM agar and EMM agar containing 1mM Spermidine.
12 Plates were incubated at either 32°C or 37°C until fully grown and imaged using
13 a flatbed scanner.

14

15 *Comparing Phenotypes of Natural Isolates*

16 Variant calls of 161 natural isolates, along with data detailing their growth rate
17 on solid media (normalized colony size) was downloaded from published
18 supplementary data (Jeffares *et al.* 2015). A python script was used to group
19 strains by the presence or absence of each variant. For each growth condition,
20 Wilcoxon tests were then used to compare the growth of all strains with the
21 *ppk31* insertion to all strains with the wild-type allele. P-values were corrected
22 using the Benjamini-Hochberg procedure.

23

24 *Statement on Reagent and Data Availability*

1 All strains are available upon request. The bam file of parental strain Y0036 is
2 available at the European Nucleotide Archive under accession number
3 ERX007395. The bam file of parental strain DY8531 is available at the NCBI SRA
4 under accession number SRX1052153. Supplemental file available at FigShare.
5 File S1 contains 9 supplemental figures and one supplemental table. File S2
6 contains the final, filtered scores from the modelling of allele-frequency changes
7 (i.e., unsmoothed data for Fig. 2B). The vcf files used for all experiments,
8 containing raw allele frequencies at segregating sites (data used to generate
9 scores for Fig. 2B, raw data for Supp. Fig. 1, and raw data for Supp. Fig. 4), will be
10 made available on the European Variation Archive (accession numbers TBD).
11 Sequence data from Jeffares et al. (2015) are available from the European
12 Nucleotide Archive under the accession numbers PRJEB2733 and PRJEB6284,
13 and growth data are listed in Supplementary Table 4 (all phenotypes with the
14 prefix “smgrowth”).

15

16

17 **RESULTS & DISCUSSION**

18 *A Long-Lived Strain of S. pombe*

19 To sample the natural variation in cellular longevity, we measured the
20 chronological lifespan of two strains of fission yeast. One of these strains, a
21 winemaking strain from South Africa (Y0036), has been analyzed for previous
22 QTL-mapping studies (Clément-Ziza *et al.* 2014). The other strain (DY8531) is a
23 derivative of the standard laboratory strain 972 *h* that has been engineered to
24 feature the large inversion present in most other *S. pombe* strains, including
25 Y0036 (Hu *et al.* 2015). With ~4500 polymorphisms between them, including

1 single-nucleotide polymorphisms (SNPs) and small insertions/deletions (Indels),
2 these strains are approximately as divergent as two humans (~0.1%; Jorde and
3 Wooding 2004; Clément-Ziza *et al.* 2014). This close relatedness should reduce
4 the genetic complexity of the segregant pool, facilitating the detection of causal
5 phenotypic associations. Y0036 was reproducibly longer-lived than DY8531 with
6 respect to both median and maximal lifespan (Fig. 1A). We conclude that even
7 among the two closely related strains tested, differences in chronological
8 lifespan are evident, with Y0036 showing extended lifespan compared to the
9 standard laboratory strain.

10

11 *Identification of Candidate Locus that Impacts Longevity*

12 To uncover natural genetic variants underlying the difference in lifespan
13 between Y0036 and DY8531, we designed an iQTL experiment involving bulk
14 segregant analysis of large numbers of individuals from advanced intercross
15 lines (Parts *et al.* 2011; Liti and Louis 2012). Selection, in the form of
16 chronological ageing, was applied to non-dividing segregant pools from repeated
17 crosses amongst the progeny of the long-lived Y0036 and short-lived DY8531
18 strains. We expected that variants that support longevity will increase in
19 frequency among the pooled cells as a function of age (Fig. 1B). We generated an
20 AIL between Y0036 and DY8531 by intercrossing for twenty generations
21 (Materials & Methods). Genome sequencing after five, ten and fifteen cycles of
22 intercrossing (F5, F10, F15) revealed a substantial and increasing skew in allele
23 frequencies at many loci towards one or the other parental allele (Supp. Fig. 1).
24 After ten generations, several alleles had already approached fixation (Supp. Fig.
25 1). This effect likely reflects strong selection during competitive growth in liquid

1 medium after each cycle of intercrossing (Ed Louis, personal communication;
2 Materials & Methods). To minimize loss of any variants affecting lifespan, we
3 henceforth used the F6 pools, after six cycles of intercrossing.

4 The bulk segregant analysis relied on selection of long-lived cells during
5 chronological ageing. We first checked whether such selection occurred by
6 sampling non-dividing F6 segregant pools at different times. This experiment
7 revealed that the population of cells sampled at later times did indeed show a
8 subtle increase in average lifespan (Supp. Fig. 2; Materials & Methods). We then
9 inoculated eight independent F6 pools from the same AIL and let them grow into
10 stationary phase for chronological ageing. We harvested samples from the pools
11 on six consecutive days, from Day 0 (when cultures had stopped growing) to Day
12 5 (when cells showed ~15-30% viability) (Fig. 2A; Supp. Fig. 3A).

13 The genomes in all 48 samples were sequenced to determine the
14 proportion of parental alleles at different loci and time points. To prevent bias
15 from re-growth, DNA was extracted directly from aged cells. Indeed, preliminary
16 analyses suggested that antagonistic pleiotropy would otherwise skew our
17 results, i.e. QTL that cause longevity in non-proliferating cells also tend to cause
18 slow growth in proliferating cells (Supp. Fig. 4; Materials & Methods). To identify
19 alleles that were subject to selection during chronological ageing, we required an
20 accurate representation of the true underlying allele frequencies in each
21 population. To this end, we estimated allele frequencies using the filterHD
22 algorithm (Fischer *et al.* 2014), which applies probabilistic smoothing to allele
23 frequency likelihoods across the genome. For each locus, we then used the allele
24 frequency at each timepoint to infer a trajectory representing the change in allele
25 frequency over time. We contrasted these observed trajectories with a null

1 model assuming no change. For each locus, the difference in score between the
2 two models describes the extent to which the allele frequency changed with age,
3 with trajectories that were found repeatedly across replicates scoring higher.
4 After filtering outliers (Materials & Methods), scores were visualized across the
5 genome. We applied a threshold of 1.5-fold the inter-quartile range (IQR) above
6 the upper quartile to identify putative QTL.

7 Our analysis revealed a strong signal of selection in a ~100kb region of
8 Chromosome II, featuring eight variants that exceeded the threshold (Fig. 2B &
9 2C; Supp. Fig. 3B). This result suggests that at least one variant within this region
10 can promote lifespan, and contributes to the increased survival probability for
11 Y0036 cells during chronological ageing.

12

13 *Candidate Variants Implicated in Lifespan Regulation*

14 Of the eight variants exceeding the threshold on Chromosome II, six lead to
15 synonymous substitutions in coding sequences or are located within introns or
16 intergenic regions (Fig. 3A and Supp. Tab. 1), so were not strong candidates for
17 causal variants. Two alleles, however, lead to a small insertion and a small
18 deletion in the 5' untranslated regions (UTRs) of two genes: *ppk31* and
19 *SPBC409.08* (Fig. 3). Besides these two Indels, several of the other high scoring
20 SNPs were also associated with *ppk31* and *SPBC409.08* (Fig. 3A and Supp. Tab. 1).
21 Due to the neutral predicted effects of these variants, we considered them more
22 likely to be passenger alleles, although their contribution to longevity as a
23 quantitative trait cannot be ruled out.

24 *SPBC409.08* encodes a predicted spermine transmembrane transport
25 protein, whereas *ppk31* encodes an orthologue of budding yeast Rim15, a

1 conserved kinase involved in metabolic signaling (Cherry *et al.* 1998, 2012;
2 Wood *et al.* 2012; McDowall *et al.* 2015). Both spermidine, a precursor of
3 spermine that can be formed by spermine's degradation, and Rim15 have been
4 implicated in ageing. Spermidine, and polyamine metabolism in general, is
5 involved in lifespan regulation (Scalabrino and Ferioli 1984; Vivó *et al.* 2001;
6 Fraga *et al.* 2004; Nishimura *et al.* 2006; Liu *et al.* 2008; Eisenberg *et al.* 2009,
7 2016). Anti-ageing effects of spermidine are mediated by its capacity to induce
8 cytoprotective autophagy (Madeo *et al.* 2018). Rim15 plays an important role in
9 transcriptional regulation downstream of TORC1 (Wei *et al.* 2008) and, like
10 spermidine, is involved in the induction of autophagy (Bartholomew *et al.* 2012;
11 Bernard *et al.* 2015). Rim15 is antagonistically pleiotropic with respect to
12 fermentation and stress response (Kessi-Pérez *et al.* 2016). Many traits that are
13 beneficial for longevity and stress response are detrimental for growth, leading
14 to antagonistic pleiotropy (Williams 1957; López-Maury *et al.* 2008; Teresa
15 Avelar *et al.* 2013; Rallis *et al.* 2014). This feature further supports the
16 involvement of this locus as a QTL for chronological lifespan. Antagonistic
17 pleiotropy could explain why alleles that are beneficial for chronological lifespan
18 might be present in one strain that has evolved under one set of selective
19 pressures, but not in another strain. Because of their high scores in our
20 modelling, as well as published findings in other species, we further pursued the
21 variations in the 5' UTRs of *SPBC409.08* and *ppk31* as candidate QTL.

22

23 *Validation of Candidate Alleles*

24 To test whether these two variants can modify lifespan, we used a CRISPR/Cas9-
25 based allele-replacement approach to engineer the candidate Y0036 Indels into

1 the laboratory strain genetic background (DY8531), without any scars or
2 markers (Supp. Fig. 5 & 6; Materials & Methods). The Ppk31 allele led to a subtle
3 but reproducible lifespan extension of the DY8531 strain, especially at later
4 timepoints (Fig. 4A & B). The SPBC409.08 allele, on the other hand, showed more
5 variable effects on lifespan, but also appeared to slightly extend lifespan at later
6 timepoints (Fig. 4B). This result supports a partial contribution for these alleles
7 to the long-lived phenotype of Y0036 cells.

8 Both spermine metabolism and Ppk31 have been previously been
9 implicated in autophagy induction and lifespan regulation. However, whether
10 there is any crosstalk between Ppk31 signaling and spermidine metabolism, and
11 whether spermidine's ability to extend lifespan is dependent on Ppk31, is not
12 known. To further examine the functional relationship between the variants in
13 *SPBC409.08* and *ppk31*, we generated a double-replacement strain that harbors
14 both Y0036 variants in the DY8531 background. Similar to the SPBC409.08
15 single replacement strain, the chronological lifespan of this double-replacement
16 strain was slightly increased at later timepoints (Fig. 4A & B). Although such
17 lifespan assays are inherently variable, our results show that the double-
18 replacement strain does not feature an extended lifespan compared to the single-
19 replacement strains. This finding provides some support to the notion that the
20 two variants in *SPBC409.08* and *ppk31* affect cellular processes that function
21 together in the same pathway. Epistasis has been predicted to drive linkage of
22 variants in a sexual population (Liti and Louis 2012). Our data does not suggest
23 that the two variants genetically interact with respect to lifespan, yet they are
24 quite tightly linked. Ppk31 appears to have multiple functions (see below), and
25 the two genes might genetically interact with respect to a different phenotype.

1 Longevity is unlikely to have been under strong selection in the wild

2 (Charlesworth 2000), and the two variants are not necessarily a direct

3 consequence of selection of longevity.

4 To further test whether spermine import and Ppk31 are functionally

5 linked, we assessed growth of the *ppk31Δ* deletion strain with or without

6 spermidine. A subtle phenotype was evident: at 37°C with 1mM spermidine,

7 *ppk31Δ* cells grew better than wild-type cells (Fig 4C). Intriguingly, *SPBC409.08Δ*

8 deletion cells showed similar improved growth with spermidine at 37°C (Fig.

9 4C). The observation that the *ppk31Δ* and *SPBC409.08Δ* deletion strains share the

10 same phenotype further argues for a model in which both genes affect the same

11 cellular process. The reduced growth of wild-type cells with spermidine at 37°C

12 suggests that spermidine is toxic in this condition.

13

14 *How Might Variants in ppk31 and SPBC409.08 Extend Lifespan?*

15 The 5' UTR of *ppk31* features an unusually high number of small upstream ORFs

16 (uORFs; Fig. 3C). This peculiarity is also evident in related *Schizosaccharomyces*

17 species (up to 16 in *S. cryophilus*; >3 amino acids). The small insertion in the 5'

18 UTR of *ppk31* leads to a nonsense mutation in a uORF (Fig. 3D). Ribosome

19 profiling data suggest that this uORF is not translated in proliferating or meiotic

20 cells (Duncan and Mata 2014). It is possible, however, that this uORF is

21 specifically translated in non-dividing, ageing cells. Such condition-specific

22 translation is evident for another uORF of Ppk31 (uORF7; Fig. 3C), which is

23 highly meiosis-specific (Duncan and Mata 2014). Typically, uORFs modulate

24 ribosome access to the large downstream ORF (Andrews and Rothnagel 2014).

1 Thus, the insertion we identified could affect the post-transcriptional regulation
2 of *ppk31*.

3 How might the small deletion in the 5' UTR of *SPBC409.08*, encoding a
4 predicted spermine transmembrane transporter, lead to lifespan extension?
5 Given the deletion's location in the UTR, another effect on post-transcriptional
6 regulation is plausible. However, the deletion does not appear to change the
7 coding sequence of any existing uORF or create a new uORF, nor does it lead to
8 any predicted change in RNA secondary structure (Supp. Fig. 7; Materials &
9 Methods). UTRs in *S. pombe* are under quite strong selective constraints, and
10 Indels in UTRs appear to contribute to phenotypic changes, probably by affecting
11 transcript regulation (Jeffares *et al.* 2015). Any modulation of spermine
12 transmembrane transport could be expected to affect chronological lifespan.
13 Spermidine levels are known to decrease with age (Scalabrino and Ferioli 1984).
14 This decrease appears to be detrimental, as supplementing spermidine extends
15 lifespan from yeast to mammals (Eisenberg *et al.* 2009, 2016; Madeo *et al.* 2018).
16 We propose that the genetic variant identified dampens the age-associated
17 reduction in spermidine by increasing intracellular spermine levels.

18 Although subtle, our data suggest that the two alleles identified function
19 together to extend lifespan via the same process. Intriguingly, budding yeast
20 Rim15 shows a positive genetic interaction with the polyamine transmembrane
21 transporter Tpo4 (Costanzo *et al.* 2010). However, we can only speculate how
22 changes in spermine transmembrane transport via *SPBC409.08* might affect
23 Ppk31 function or *vice versa*. One possibility is that translation of Ppk31 is
24 affected by spermidine, given that there are several documented examples of
25 polyamines affecting translation. For example, polyamines like spermidine

1 contribute to global translation through modification of the translation factor
2 eIF5A (Benne and Hershey 1978; Gregio *et al.* 2009; Saini *et al.* 2009; Patel *et al.*
3 2009; Landau *et al.* 2010). During this modification, spermidine is used to
4 directly convert lysine present in eIF5A to hypusine, and this modification is
5 essential for the biological activity of eIF5A (Park *et al.* 2010). Polyamines also
6 affect translation by other means. For example, frameshifting during translation
7 of antizyme mRNA, necessary for the production of full-length protein, depends
8 on spermidine concentrations (Gesteland *et al.* 1992; Rom and Kahana 1994;
9 Matsufuji *et al.* 1995). Furthermore, polyamines are associated with RNA for
10 other reasons (Igarashi and Kashiwagi 2010; Mandal *et al.* 2013). An intriguing
11 example is the polyamine-responsive uORF in the S-adenosylmethionine
12 decarboxylase mRNA, translation of which leads to repression of the
13 downstream ORF (Ruan *et al.* 1996; Raney *et al.* 2000). During translation,
14 polyamines directly interact with nascent peptides to stall the ribosome at the
15 uORF (Andrews and Rothnagel 2014).

16 If these genes do act in the same pathway, what is the nature of their
17 relationship? We found that *SPBC409.08Δ* cells grow better in toxic
18 concentrations of spermidine (Fig. 4C), most likely because they do not import
19 enough of the polyamine (or its precursor, spermine) to reach toxic levels. The
20 improved growth we observed in *ppk31Δ* cells could then be explained by two
21 models. In one model, translation of Ppk31 might be regulated by spermidine
22 levels, e.g. via polyamine-responsive uORFs (Ruan *et al.* 1996; Raney *et al.* 2000;
23 Andrews and Rothnagel 2014), which leads to toxicity under certain conditions.
24 This scenario puts Ppk31 downstream of SPBC409.08 and spermidine import. In
25 another model, Ppk31 might act upstream of the SPBC409.08 transporter, and its

1 deletion leads to a reduction in polyamine import, thus mirroring the deletion of
2 *SPBC409.08*.

3 A scan of 161 sequenced strains of *S. pombe* (Jeffares *et al.* 2015) shows
4 that whilst 55 strains (34%) have the *ppk31* insertion, only 8 (5%) have the
5 deletion in *SPBC409.08*. The latter strains always harbor the *ppk31* insertion as
6 well, although this could reflect the very close relatedness of all eight strains to
7 Y0036 (Jeffares *et al.* 2015). The small number of strains with the *SPBC409.08*
8 deletion limited further analyses, however we tested the 55 strains with the
9 *ppk31* insertion for enrichments in any quantitative phenotypes assayed by
10 Jeffares *et al.* (2015). Intriguingly, these 55 strains show sensitivity to various
11 chloride salts compared to the 106 strains without *ppk31* insertion (Supp. Fig. 8).
12 Thus, the *ppk31* insertion might have pleiotropic effects on the import of other
13 cationic substances, besides the proposed changes in polyamine import. Strains
14 with the insertion also show a trend for improved growth in the presence of
15 various drugs, such as caffeine which inhibits TORC1 signaling (Supp. Fig. 8).

16 TORC1 inhibition can increase lifespan through a number of downstream
17 effectors (Fontana *et al.* 2010; Johnson *et al.* 2013). In budding yeast, the
18 orthologue of Ppk31, Rim15, is one such effector (Wei *et al.* 2008), and its
19 activation upon TORC1 inhibition leads to the transcription of genes involved in
20 entry into quiescence (Reinders *et al.* 1998; Pedruzzi *et al.* 2003; Wanke *et al.*
21 2005; Urban *et al.* 2007) and stress response (Cameroni *et al.* 2004; Wei *et al.*
22 2008). Accordingly, the insertion variant could lead to increased levels of Ppk31
23 protein, in the presence and/or absence of TORC1 signalling, thus improving
24 stress-resistance and lifespan of non-dividing cells. Furthermore, spermidine is
25 known to cause TORC1 inhibition (Madeo *et al.* 2018). Another possibility,

1 therefore, is that the regulation of intracellular spermidine levels by *SPBC409.08*
2 indirectly leads to Ppk31 regulation via TORC1.

3 We conclude that two known lifespan extending interventions, Rim15
4 regulation and spermidine metabolism, may be intertwined at the molecular
5 level. Spermidine extends lifespan by enhancing autophagic flux, which is
6 mediated via phosphorylation of many proteins, including key autophagy
7 regulators, such as Akt and AMPK (Eisenberg *et al.* 2009, 2016; Madeo *et al.*
8 2018). The kinase(s) responsible for this spermidine-dependent
9 phosphorylation, however, remain(s) elusive. Intriguingly, Rim15 also positively
10 regulates autophagy through phosphorylation of Ume6 (Bartholomew *et al.*
11 2012). These parallels raise the enticing possibility that spermidine's effect on
12 autophagy, and therefore its mode of action for extending lifespan, is exerted via
13 the Ppk31 kinase.

14

15 **CONCLUSION**

16 We applied selection, in the form of ageing, to large, inter-crossed populations of
17 non-dividing *S. pombe* cells with standing genetic variation. We then used deep
18 sequencing to detect genetic variants that became reproducibly enriched as
19 pools aged. In a region of Chromosome II that appeared to be under selection, we
20 identified indels in the 5' UTRs of *ppk31* and *SPBC409.08* as the most promising
21 causal variants. Using CRISPR/Cas9-based gene editing, we created allele
22 replacement strains that revealed subtle effects of the two variants on longevity.
23 Both Ppk31 and spermidine metabolism (predicted biological process associated
24 with *SPBC409.08*) have previously been implicated in cellular ageing. Our results
25 point to natural genetic variations that influence the regulation of these loci, and

1 that may contribute to the variation in chronological lifespan in wild *S. pombe*
2 strains. Experiments using a double allele replacement strain and single deletion
3 mutants suggest that Ppk31 and SPBC409.08 function in the same process to
4 modulate lifespan, possibly via spermidine-based regulation of Ppk31 or via
5 Ppk31-regulated spermidine homeostasis. The finding that even the strongest
6 candidates for causal alleles produced only subtle effects suggests that the
7 longer-lived strain must contain many other alleles with weak effects,
8 highlighting the complex genetics underlying cellular lifespan.

9

10

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- 9
- 10

1 **Figure legends**

2

3 **Figure 1. An industrial isolate of *S. pombe* is long-lived compared to a**
4 **laboratory strain.**

5 A: Lifespan curves of the two parental strains – DY8531 (red) and Y0036 (blue).

6 Lines correspond to the mean \pm shaded standard deviations (N=3).

7 B: Experimental design (see main text for details).

8

9

10 **Figure 2. Selection for parental alleles in region of Chromosome II with age.**

11 A: Lifespans of each replicate AIL pool. Sampling time points colored

12 corresponding to C.

13 B: 50kb sliding median of the scores at each locus. Scores describe the extent to

14 which allele frequency changed with age, with high scoring variants displaying

15 similar trajectories repeatedly across replicates (Materials & Methods). Red

16 dotted line represents the threshold used for peak calling (upper quartile + 1.5x

17 inter-quartile range).

18 C: Allele frequency at each time point, for each locus within 300kb surrounding

19 the Chromosome II peak. Each dot represents a single allele. Allele frequencies

20 are the mean of all eight replicates. The color of each point represents the

21 sampling time (see key). Arrows highlight above-threshold variants. Dotted lines

22 highlight the location of *SPBC409.08* and *ppk31*.

23

24

1 **Figure 3. Genomic context of variants exceeding the threshold in peak**
2 **region.**

3 A: Broad genomic context of all above-threshold variants on Chromosome II. The
4 location of Indels in the 5' UTRs of *SPBC409.08* and *ppk31* are labelled 1151936
5 and 1216499, respectively. CDS in dark blue, UTRs in light blue.

6 B: Local genomic context of the deletion in a short, repetitive stretch in the 5'
7 UTR of *SPBC409.08*.

8 C: Predicted uORFs in the 5' UTR of *ppk31*.

9 D: An insertion in uORF3 (red) leads to a frameshift in the predicted peptide and
10 premature stop codon. Amino acids in grey are unique to the protein of strain
11 Y0036.

12

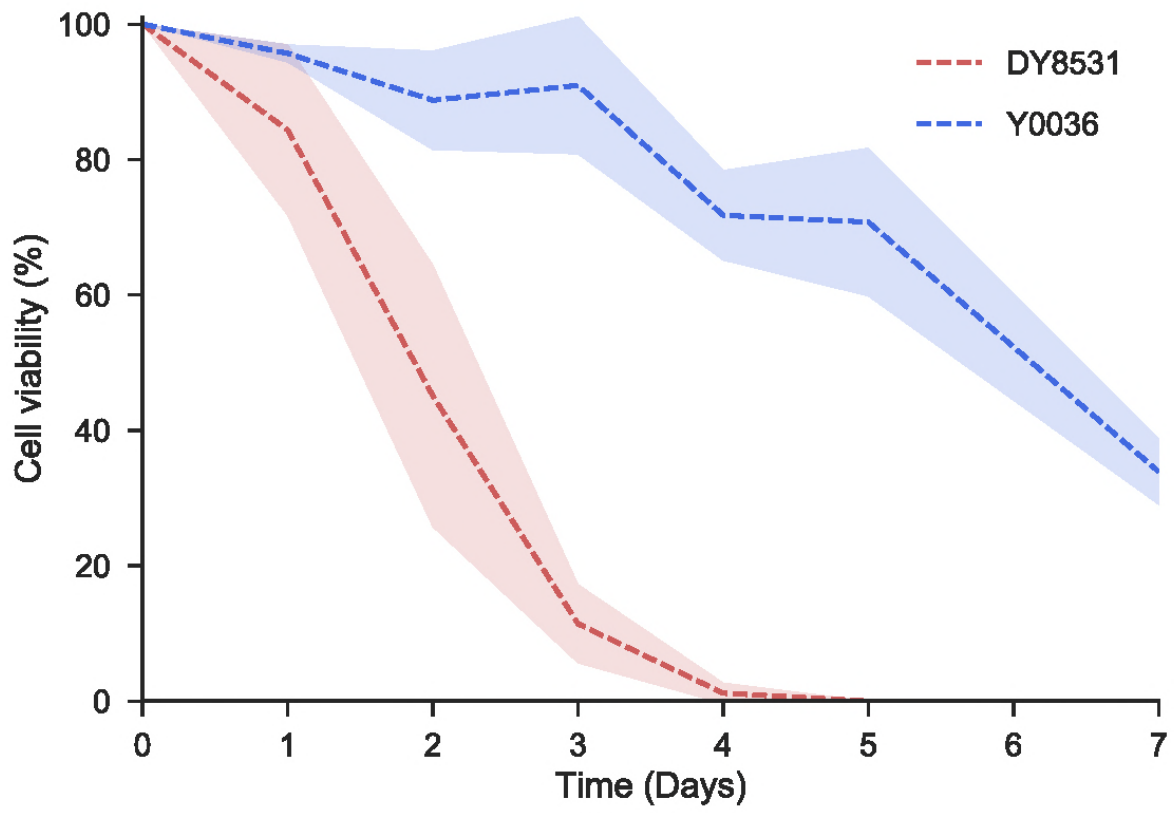
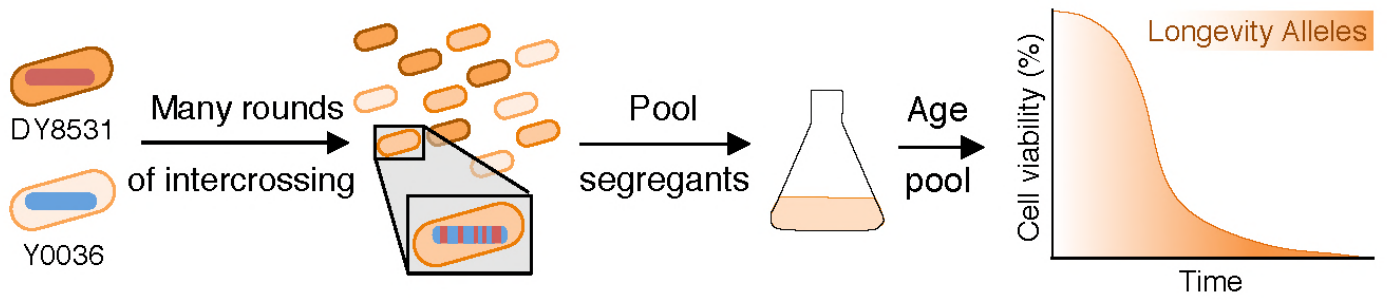
13 **Figure 4. Allele replacement with candidate variants and gene deletion at**
14 **both *ppk31* and *SPBC409.08* give similar, subtle phenotypes.**

15 A: Lifespan curves of the single and double allele replacement strains (solid
16 lines) compared to the two parental strains (dashed lines) as indicated. Lines
17 correspond to the mean \pm shaded standard deviation (N=3).

18 B: Same lifespans as in part A, shown as normalised \log_{10} colony forming units
19 (median \pm standard deviation). Lifespans shown up to day four, where all strains
20 still showed some viability.

21 C: Spot assays comparing the growth of *ppk31* Δ and *SPBC409.08* Δ to WT. Rows
22 show 5-fold serial dilutions of each strain grown with and without 1mM
23 spermidine, at 32°C and 37°C.

24

A**B****Figure 1.**

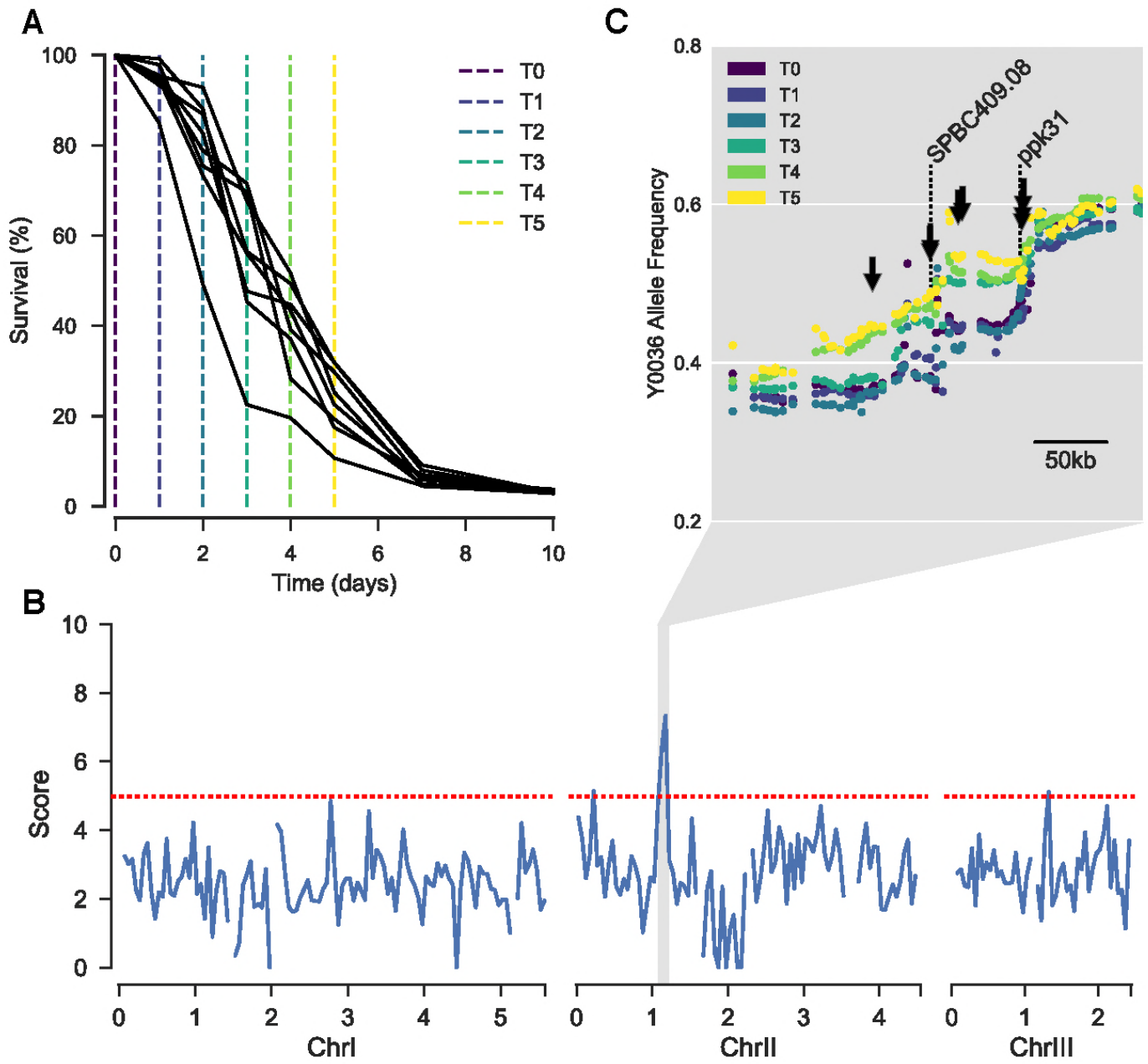
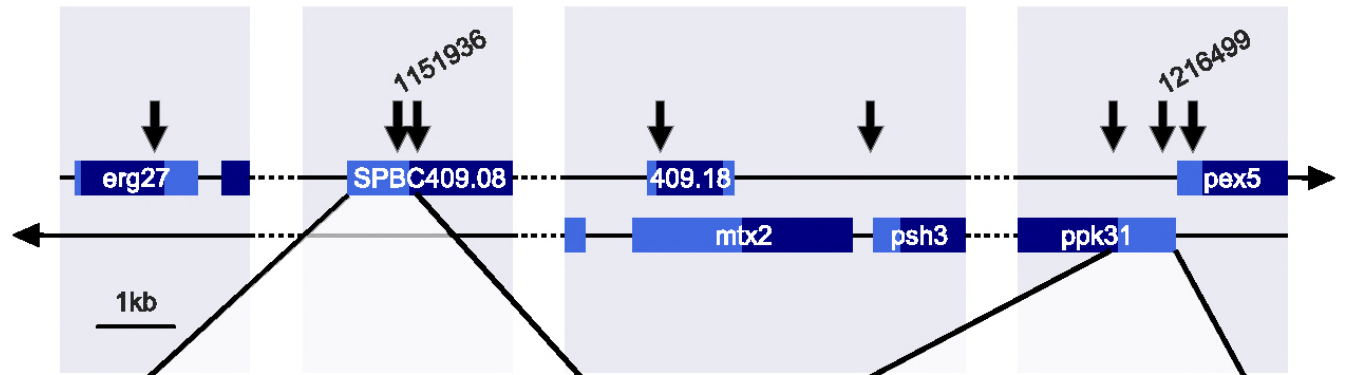
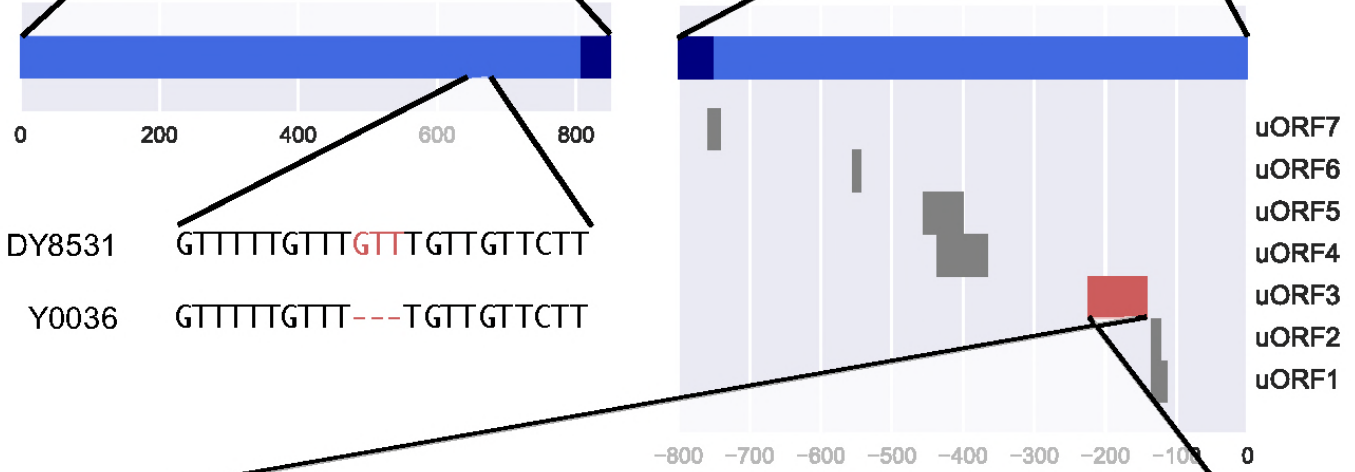


Figure 2.

D



B



DY8531 GTTTTTGTTGTTGTTGTTCTT
 Y0036 GTTTTTGTT---TGTGTTCTT

DY8531 M I Q R K K S I L F L / S S F Q I S V I R K L N Q G L D *
 ATGATACAGAGAAAAAATCCATATTATTTCT-AAGTTCCTTTCAAATTAGTGTGATTAGAAAGTTAAACCAAGGTTGGATTGA

Y0036 M I Q R K K S I L F L K F F S N *
 ATGATACAGAGAAAAAATCCATATTATTTCTAAGTTCCTTTCAAATTAGTGTGATTAGAAAGTTAAACCAAGGTTGGATTGA

Figure 3.

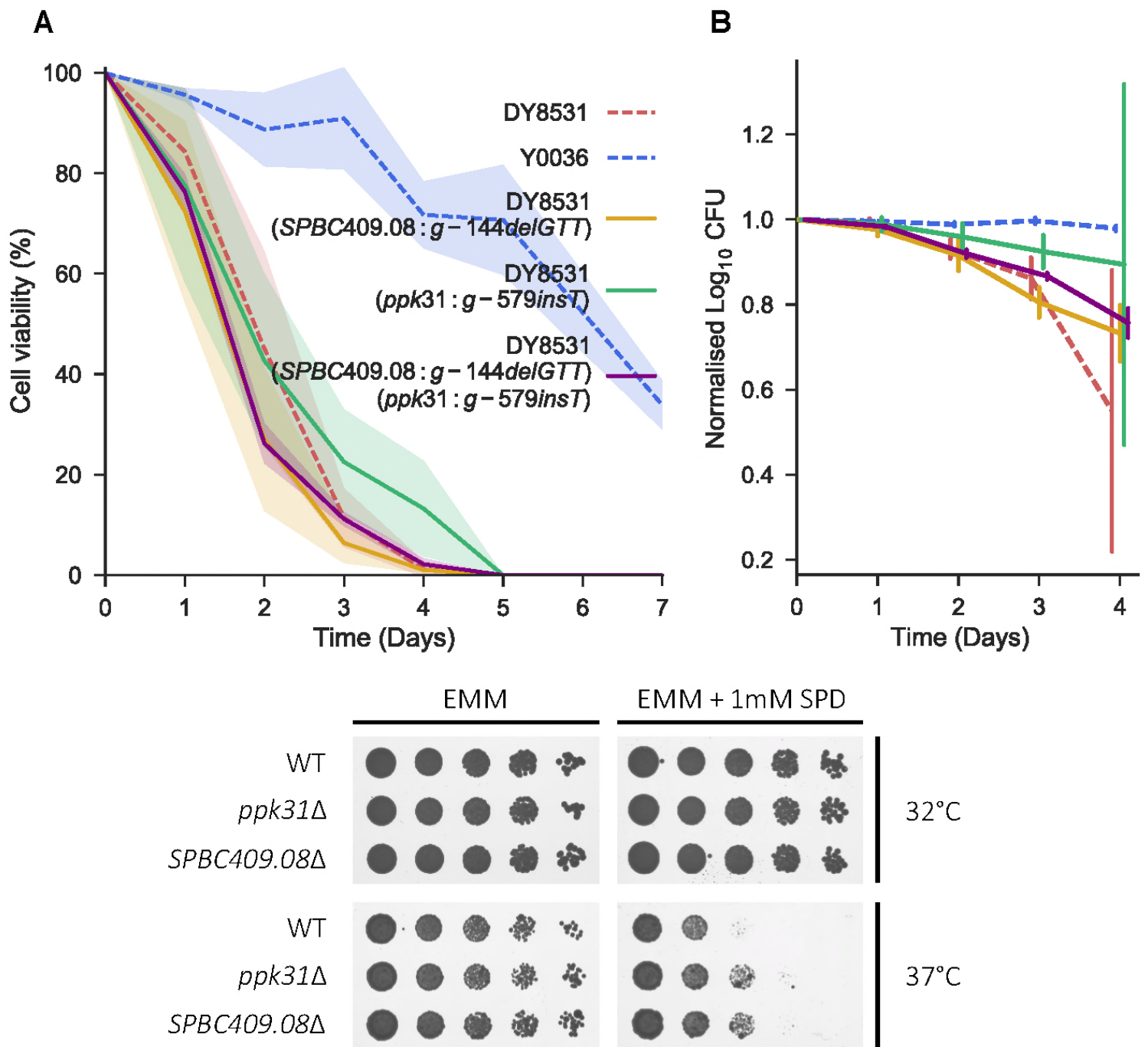


Figure 4.