A Genome-Wide Analysis Reveals Significant Overlap of Transcription and DNA Repair in Stationary Phase Yeast

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One-sentence summary: Non-propagating yeast cultures exhibit significant DNA synthesis, which occurs preferentially in genes that undergo constant transcription.

Abbreviations: SP – stationary phase; SMM – supplemented minimal medium; BrdU - 5-bromo-2-deoxyuridine; ORF – open reading frame; O.D. – optical density; IP – immunoprecipitation; RE – repaired-expressed; E – expressed; R – repaired; ARS – autonomously replicating sequence; ORC – origin recognition complex; GO – gene ontology; ML – maximum likelihood; TCR – transcription-coupled repair; RNAP – RNA polymerase

Abstract: The association between transcription and DNA repair is acknowledged as a player in the generation of mutations in a non-random fashion in prokaryotes and eukaryotes. Previous studies demonstrated that the transcription complex is capable of directing DNA repair to sites of transcription. This process is especially important to growth-arrested cells, in which many DNA repair capacities are diminished; it may also lead to mutations preferentially in transcribed genes. Using microarray analysis of growth-arrested yeast cultures, we demonstrated on a genomic scale, the co-localization of a DNA-turnover marker, indicative of DNA-repair-associated DNA synthesis, with genes persistently transcribed during stationary phase. This may serve as a clue regarding the non-random manner in which non-dividing cells may potentially mutate in the absence of replication, solely as a result of their inherent, transcriptional stress response.

Prolonged stressful conditions, which often induce extensive periods completely devoid of DNA replication, are constantly encountered by the majority of the microorganisms in Nature [1]. However, these cells retain their viability and genome integrity even for exceedingly long stationary periods, owing apparently to persistent DNA repair mechanisms, and specific modifications of the cellular morphology [2, 3]. In addition, microorganisms have the capability of adapting to environmental challenges by transcriptional activation and inactivation of defined sets of genes [4-6]. In *Saccharomyces cerevisiae*, the transcriptional response to various stressful conditions and the molecular and morphological qualities required for subsistence during stationary periods are well studied [2, 7]. Upon the depletion of carbon source, *S. cerevisiae* cultures readily enter stationary phase (SP), and can retain nearly 100% viability for periods as long as several months. When nutrients are eventually replenished, the culture recommences propagation, exhibiting normal, healthy growth characteristics [1]. Several studies have concluded that non-dividing cells of stationary phase yeast cultures are capable of introducing mutations specifically in stress-related genes, in a strictly non-random manner, conceptually similar to bacterial stationary phase mutation [8-11]. Eventually, such capacity has the potential of creating a mutation pattern that is not random with respect to the mutation's position along the genome.

Even though the mechanism that may underlie the non-randomness of the observed mutational spectrum remains elusive for the time being, several independent hypotheses suggested that the cell's inherent transcriptional response to stress conditions can facilitate a higher incidence of mutations in transcriptionally active chromosomal regions compared to inactive ones [12-17]. We suggest that such a 'mutational bias' can be achieved,

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at least in theory, by coupling error-prone DNA repair mechanisms to relatively

unprotected, damage-susceptible DNA segments, involved in persistent, stress-induced

transcription, whereas inactive regions remain packed and comparatively more protected

from spontaneous damage. This can also be the result of error-free DNA repair (such as

transcription-coupled repair; TCR) [18-22], as long as the incidence of spontaneous DNA

damage is indeed higher in active compared to inactive regions. If this is indeed the case

in arrested cultures, one should be able to observe considerable DNA turnover in genes

that are constantly active, in a pattern that significantly deviates from randomness.

To test this hypothesis, we utilized stationary cultures of a transgenic yeast strain

(MAT**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL URA3*::*GPD-TK*$_{7X}$), denoted

E1000, capable of efficient incorporation of very small concentrations (~50 μg/ml) of

external BrdU into their DNA [23], as markers of DNA synthesis. In the absence of cell

division, DNA nucleotide turnover can only be the result of DNA maintenance (i.e.

repair) or unscheduled replication [24-26]. Therefore, genes that were labeled with BrdU can

be regarded as genes that underwent unscheduled replication or DNA repair, and can

most probably be distinguished based on their chromosomal positions (see below).

Logarithmically growing cultures of *S. cerevisiae* were washed, transferred to

supplemented minimal medium (SMM) without a carbon source, and further incubated

for 48 hours until complete arrest. Stationary phase entry was monitored by measuring

O.D. at 600 nm (observed constant O.D.; Fig. 1C) and light microscopy (budding index

~0%, not shown). BrdU was then added to a final concentration of 400 μg/ml [23], and

DNA samples were removed at T_0 (time of transfer to SMM w/o dextrose), 12 hrs, and

from 1 day to 21 days at 24 hrs intervals. DNA was extracted, digested with Sau3AI, and

restriction fragments were ligated to Sau3AI linkers ([27], and in http://www.flmnh.ufl.edu/soltislab/Protocols.htm; see link for "Microsatellite Capture Protocol" therein). Genes that contain BrdU were identified by immunoprecipitation (IP) using an anti-BrdU antibody [28], and subsequent amplification, labeling and hybridization onto a glass cDNA microarray [29], containing full-length open reading frames of the entire *S. cerevisiae* genome (both verified and predicted open reading frames, each feature is represented once) [30]. In parallel, we performed global expression analysis of these stationary cultures (Fig. 1A). RNA samples were removed at T_0, 1 hour, 2 hrs, 3 hrs, 4 hrs, 6 hrs, 8 hrs, 24 hrs, and from 2 days to 21 days at 24 hrs intervals (Fig. 1C). Growth arrest and viability were monitored throughout the entire time course of the experiment by measuring the O.D. at 600 nm and CFU counting (Fig. 1C). Both the O.D. and the number of CFU remained constant for the 21-day stationary period (Fig. 1C), and no budding was observed (not shown). From the entire time course of the experiment, we selected and analyzed the first six days of the stationary phase, based on six expression time points and three DNA-IP samples (in biological duplicates, each in triplicate).

Data normalization of the Cy3/Cy5 signals per hybridization was performed as described in Bolstad's quantile normalization procedure for oligonucleotide microarrays [31]. The outputs of the normalization procedure are per spot Cy3/Cy5 log-differentials. The expression level of a feature was determined by the average log-differential (significance calculated by t-test) across all expression samples, and we generated sets of genes that are significantly expressed during the first six days or any five out the first six days of the stationary phase. A DNA repair event was determined based on a significant

difference (calculated by t-test) between the average log-differential of DNA repair at the two later time points and the DNA repair log-differential at T_0.

Our analysis revealed, for the first time, genome-wide occurrence of DNA repair during stationary phase. We selected features that exhibited significant BrdU labeling (728 genes, classified as repaired genes), and compared this data with features that are exclusively expressed at all six time points; we performed identical, separate analysis of genes that are expressed only during five out of six days of the sampled time course. Based on the proportions of expressed and repaired features, one can test whether the number of overlapping features (both expressed and repaired) deviates from the expected under the assumption of independence, by a simple analysis of the corresponding 2×2 contingency table. We found a significant positive association of these two features, manifested as excess of repaired and expressed features ($p<5\cdot10^{-6}$ for 6 days expression and $p=0.000012$ for 5 days expression).

The same conclusion was reached when we used a different approach to evaluate the expression-repair association. We assumed that the probability for a gene with high DNA repair index to be present in a segment of a list of features, sorted by expression level, is proportional to the segment length, and is independent on a position of that segment in the sorted list. Under this assumption, the probability to have $\geq k$ repair events in the first segment of length n (highest expression levels), is distributed binomially:

$$(1) \qquad P(\geq k \mid n) = 1 - \sum_{i=0}^{k-1} \frac{n!}{i!(n-i)!} p^i (1-p)^{n-i}$$

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where *p* is the fraction of features with high DNA repair index. Since *p* is small (~0.014) and the number of features in each array is large (~7000), the probability (1) can be approximated by the following expression, based on Poisson distribution:

$$(2) \quad P(\geq k \mid n) < \sum_{i=k}^{\infty} \frac{\lambda^i}{i!} e^{-\lambda} < \frac{\lambda^k}{k!} e^{-\lambda} \cdot (1 + \frac{\lambda}{k+1} + \frac{\lambda^2}{(k+1)^2} + \ldots) = \frac{\lambda^k}{k!} e^{-\lambda} \frac{1}{(1 - \frac{\lambda}{k+1})},$$

where $\lambda = p \cdot n$.

For each starting segment of sorting of size *n* we can calculate the number of features (*k*) with high repair index within this segment, and then the probability $P(\geq k \mid n)$. If the probability $P(\geq k \mid n)$ is sufficiently small, the overpopulation (hence, the association) is significant. Meaning, the starting segment of highly expressed features is markedly overpopulated by features with high DNA repair index. For the list of 800 features with highest average expression (first 800 features of the sorting), the overpopulation with features with high DNA-repair index was indeed highly significant ($P(\geq k \mid n) = 0.000004$) (Fig. 2).

For each of the two groups of expressed features (5 and 6 days expression), we composed a list of overlapping repaired-expressed (RE) features, comprising 122 and 186 features, respectively (Fig. 3A). Next, we analyzed the positional pattern of all repaired (R), expressed (E), and RE sites in the yeast genome, of both expression groups (5 and 6 days). Log-linear analysis revealed that the distribution of R, E, and RE sites among chromosomes significantly deviates from the proportions expected based on the yeast genome map (Fig. 3B & C); namely, several chromosomes are over- or under-represented with respect to the occurrence of R and E sites. Reproducible, highly significant paucity

and excess of RE sites was found in chromosomes 2 and 8, respectively (Fig. 3B & C); weaker (albeit similar in both 5 and 6 days) deviations were observed in other chromosomes as well.

A considerable proportion of RE features (20.5%) were located in the vicinity (up to 5000 bp) of autonomously replicating sequences (ARS), which point to a high level of activity near ARS during stationary phase. This finding is strengthened by a recent study, concluding that genes induced by environmental stress are located near origin recognition complexes (ORC) recognition sequences at a higher frequency (22%) compared to all genes (16.4%) [32]. In addition, it was recently reported that genes that are repressed by starvation are located in the proximity to ORC-binding sites less frequently compared to all genes [33]. These data may come in support to our finding of the non-random distribution of RE features across yeast chromosomes, as well as for the high abundance of RE sites near ARS. To exclude the possibility that the enrichment of genes in the vicinity or ARS found by our analysis, is the result of unscheduled replication (or even replication *per se*), we calculated the average tract size of consecutive ORFs near ARS elements that were labeled with BrdU. If unscheduled or regular replication had indeed taken place, then we would have detected BrdU labels of several consecutive ORFs near ARS elements, since the experiment time course allowed several rounds of genome replication. In addition, since our DNA samples represent the entire population, the different origin firing of all cells sampled together, will add up to a long BrdU-labeled tract in the vicinity of ORF sequences. This, however, was not the case: the average length of consecutive ORF tracts residing near ARS was found to be 1.14 ORFs. Hence,

we can conclude that the signal of R features was not the result of replicative DNA polymerization.

Our analysis showed that 14.8% of RE features were located inside telomeric regions. In accordance with that, the RE set was found to be significantly enriched (SGD's GO Term Finder analysis; p=0.00138) with nine (YBL113C, YRF1-2, YIL177C, YLL066C, YRF1-4, YRF1-5, YML133C, YRF1-6, YPR204W; 6-days expression) or eleven (6-days expression set plus YHL050C and YLL067C; 5-days expression) helicases. Seven and nine (five and six days expression, respectively) of these helicases, are encoded inside telomeres or specifically by Y' elements of sub-telomeric regions, and are involved in telomere maintenance via recombination. Helicases are also required in TCR, needed for the remodeling of the RNAP complex at the lesion site, enabling the progression of DNA repair [34]. It is to note that recent findings conclude that transcriptional activity within mammalian telomeres is essential to maintain telomere and genome integrity [35]. In this respect, it may be possible that the observed high level of telomere activity might be necessary for the yeast to maintain proper genome integrity, fitness and cellular longevity during the stationary phase, as a preparatory step for potential upcoming periods of propagation.

The marked presence of RE sites within telomeric regions may imply that these sites are major contributors to the high expression-repair association we found. Therefore, is important to test whether this association is maintained if we exclude these sites from our analysis. For that, we re-calculated the R and E association without taking into account telomere-residing or near-telomere features. We found that even in the absence of telomeric features, we still obtained significant positive association of these two sets

(p=0.000027 for 6 days expression and p=0.00043 for 5 days expression). The new p values are indeed larger, however the association of R and E is still highly significant, and we can conclude that our observation represents a valid link between transcription and DNA repair that spreads beyond telomeres.

Taken together, these findings show a genome-wide co-incidence of expression and repair events in SP, which provide an insight into the dynamics of seemingly static chromosomes in stationary cultures. Although non-dividing cells can appear to dispense the task of maintaining the majority of their non-essential, transcriptionally inactive genome, it is critically important to maintain the integrity of transcribed genes.

In yeast and bacteria, it has already been found that the mutation rate is directly proportional to the level of transcription [12, 14, 15]. However, these studies referred only to dividing cells, in which the replication process and sometimes specifically the progression of the replication fork [14] are indispensable players in the mutational process. In addition, only a small number of genes was studied [9, 14, 15]. Our study is the first to demonstrate a link between transcription and the dynamics of DNA in non-dividing cells, namely, without the requirement for DNA replication or replication-dependent repair processes, using genome-wide assay. We detected significant, non-random co-localization of transcription and DNA repair events in stationary yeast cultures, suggesting a reasonable mechanistic foundation for the debatable phenomenon of the observed stationary phase mutation in yeast. A straightforward continuation of this study will be to perform a genome-wide sequence analysis of RE sites in search of differences between RE and non-RE sequences in SP and normally propagating cultures. If mutations

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will eventually be discovered, then these studies are highly likely to establish the importance of stationary phase DNA dynamics to the process of adaptation and evolution.

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Figure captions

Fig. 1. An outline of the experimental strategy for the identification of expressed-repaired sites in stationary phase yeast cultures. **A.** fragmented DNA from stationary and dividing cultures labeled with BrdU was enriched by performing IP using an anti-BrdU antibody; BrdU-containing DNA segments were identified by microarray hybridization. **B.** IP of both cycling and arrested BrdU-incorporating and non-incorporating yeast strains (E1000 and E001, respectively), grown in SMM; no labeled DNA is present in E001 and in E1000 grown in SMM with or w/o BrdU, signifying low level of non-specific "noise". **C.** O.D. (magenta graph) and CFU count (blue graph) of two independent cultures (squares and triangles) during the experiment's time course; arrows denote sampling of DNA (upper arrows) and RNA (lower arrows); asterisk denotes T_0.

Fig. 2. Highly significant overpopulation of the set of highly-expressed features with features having a high DNA repair index. The graph shows the logarithm of probability of having $\geq k$ repair events within the first segment of a length n in the ordered list of expressed features, assuming no association (H_0 hypothesis) between expression and repair; dashed line indicates n=800.

Fig. 3. Site distribution of feature sets. **A.** Venn diagram depicting sets of features chosen for analysis from 5 and 6 days expression groups. RE, repaired-expressed (RE5 and RE6, five- and six-day expression, respectively); E, expressed; and R, repaired features. **B.** Chromosomal locations of RE (red lines), R (green arrows) and E (brown arrows) features in 5 (upper pane) and 6 (lower pane) days expression groups. **C.** Analysis of distribution among chromosomes of all sets of features, in the 5 and 6 days expression

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groups. ML-χ^2, maximum likelihood log-linear test of between-chromosome homogeneity of feature distribution (for 2×n table).
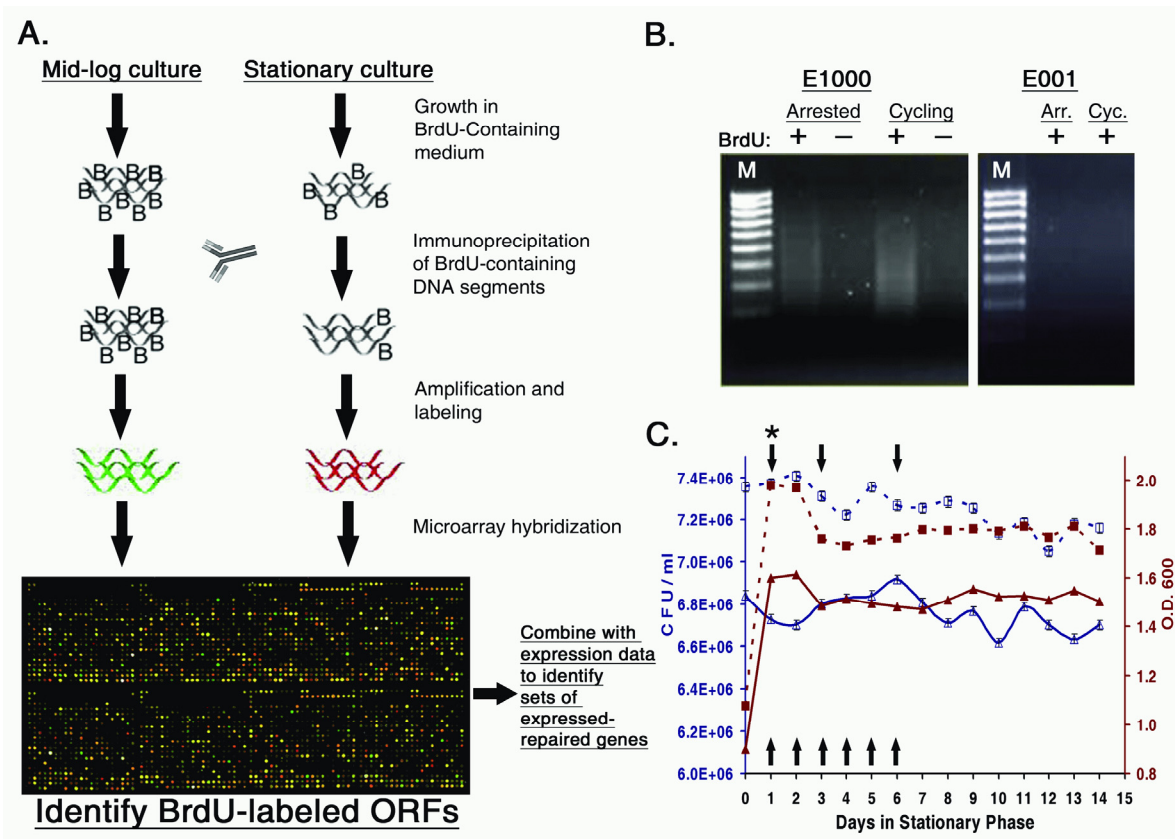
Fig. 1
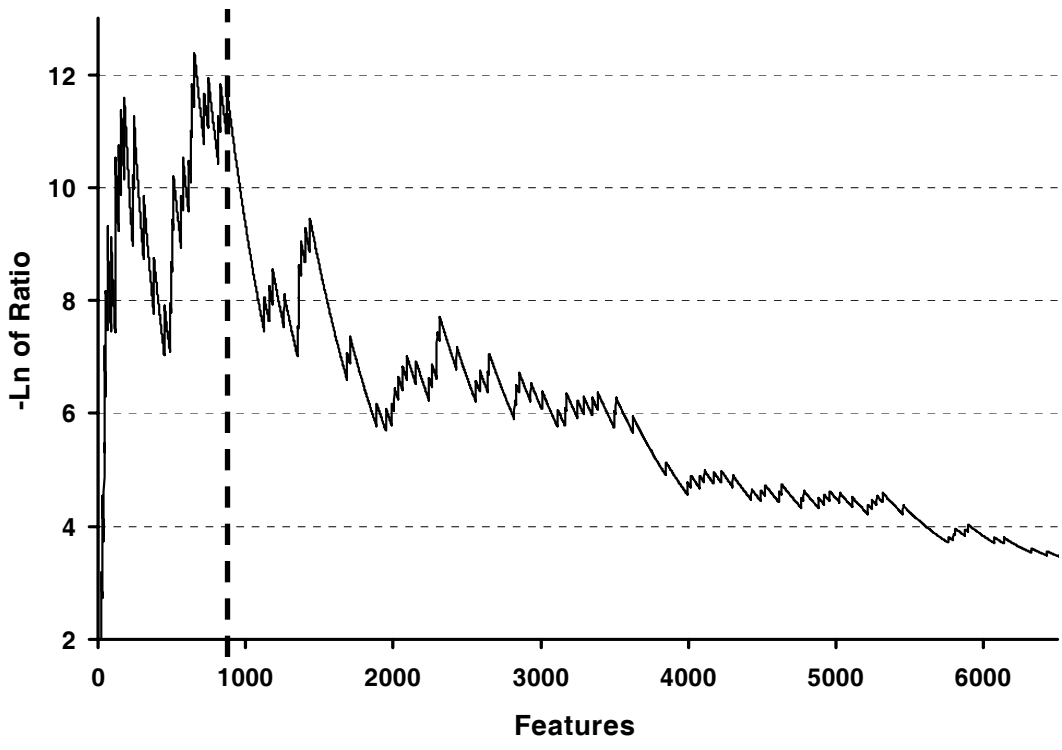

Fig. 2

A.

5 Days Expressed Features n=1266

6 Days Expressed Features n=735

RE 6 n=122

RE 5 n=186

Repaired Features n=728

C.

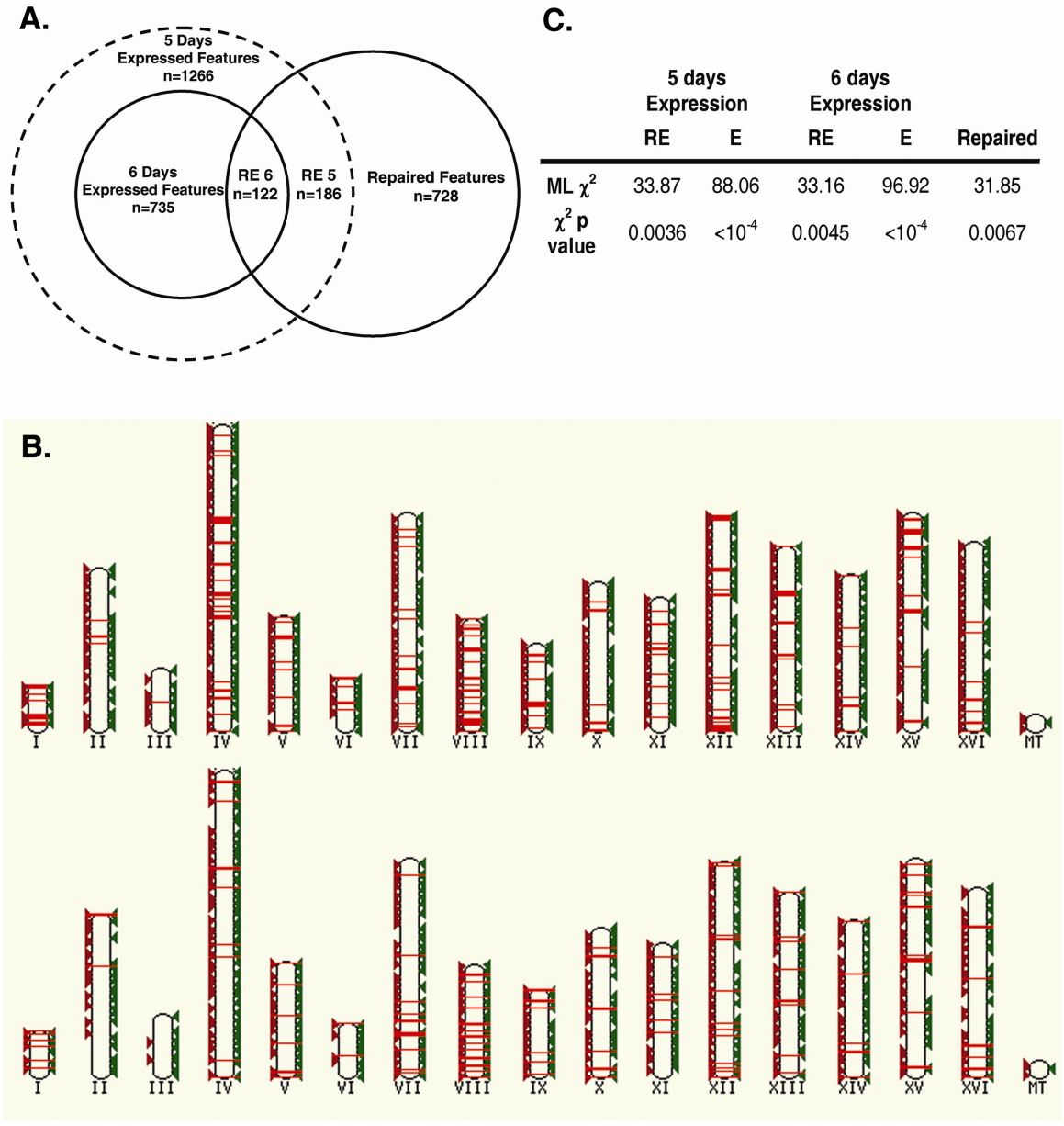|	5 days Expression		6 days Expression		
	RE	E	RE	E	Repaired
ML χ^2	33.87	88.06	33.16	96.92	31.85
χ^2 p value	0.0036	$<10^{-4}$	0.0045	$<10^{-4}$	0.0067

B.

Fig. 3.

MATERIALS AND METHODS

Yeast strains and media

In this study we used the following strains: E1000 [1], capable of uptaking and incorporating exogenous BrdU into its DNA. E1000 (MAT**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL URA3*::*GPD-TK$_{7X}$*), contains seven copies of the Herpes simplex thymidine kinase (TK) gene under control of the GPD promoter at the URA locus; E001, auxotrophic for uracil (MAT**a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL*) identical to E1000, however unable to utilize BrdU [1]; S288c (MAT**α** *SUC2 gal2 mal mel flo1 flo8-1 hap1*). Yeast were grown either in 1% yeast extract/2% peptone/2% dextrose (YPD) medium or SMM, supplemented with Adenine sulphate, L-Tryptophan and L-Histidine (20 mg/litre), and L-Leucine (100 mg/litre) [2].

Yeast growth and maintenance

Frozen (-80°C) yeast from strain E1000 were thawed and plated on SMM-agar plates supplemented with all necessary auxotrophic nutrients except uracil [1]. Single colonies were picked and used to inoculate 5 ml SMM starter cultures, grown overnight in an orbital shaker at 30ºC, 200rpm. Cells were then transferred into a 50 ml SMM culture, grown overnight at 30ºC, 200 rpm. This culture was used to inoculate a larger, 1000 ml SMM culture, incubated overnight at 30ºC, 200 rpm. Cells (O.D.$_{600}$ ~1.0) were then pelleted, washed with sterile ddW and transferred into 500 ml SMM-containing Ehrlenmeyer flasks without a carbon source, in order to induce growth arrest and stationary phase entry. Cultures were maintained at 30ºC for another 48 hours, then BrdU

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(Acros Organics, Belgium) was added to a final concentration of 400 μg/ml [1]. Cultures were incubated for additional 4 weeks. O.D.$_{600}$ and CFU count (by plating on YPD-agar plates) were monitored throughout the experiment. BrdU was replenished every 4 days. Dividing yeast cultures, started from single colonies were grown in SMM supplemented with 400 μg/ml of BrdU (E1000, E001) or YPD (S288c).

DNA and RNA sampling

Samples were removed by fast vacuum filtration of 10-12 ml of the yeast culture through sterile nylon filters (pore size 0.45 μm; Whatman, Maidstone, UK). Filters were immediately frozen in liquid nitrogen and stored frozen at -80ºC until further processing. Stationary phase cultures were sampled for RNA at T_0 (time of transfer to SMM w/o dextrose), 1 hour, 2 hrs, 3 hrs, 4 hrs, 6 hrs, 8 hrs, 24 hrs, and from 2 days to 21 days at 24 hrs intervals. Stationary phase samples for DNA extraction and analysis were removed at T_0, 12 hrs, and from 1 day to 21 days at 24 hrs intervals. Samples for DNA and RNA extraction were also removed from proliferating cultures of E1000, E001 and S288c yeast at mid-log phase.

DNA and RNA extraction

The frozen nylon filters were quickly thawed in sterile, cold ddW, and cells were briefly pelleted in a cooled (4ºC) centrifuge. Total RNA was extracted using Epicentre's (Madison, WI, USA) MasterPure® Yeast RNA kit, according to the manufacturer's instructions. The extracted RNA was treated with RNase-free DNaseI to remove any traces of interfering DNA. Total yeast DNA was extracted using Epicentre's MasterPure®

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Yeast DNA kit, following manufacturer's instructions. The extracted DNA was treated with DNase-free RNaseA to remove any contaminating RNA. DNA and RNA were dissolved in nuclease-free water and stored frozen until use.

DNA processing, immunoprecipitation and labeling

Equimolar amounts of DNA from all samples were processed as follows: total yeast DNA was digested and linked to Sau3AI linkers, as described in ([3]; http://www.flmnh.ufl.edu/soltislab/Protocols.htm see link to 'Microsatellite Capture Protocol' therein). Briefly, 20 µg of total yeast DNA was digested overnight at 37ºC with Sau3AI (Takara Bio Inc., Europe). Digested DNA was cleaned and ligated to the following Sau3AI linkers: A- 5'-GCGGTACCCGGAAGCTTGG-3'; and B- 5'-GATCCCAAGCTTCCGGGTACCGC-3'). Ligation was verified with PCR using the linkers as specific primers. DNA Immunoprecipitation with an anti BrdU antibody (Beckton Dickinson, USA) was performed as described [4]. The resulting DNA was cleaned and linearly amplified with Klenow exo⁻ fragment (New England Biolabs, USA) as described [5]. The DNA was then cleaned and labeled with either Cy5-dUTP (stationary phase cultures), or Cy3-dUTP (mid-logarithmic cultures, reference samples) (GE Healthcare, UK), using the linkers as specific primers [5]. Unincorporated dye was removed (Zymo Research, DNA Clean and Concentrator 5), and samples ware eluted in 7 µl of TE (pH 8). The efficiency of dye incorporation was measured using NanoDrop® spectrophotometer (NanoDrop Technologies, USA).

RNA labeling

Total yeast RNA (10 μg per sample) was labeled with reverse transcription, essentially as described in [6], with either Cy5-dUTP (stationary phase cultures), or Cy3-dUTP (logarithmic cultures, reference samples) (GE Healthcare, UK), using a $dT_{(20)}VN$ oligonucleotide as a primer (Sigma Genosys, Israel). Unincorporated dye was removed (Zymo Research, USA), and samples ware eluted in 6 μl of TE (pH 8). The efficiency of dye incorporation was measured using NanoDrop® spectrophotometer (NanoDrop Technologies, USA). In addition, to control for arbitrary loss of mRNA from samples during processing, and for signal variation arising from differences in labeling efficiency, hybridization and scanning, an external control normalization mix consisting of five different *in vitro*-transcribed *Bacillus subtilis* RNAs (ATCC clones: *LysA* (clone no. 87482; final concentration 2 pg/μl), *Phe* (no. 87483; 8 pg/μl), *Thr* (no. 87484; 6 pg/μl), *Trp* (no. 87485; 4 pg/μl), and *Dap* (no. 87486; 5 pg/μl) was added to each of the samples immediately after cell lysis.

Target preparation, microarray hybridization and scanning

The labeled RNA and DNA were hybridized to a glass cDNA microarrays containing full-length open reading frames of the entire *S. cerevisiae* genome (both verified and predicted yeast open reading frames, each feature is represented once), and 120 features for external control normalization, printed in a spatially-even manner across the entire grid of the array. The microarrays were printed by Dr. Yoav Arava, Faculty of Biology, Technion- Israel Institute of Technology, Israel. Three independent repetitions (microarray hybridizations) were done for each selected time point, each from 2 different cultures. Stationary phase DNA and RNA were labeled with Cy5-dUTP, whereas mid-log

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total RNA and total yeast DNA (taken from a common pool) were labeled with Cy3-dUTP. To quantify the background signal of the DNA IP, total yeast DNA which was not labeled with BrdU was immunoprecipitated, processed as described above labeled with Cy5-dUTP, and hybridized. Slides were washed with SDS 0.1%, denatured for 3 minutes in boiling ddW, washed with 70% ethanol and dried. Each slide was then incubated in a filtered 50 ml pre-hybridization solution (10 mg/ml BSA, SSC×5, 0.1% SDS) at 42°C for 1 hour and washed with ddW. Purified targets (10 μl; Cy3+Cy5) were mixed with 4.5 μl 20×SSC, 15 μl formamide, 0.3 μl SDS 10%, and 3 μl yeast tRNA (10 mg/ml; Ambion, UK), denatured for 3 minutes at 95°C and supplemented with 3 μl BSA 10%. 25 μl of the target mix was placed on each microarray slide and covered with a glass coverslip. Microarray slides were placed in hybridization chambers and incubated for 10-12 hours in a water bath at 42°C. Slides were then washed with SSC×2/SDS 0.05%, SSC×1 and SSC×0.1, and scanned with an Axon Instruments Scanner 4000B (Molecular Devices, USA). Data were collected using Axon Instruments GenePix® Pro 5.1 program (Molecular Devices, USA).

Data normalization and treatment-control spots differentials

The data normalization was performed according to Bolstad's quantile procedure for oligonucleotide arrays [7]. Since a significant number of array spots were either control or empty spots, the distribution of high and low biological signals is essentially mixed with the control and "empty signal" populations. Such a mixture may bias the position of the "real", experimental signals in the cumulative distribution of the hybridization. Therefore, control and empty spots were filtered out before normalization. In addition, in cDNA-

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spotted arrays, variations in amount of probe printed on the glass slide necessarily introduce signal variations across hybridizations. Therefore, the normalization of the signals from the two channels was hybridization-specific. Namely, per hybridization, the cumulative distributions of Cy3 and Cy5 log-signals were aligned using a quantile normalization [7] method with Cy3 (control sample) as the reference. The normalization procedure yielded per spot Cy3/Cy5 log-differentials (natural logarithm of the signal ratio). The significance of the experiment-control log-differential was determined by a t-test. Spot-specific standard deviations (SD) were estimated according to pooled intra-replicate group variability of log-differentials of the spot across all hybridizations. The rational for such pooling is as follows: we may assume that the majority of genes (spots) are not induced by the experimental conditions (or treatment). Therefore, the intra-replicate variability of differentials for these spots reflects a noise component of the microarray, factorized by the sequence specificity of the spot-printed material. Apparently, the after-normalization noise component of the microarray is independent of the nature of hybridized samples. In the case of treatment-induced signals, the intra-replicate-group variability is higher than the microarray noise component due to higher differentials. Therefore, if the replication group-specific SD estimations are used, or SD estimations calculated according to hybridizations of the given nature, then the significance of the differential will be underestimated. Thus, the pooling of all replicate-groups diminishes the SD, making it more microarray noise-like, as the influence of the given spot by the treatment is not expected to occur for all replication-groups or not for all treatment-nature types.

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