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The role of genomic duplications in adaptive evolution

תפקידן של כפילויות גנומיות בהסתגלות אבולוציונית

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1. List of abbreviations

WT = wild-type.
Chr = chromosome.
ORF = open reading frame.
HSP = heat shock protein.
Arg = arginine.
Ser = serine.
Ura = uracil.
VHL = the human gene von Hippel-Lindau.
TSG = tumor suppressor gene.

2. Thesis Overview

Experimental evolution of microorganisms, performed in laboratory conditions, provides a unique opportunity for "real-time" inspection of evolutionary processes. Obtaining an evolutionary log of a microbial population adapting to a new challenge can be achieved by frequent sampling along the course of the evolutionary process.

I studied the evolutionary dynamics of adaptation by experimental evolution preformed on the budding yeast *Saccharomyces cerevisiae*. Here I conducted experimental evolution by serial dilutions, under well defined selective pressures. In each evolution experiment, phenotypic measurements comparing the ancestral strain to the evolving population are regularly preformed until a significant phenotypic improvement is detected in the evolved population. Afterwards, a characterization of the underlying molecular mechanism is being pursued, mainly by gene-expression measurements and genome sequencing.

In this work, I aimed to use experimental evolution not only to study dynamics of evolutionary processes, but also as a tool that can provide novel biological insights on genomic features like stress genes (Yona et. al *PNAS* 2012) or the translation machinery (Yona et. al *eLife* 2013). A common experimental approach to learn about genomic features is to introduce a perturbation, e.g. gene deletion, and then look for the phenotypic change caused by the deletion (c.f. Giaever et. al. 2002). Here, by using experimental evolution, we start by selecting for a desired phenotypic change, and then we look for the genetic alterations that underlie adaptation. In this way, the linkage between genotype to phenotype is predominantly driven by the detection of the desired

phenotype without an a priori focus on particular genomic loci. In this manner, I perused two lines of research, each consists of a different set of evolutionary experiments: first, I evolved yeast cells under environmental stresses in order to characterize the role of genome structure changes (like large-scale genomic duplications), in comparison to changes at the level of specific genes. I aimed to study the interplay between these two adaptation means in different regimes of selective pressures. When I evolved yeast populations under constant heat, I found that adaptation was repeatedly based on expression changes that involved hundreds, which was the result of chromosomal duplications (aneuploidy). The most common of these duplications was the duplication of chromosome III which occurred in all evolved repetitions. However, parallel evolution experiments in which heat was applied in a gradual manner during evolution rather than abruptly from normal to high temperature, showed adaptations that were not based on genomic duplications, and were characterized by expression changes that affected a much smaller set of genes with comparison to chromosomal-duplication based adaptations. Moreover, the gradual adaptation to heat resulted in better heat-tolerance, compared to the abrupt adaptation regime. Thus, we suggest that adaptation to constant environmental challenge that occurs abruptly may lead to fixation of gross genomic solutions like aneuploidy (non canonical chromosome number). On the one hand, aneuploidy-based solutions were recurrently fixed in our stress-evolved populations, and were proven to be advantageous under the stress. On the other hand, we demonstrated the substantial burden that is known to be associated with aneuploidy (Torres et. al. 2008). This dual nature of aneuploidy is at the center of an ongoing scientific debate that was coined “the aneuploidy paradox” (Sheltzer et. al. 2011) or “Dr. Jekyll and Mr. Hyde” (Pavelka et. al. 2010). Here, we aimed to solve this question by following long-term evolution experiments under stressful conditions. Although it was appreciated already that aneuploidy is an available and often adopted evolutionary solution to stress that is used to elevate the expression of genes needed for increased survivability (c.f. Hughes et. al. 2000), our study showed that such adaptations are surprisingly only transient evolutionarily. We showed that these chromosomal duplications are quickly reversed when the stress is relieved. Moreover, by following the aneuploidic cells for extended evolutionary time under the stress, we further found that aneuploidy is eliminated and replaced by more efficient focal solutions at the individual gene level. We thus concluded that chromosomal duplications are a first evolutionary means to

retain survivability under strong and abrupt selective pressures, yet they merely serve as a crude “temporary-fix", while more refined and sustainable solutions take over.

In the second set of evolutionary experiments, I focused on the translation machinery of the cell and in particular on the tRNA genes. The motivation to study the tRNA genes was driven by the multi-copy architecture of the tRNA genes. In the budding yeast *S. cerevisiae* each of the 42 different tRNAs is expressed from a gene-family of duplicated gene copies (up to 16 copies) that are scattered in the genome. This duplicated genes architecture, which is common to the vast majority of species known to date, suggests a potential evolutionary advantage, which we aim to study. Previous studies observed that highly expressed genes tend to use abundant codons that correspond to high gene-copy number tRNAs (c.f. Dos Reis et. al. 2004). This correspondence, termed here as the translational balance, is important for efficient protein synthesis in the cell. Yet, little is known on how this translational balance is restored once the organism migrates to a new ecological niche, in which the repertoire of highly expressed genes is modified. To tackle these questions, we deleted a single-tRNA gene that is found in a single copy in the yeast genome. This deletion interrupted the demand-to-supply balance in the cell and compromised growth. Surprisingly, evolution experiments performed on the deleted strain rapidly restored the translational balance by mutations in other tRNA genes that mutated their anticodon to become identical to that of the deleted tRNA. We also found evidence for this kind of mutations, in which tRNA genes mutated their anticodon, in a variety of contemporary genomic sequences. The applicability of anticodon mutation as means to rapidly alter the tRNA pool can be mainly attributed to the multi-gene-family architecture that characterize tRNA genes. One tRNA gene can transform to perform like another tRNA, while the other family copies remain functional and maintain the tRNA pool stability. We also observed a selective pressure that has a potential role in shaping the sizes of tRNA gene families. In particular, we demonstrate that artificial increase of tRNA gene copy number has a subtle effect on high-copy tRNA families, but a deleterious effect in case of low-copy tRNA families, where increase in tRNA dosage results in proteotoxic stress. Hence, we demonstrate how tRNA genes adapt to meet translational demand on the one hand while maintaining a balanced gene family size on the other.

An overview of the various evolution experiments presented in this work elucidate the role of genomic duplications in genome evolution. First, large-scale duplications provide the cell with a rapid means to boost-up expression on needed genes under harsh physiological conditions. Such gross genomic solutions, based on duplications, are especially beneficial under fluctuating environments as the duplications can also be rapidly eliminated when the stress is relieved. Nonetheless, also when the stress persists, genomic duplications allow the cell to acquire stress tolerance that sustains proliferation until more efficient solutions emerge. In the translation machinery as well, the tRNA pool architecture, in which many tRNA gene exists as gene families with several copies scattered throughout the genome, allowed the duplicated copies not only to dictate the composition of the tRNA pool, but also to enable the rapid recovery observed in our evolution experiments. The fact that the 42 different tRNA genes are found in a total number of 247 copies enhances their ability to rapidly mutate from one tRNA to another. This makes the translation system highly evolvable and thus susceptible for alterations that can meet new translational demands.

3. The Evolutionary Path of Environmental Stress Adaptations

3.1 Abstract

Aneuploidy, an abnormal number of chromosomes, is a wide spread phenomenon found in unicellulars such as yeast, as well as in plants and in mammalians, especially in cancer. Aneuploidy is a genome-scale aberration that imposes a severe burden on the cell, yet, under stressful conditions, specific aneuploidies confer a selective advantage. This dual nature of aneuploidy raises the question of whether it can serve as a stable and sustainable evolutionary adaptation. To clarify this we conducted a set of lab-evolution experiments in yeast and followed the long-term dynamics of aneuploidy under diverse conditions. Here we show that chromosomal duplications are acquired first as a crude adaptation to stress, but are only transient solutions that are eliminated and replaced by more efficient solutions obtained at the individual gene level. This transient dynamics of aneuploidy was repeatedly observed in our lab-evolution experiments: chromosomal duplications gained under stress were eliminated not only when the stress was relieved, but even if it persisted. Furthermore, when stress was applied gradually rather than abruptly, alternative solutions appear to have emerged, but not aneuploidy. Our findings indicate that chromosomal duplication is a first evolutionary line of defense, that retains survivability under strong and abrupt selective pressures, yet it merely serves as a "quick-fix" while more refined and sustainable solutions take over. Thus, in the perspective of genome evolution trajectory, aneuploidy is a useful, yet short-lived intermediate that facilitates further adaptation.

3.2 Introduction

Adaptation to stressful conditions often requires modified expression of certain genes that can generally be obtained by genome sequence changes. In addition, structural re-arrangements of the genome, and in particular chromosomal duplications, that lead to aneuploidy, may offer a simple means to boost expression level [1,2]. Indeed, cancer cells often exercise massive genomic duplications, particularly of regions that harbor growth-promoting genes [3]. Unicellular organisms too, can duplicate chromosomes that contain genes needed at a given condition [4–9]. The high prevalence of chromosomal duplications, especially under stress [10], accounts for the frequent acquisition of aneuploidies on a short evolutionary time-scale [11]. Chromosomal duplication may indeed offer the advantage of simultaneous elevation of a large set of genes, some of which may be beneficial under a particular selective pressure. Whole genome duplications too can offer selective advantage under specific conditions [12], yet genome analysis has suggested that they also survive only under specific conditions [13]. Indeed, whole genome and chromosomal duplication constitute crude solutions with significant overheads on the cell that are in part associated with increased copies of DNA, RNA and proteins [1,2]. Duplication of particular chromosomes (i.e. aneuploidy) creates in addition a stoichiometric imbalance between genes' products [14,15] and promotes further genome-destabilizing events [16,17]. Thus, it appears that aneuploidy is at the same time advantageous and highly costly [18,19]. Therefore, it is not clear under what conditions and to what extent organisms will adopt this solution. Here we offer an explanation of the dual nature of aneuploidy, in the form of dynamic and prolonged lab-evolution experiments in yeast. We show that aneuploidy gained rapidly under stress is a transient solution, subsequently replaced by focal, refined, and sustainable solutions that require more time to evolve.

3.3 Results

Towards evolving stress-tolerant yeast strains, we applied the well established methodology of lab-evolution through serial dilution [20]. In this procedure, populations of yeast cells are grown under a certain condition and are diluted daily into fresh medium, still under the same condition. Our lab-evolution experiments all together create a highly branched evolutionary scheme (Fig. 1).

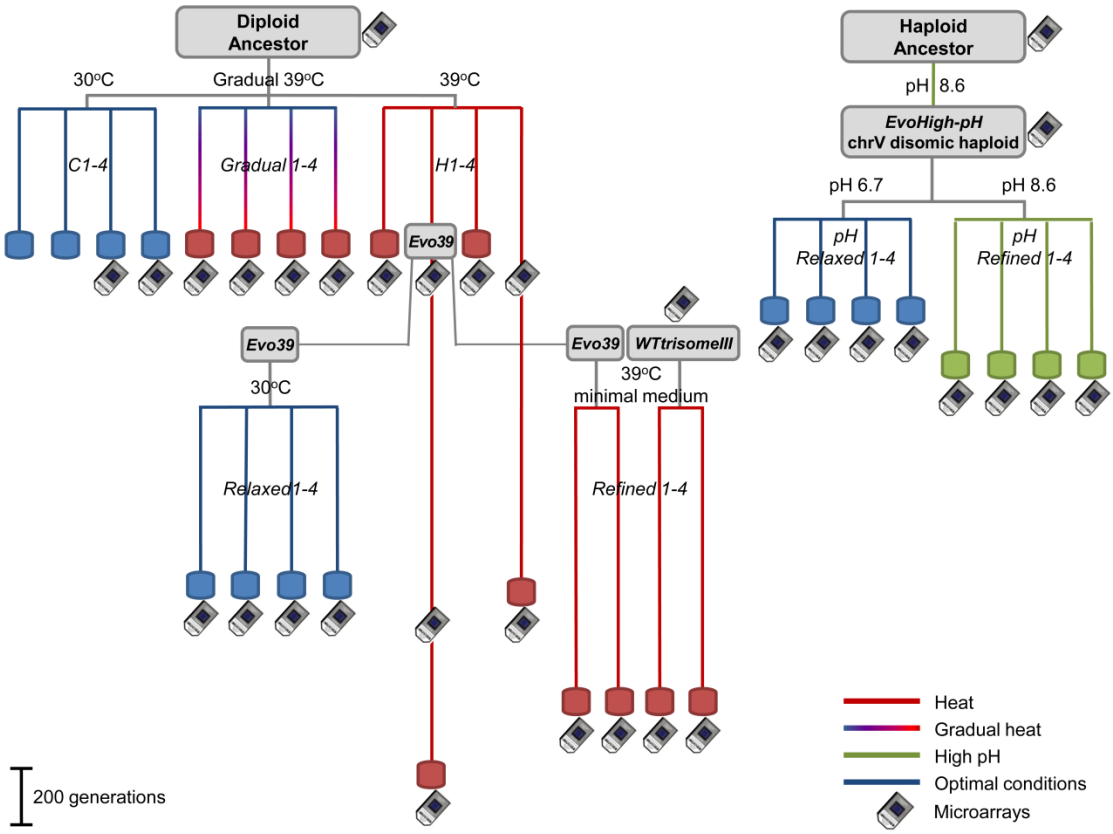

Figure 1. **Lab-evolution tree describing the experimental outline.** Each evolution experiment starts with an ancestor strain (grey box) that was subjected to certain growth conditions: high temperature, 39°C (red), permissive temperature, 30°C (blue), gradually increasing temperature from 30°C to 39°C (gradient line) and high pH=8.6 (green). Parallel lines splitting from the same branch represent independent repetitions and their length is in scale with the number of generations under the specified condition. *Evo39* strain was taken after 450 generations on high temperature (39°C) as an ancestor for another two evolutionary branches (*Refined1-4* and *Relaxed1-4*). Microarrays icons represent points during the evolution tree in which such measurement was performed.

3.3.1 Acquisition and subsequent elimination of chromosome III trisomy during adaptation to heat. We started with diploid *Saccharomyces cerevisiae* cells that were grown, in four independent repetitions, in rich medium under a constant heat stress of 39^{o}C. After an evolution period of 450 generations, all populations were examined, and duplication of chromosome III (trisomy) was detected in all four repetitions (Fig. 2). Two additional segmental duplications occurred in two repetitions on chromosomes IV and XII. We focused on the two populations in which only chromosome III was duplicated, as they serve as a good model for single acquired aneuploidy, and further evolved them under the same heat stress. Strikingly, we observed elimination chromosome III trisomy within 2350 generations (Fig. 2). We then turned to carefully study the dynamics of evolutionary gain and loss of aneuploidies under various growth conditions in which we vary temperature, nutrients availability and pH.

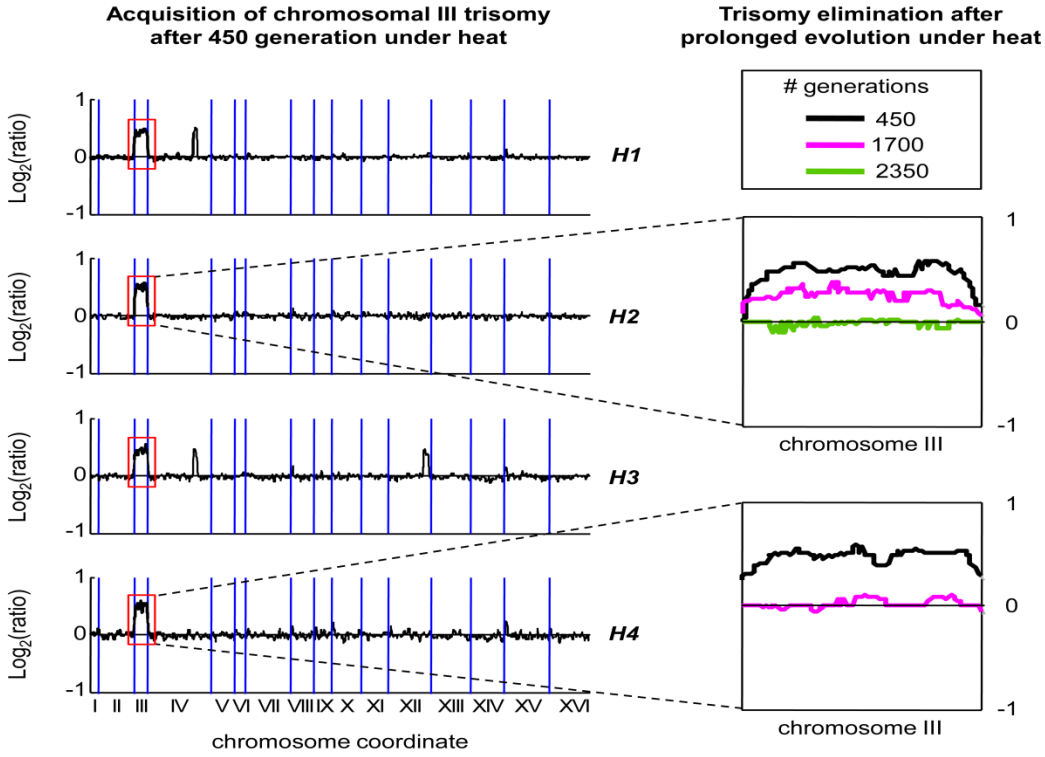

Figure 2. **Aneuploidy appears and subsequently eliminated during evolutionary adaptation to heat.** Four independent repetitions (*H1-4*) that evolved for 450 generations in rich medium and heat (39^{o}C) show chromosomal duplications (black lines). Notably, duplication of chromosome III occurred in all four repetitions. *H2* and *H4* that carry no large-scale duplication other than chromosome III trisomy were further evolved under the same conditions and after 1700 generations (magenta lines) and 2350 generations (green line) the trisomy was eliminated. All lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of heat evolved strain over a diploid wild-type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes.

3.3.2 A causal link between chromosome III copy number and heat tolerance

We began by determining whether the trisomy was selected for its direct contribution to heat tolerance, or whether it was merely hitchhiking on another beneficial mutation and thus was lost further along the evolution under heat. To that end, strains that carry chromosome III aneuploidies (without evolution under heat) were obtained and measured for their heat tolerance. One strain is a diploid with a trisomy of chromosome III, termed here *WTtrisomeIII* and the other is a diploid with a monosomy of chromosome III, termed *WTmonosomeIII*. Interestingly, *WTtrisomeIII* showed elevated heat tolerance at a level that is similar to that of the trisomic strains that evolved under heat. On the other hand, *WTmonosomeIII* exhibited lower heat tolerance than the diploid wild-type (Fig. 3*A*). These findings show a clear quantitative correspondence between the number of copies of chromosome III and heat tolerance.

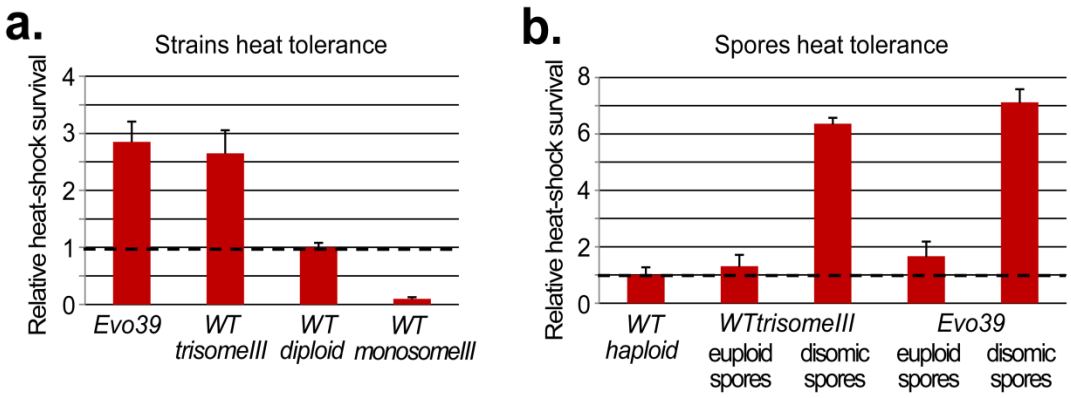

Figure 3. **An extra copy of chromosome III is beneficial under heat. (A)** Heat-shock tolerance rates are proportional to the copy number of chromosome III. Heat-shock survival fold-change of chromosome III aneuploidic strains compared to a diploid wild-type (dashed line). **(B)** The extra copy of chromosome III is the predominant genetic trait responsible for the increased heat tolerance. Heat-shock survival fold-change of spores from *WTtrisomeIII* and *evo39*, compared to a haploid wild-type (dashed line), presented separately for euploid spores and for chromosome III disomic spores (p-value < $8*10^{-5}$, for spores karyotype see Fig. 4).

To confirm that the extra chromosome is the predominant genetic change underlying the heat tolerance, tetrad analysis was performed on haploid cells of both a strain that evolved at 39^{o}C for 450 generation and became trisomic (*H2* after 450 generations, abbreviated as *evo39*) and on *WTtrisomeIII*. These two trisomic strains were subjected to meiosis and the heat tolerance of the haploid progeny (spores) was measured. The results showed that all euploid spores (normal number of chromosomes) have heat

tolerance similar to a haploid, while all disomic spores, which carry two copies of chromosome III (Fig. 4), have a marked increase in heat tolerance (Fig. 3*B*).

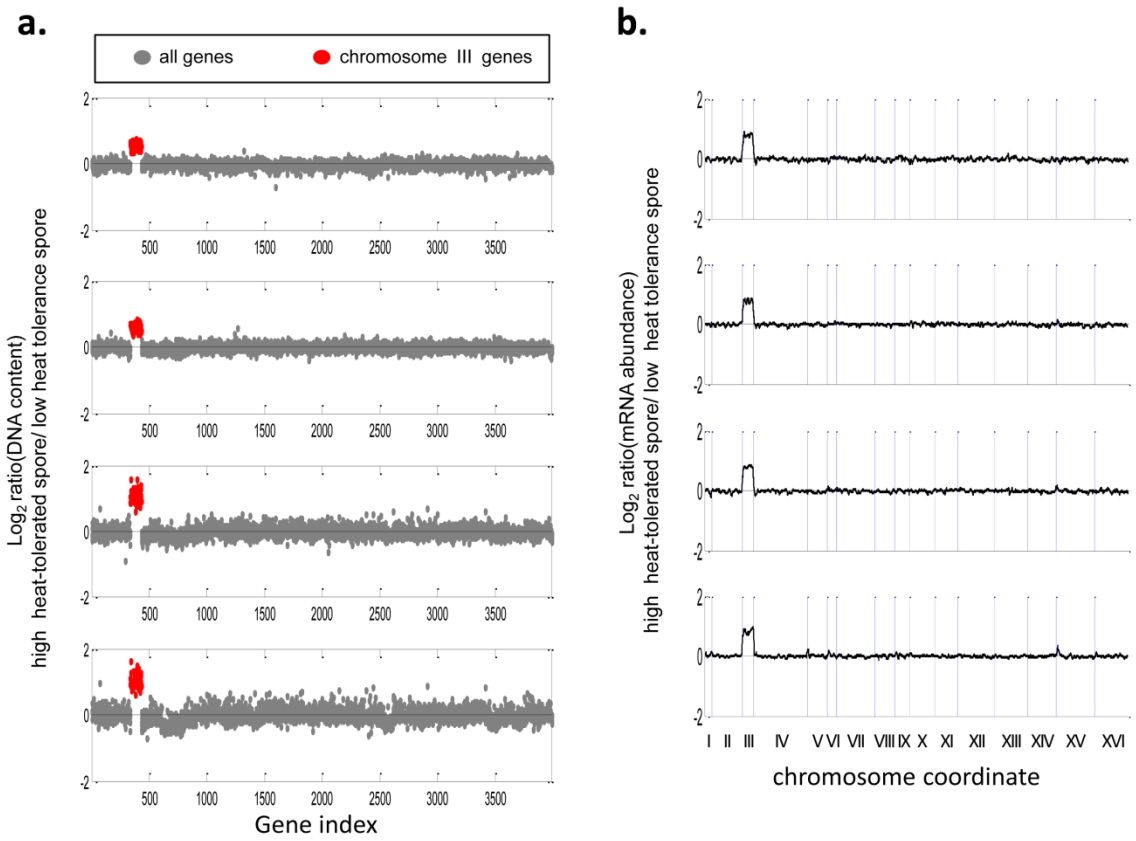

Figure 4 – Determination of extra chromosome III segregation in *evo39* and *WTtrisomeIII* spores. **a,** *WTtrisomeIII* was subjected to tetrad analysis and the heat tolerance of eight spores from two tetrads was measured. The spores were divided to pairs according to their heat tolerance; each pair consists of one spore that showed increased heat tolerance and one spore with a heat tolerance level similar to that of a haploid wild-type. Dots represent $\log_2$ intensity ratios of DNA copy number of increased heat tolerance spore over wild-type like heat tolerance spore, aligned according to chromosomal order, where red dots represent genes from chromosome III and grey from all other chromosomes. **b,** *Evo39* was subjected to tetrad analysis and the heat tolerance of eight spores from two tetrads was measured. The spores were divided to pairs according to their heat tolerance; each pair consists of one spore that showed increased heat tolerance and one spore with a heat tolerance level similar to that of the wild-type. Dots represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of increased heat tolerance spore over wild-type like heat tolerance spore, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes.

We further examined whether chromosome III trisomy confers advantage under other stress conditions by measuring the tolerance of *WTtrisomeIII* to a battery of other stresses. We found that duplication of chromosome III decreased the tolerance to all of the other stresses tested (Fig. 5).

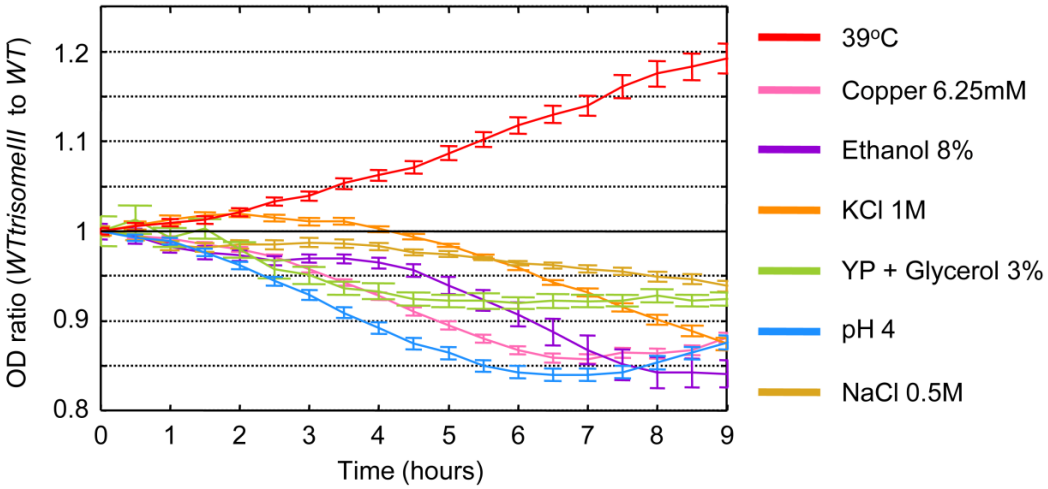

Figure 5. **An extra copy of chromosome III is beneficial under heat, yet it is costly and maladaptive under other conditions.** The growth advantage conferred by chromosome III trisomy under heat (red line) cannot be attributed to a general stress tolerance. The colored lines represent OD ratios of *WTtrisomeIII* over a diploid wild-type during continuous growth under various stresses.

Therefore, increased heat tolerance cannot be attributed to a general stress tolerance conferred by chromosome III trisomy. Together, these results establish a causal link between the relative number of chromosome III copies and heat tolerance, and may explain why this chromosomal duplication was repeatedly fixated in all lab-evolution experimental lines under heat.

3.3.3 The cost associated with chromosome III trisomy. An additional copy of chromosome III thus clearly contributes to the heat tolerance, yet its elimination later on raises the hypothesis that the substantial burden associated with chromosomal duplications [1] prevents the trisomy from serving as a sustainable solution to the stress. Therefore, a next goal was to assess the costs associated with the chromosome III trisomy and to characterize the role of these costs in the trisomy elimination. We reasoned that such costs can be measured when neutralizing the benefit, i.e. when heat is

not applied. For that reason, the cost of chromosome III trisomy was measured in terms of growth defect by comparing *WTtrisomeIII* to a diploid wild-type, at the permissive temperature (30°C). A considerable cost of the trisomy was detected in rich medium and a further cost increase was detected in minimal medium (Fig. 6 & Fig. 7).

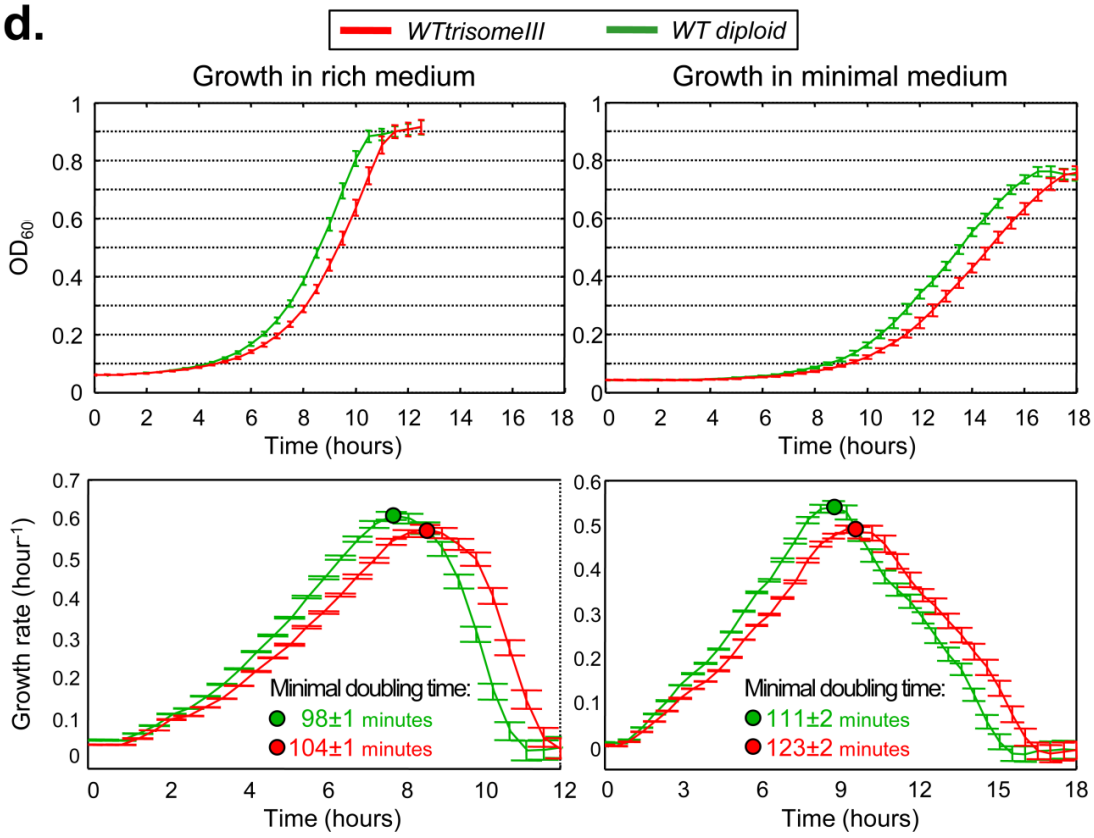

Figure 6. **The cost of carrying an extra copy of chromosome III.** The cost of chromosome III trisomy at permissive temperature (30°C) is increased on minimal medium compared to rich medium. Growth curve measurements of *WTtrisomeIII* (red) and of a diploid wild-type (green) are shown in OD values over time during continuous growth at 30°C (upper boxes), in rich medium (left) and in minimal medium (right). Lower boxes show growth rate analyses, derived from the OD values, and the differences in minimal doubling time between *WTtrisomeIII* and *WT*.

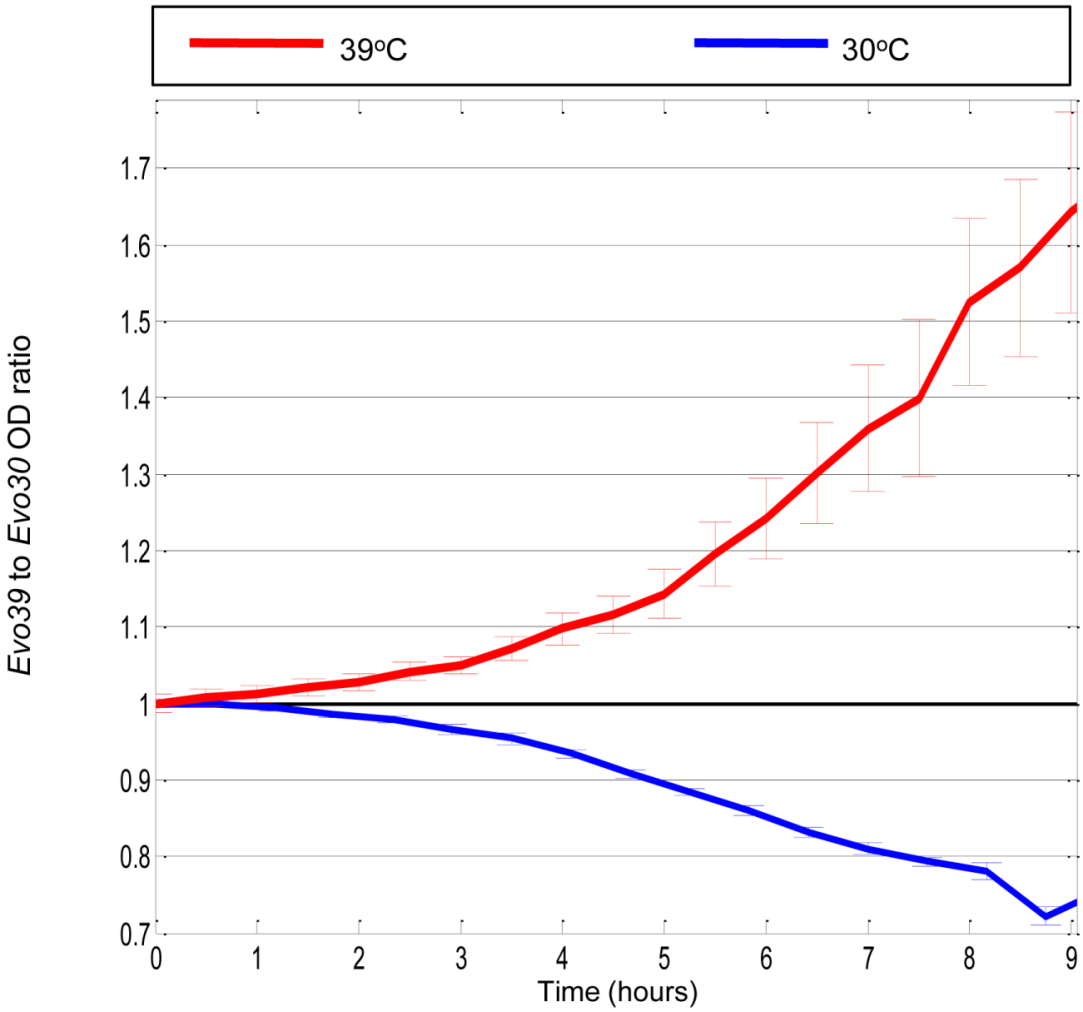

Figure 7 - The benefit and cost of chromosome III trisomy. *Evo39*, a strain that evolved 450 generations under heat and gained an extra copy of chromosome III, is growing better under heat than *evo30*, a strain that evolved at permissive temperature for the same number of generations and remained euploid. The results are reversed when measuring growth at 30°C, when heat is not applied the extra copy of chromosome III decrease the growth of *evo39* compared to the euploid *evo30*. Values represent OD ratios of *evo39* over *evo30* measured during continuous growth at 39°C (red) and at 30°C (blue). Data are presented as mean and s.e.m.

3.3.4 Optimized adaptations replace the aneuploidy-based solution. Due to the increased cost of the trisomy in minimal medium, we hypothesized that during prolonged evolution in minimal medium and heat, the trisomy would be eliminated more rapidly compared to rich medium and heat. Following this hypothesis, an additional evolution experiment was carried out. We started with two ancestral strains that carry chromosome III trisomy, namely *evo39* and *WTtrisomeIII*. Interestingly, after 1000

generations under heat and minimal medium all four repetitions (termed *Refined 1-4*) from both ancestral strains eliminated the extra copy of chromosome III (Fig. 8).

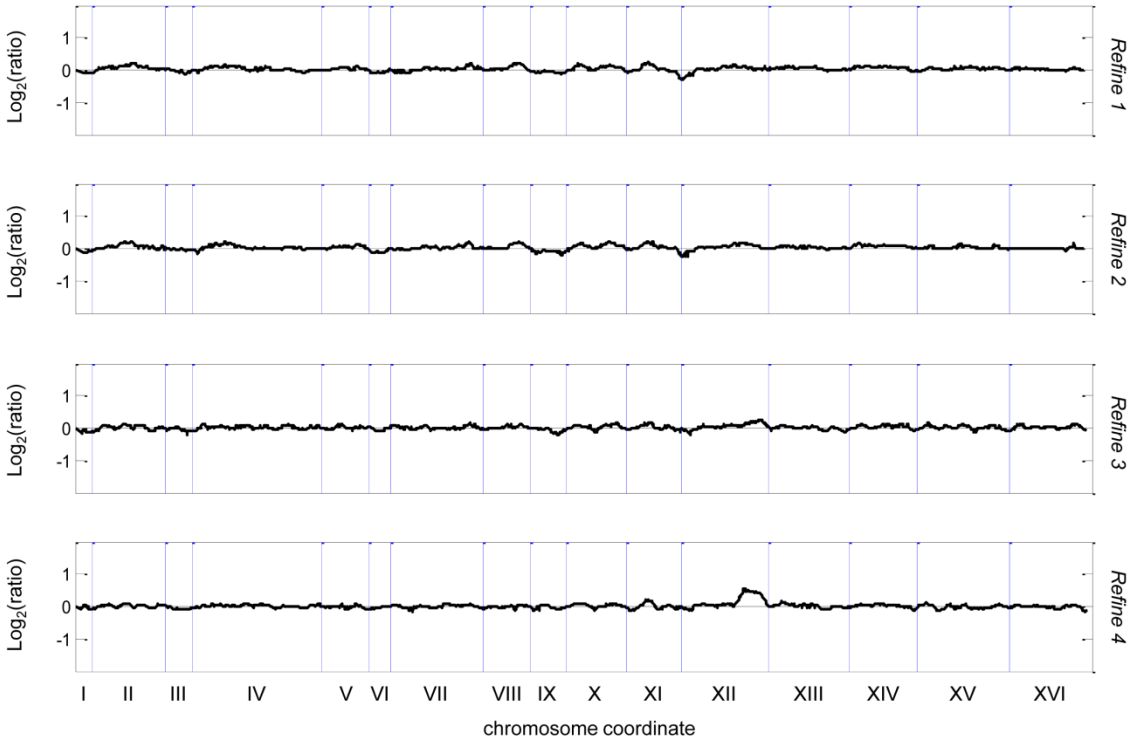

Figure 8 – Chromosome III trisomic strains further evolved under heat eliminated the trisomy. Four independent repetitions, descendants of *evo39* (*Refined1-2*) and *WTtrisomeIII* (*Refined3-4*), were further evolved for 1000 generations under heat (39^{o}C) and minimal medium. All lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of heat evolved strain over a diploid wild-type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. While chromosome III trisomy was eliminated in all four repetitions, in *Refined4* it appears that a subpopulation also duplicated a segment in chromosome XII. Interestingly, *Refined4* shows the lowest improvement under heat and the lowest cost reduction compared to the other *Refined1-3*(Fig. 9).

These evolutionarily refined strains, which have eliminated the cost associated with the trisomy, exhibit improved growth both at the permissive and the high temperatures compared to their trisomic ancestors (Fig. 9). The rapid elimination in minimal medium compared to rich medium shows that the high cost of the trisomy accelerates the evolutionary dynamics of its elimination.

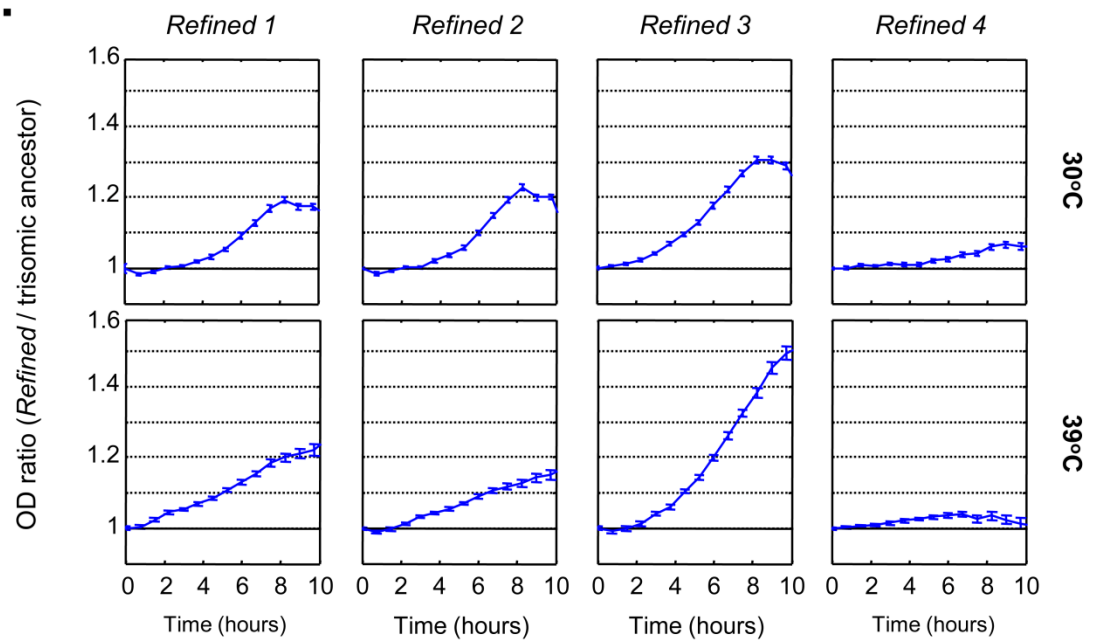

Figure 9. **The aneuploidy-based adaptation was evolutionarily eliminated and replaced by more economical solutions.** Descendants of chromosome III trisomic ancestors that were further evolved under heat and lost the trisomy (*Refined 1-4*) show improved growth under heat, yet with less cost compared to their trisomic ancestors. Each sub-graph shows the OD ratios of a refined descendant over its trisomic ancestor measured during continuous growth at 30°C (upper graphs) and at 39°C (lower graphs).

Focusing on these evolutionary refined populations, it is expected that the original contribution of trisomy to the heat tolerance was replaced by alternative solutions. We hypothesize that such refined solutions could take over the trisomy by conferring heat tolerance, while avoiding much of the cost associated with aneuploidy. In order to characterize the refined solutions, we examined the gene expression of the newly evolved refined strains from the different repetitions. A subset of 17 genes on chromosome III was found to retain elevated expression despite the elimination of the extra chromosome copy (Fig. 10).

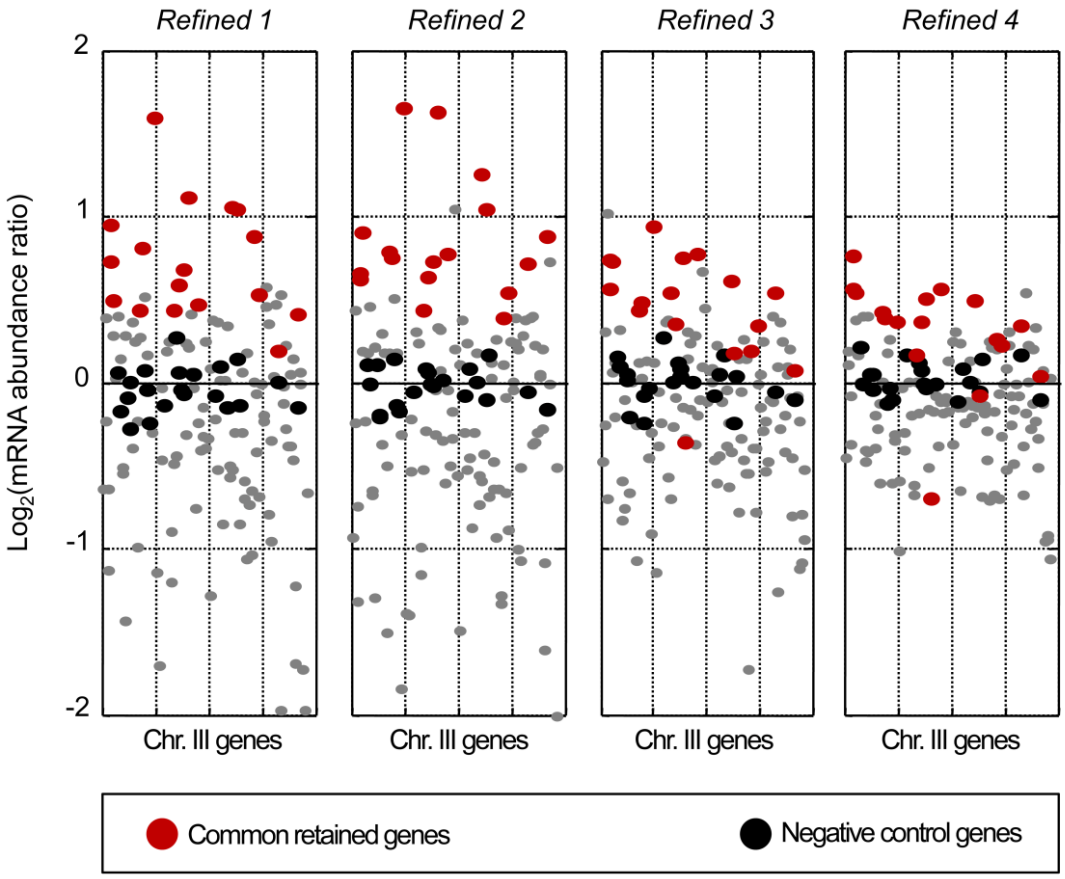

Figure 10. **The aneuploidy-based adaptation was evolutionarily eliminated and replaced by more economical solutions based on refined gene expression adaptations.** Despite elimination of the trisomy in *Refined 1-4*, a group of genes from chromosome III retained high expression levels. Dots represent $\log_2$ ratios of mRNA abundance of chromosome III genes over a diploid wild-type. Genes that retain high expression (in at least 3 out of the 4 refined evolutions) are marked in red, and from the majority of genes that went back to wild-type like expression (grey dots) a control group was selected and marked in black (used in panel c). These retained genes were also up-regulated in *H2* and *H4* that also eliminated the trisomy (χ^2 p-values $4*10^{-5}$ and $3*10^{-2}$).

Next, we checked whether these genes can contribute to the heat tolerance when introduced to a wild-type strain, one at a time. To this end, a diploid wild-type strain was transformed with centromeric plasmids [21], each containing one of these genes. As a negative control, 22 random genes from chromosome III that did not retain elevated expression were inserted in the same manner. Reassuringly, most of the genes that retained high expression demonstrated an increased heat tolerance in the transformed wild-type cells (Table 1*A*), with the highest contribution at 23.5% of the heat-tolerance of *WTtrisomeIII* (defined here as 100% heat tolerance) (Fig. 11). In contrast, none of the genes from the negative control set had a considerable effect on the wild-type heat tolerance (mean -2.4% ± 2.6%, Table 1*B*).

a.

ORF	Heat tolerance contribution	Gene Name
YCR065W	23.5 %	HCM1
YCR016W	19.9 %	Uncharacterized
YCR045C	19.3 %	RRT12
YCR102C	18.8 %	Uncharacterized
YCR071C	18.4 %	IMG2
YCL005W-A	16.0 %	VMA9
YCL059C	13.6 %	KRR1
YCL035C	10.6 %	GRX1
YCR007C	8.3 %	Uncharacterized
YCL001W	7.2 %	RER1
YCL063W	6.4 %	VAC17
YCL036W	3.7 %	GFD2
YCR003W	2.6 %	MRPL32
YCR087C-A	0.9 %	LUG1
YCR043C	0 %	Uncharacterized
YCL061C	-1.7 %	MRC1
YCL026C-B	-2.2 %	HBN1

b.

ORF	Heat tolerance contribution	Gene Name
YCL056C	-1.2 %	PEX34
YCL055W	-4.1 %	KAR4
YCL048W	-1.6 %	SPS22
YCL047C	-3.1 %	POF1
YCL045C	0.2 %	EMC1
YCL033C	0.3 %	MXR2
YCL032W	-1.9 %	STE50
YCL029C	-4.5 %	BIK1
YCL016C	1.8 %	DCC1
YCL004W	-0.5 %	PGS1
YCL001W-A	-3.1 %	Uncharacterized
YCL001W-B	1.5 %	Uncharacterized
YCR002C	-2.4 %	CDC10
YCR010C	-6.3 %	ADY2
YCR012W	-5.5 %	PGK1
YCR027C	0.4 %	RHB1
YCR031C	-5.0 %	RPS14A
YCR035C	-4.4 %	RRP43
YCR046C	1.3 %	IMG1
YCR047C	-5.2 %	BUD23
YCR086W	-4.4 %	CSM1
YCR101C	-5.4 %	Uncharacterized

Table 1 – Heat tolerance contribution of genes from chromosome III introduced separately into a diploid wild-type. **a,** Genes that retain elevated expression after the elimination of chromosome III trisomy in *Refined1-4* (see supplementary file for heat tolerance functional analysis of these genes). **b,** Genes that returned to wilt-type expression level after the elimination of chromosome III trisomy in *Refined1-4*. Heat tolerance was measured by survival after 90 minutes exposure to 45^{o}C (see methods). The contribution of each gene was calculated by subtracting the tolerance of the wild-type with an empty plasmid and then dividing by the tolerance of *trisomeIII* (in order define wild-type contribution as 0% and *trisomeIII* as *100%*).

Note that the contribution of the individual genes adds up to more than 100% (defined as the tolerance of the trisomic strain). This probably reflects the fact that the trisomic level of heat-tolerance includes the considerable cost of aneuploidy, and may also suggest a negative epistasis between the contributions of the individual genes [22].

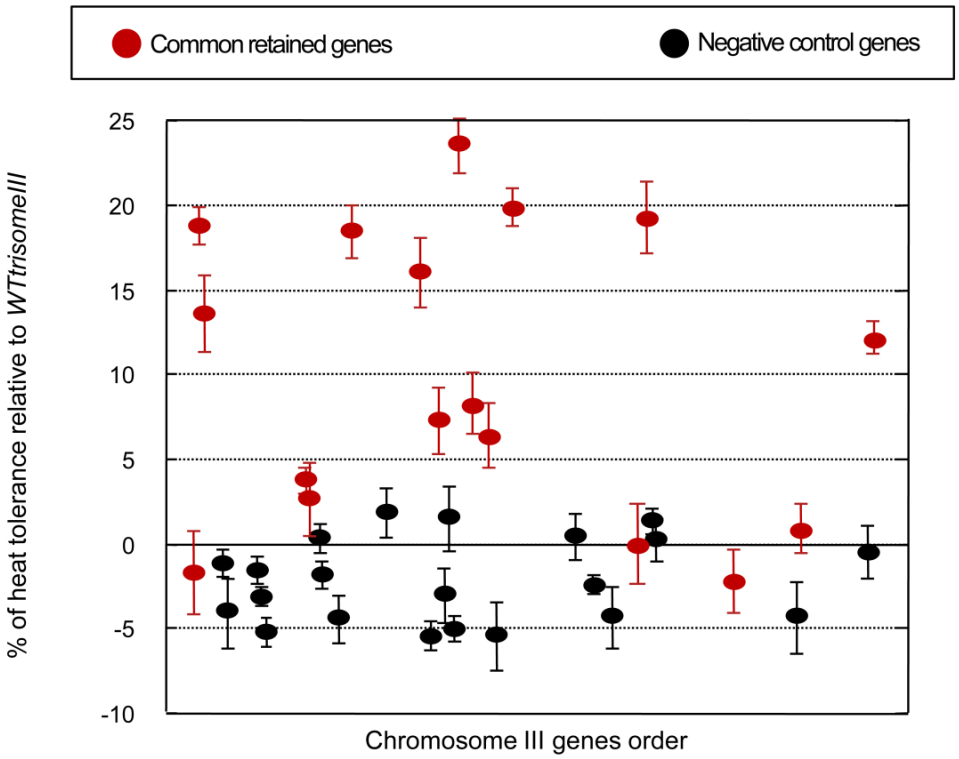

Figure 11. **The refined solution is comprised of gene expression upregulation of heat-tolerance contributing genes.** The group of genes that retain high expression levels, after the elimination of the trisomy, confers increased heat tolerance when introduced into wild-type. Each of the highly expressed genes (red) and of the negative control genes (black) was inserted into the diploid wild-type, on a centromeric plasmid, and heat-shock tolerance was compared to the heat tolerance of *WTtrisomeIII* (t-test p-value<$5*10^{-7}$, see methods).

3.3.5 Functional analysis of heat tolerances genes from chromosome III that retain high expression level after the elimination of chromosome III trisomy under heat

We have focused on the genes with the most substantial heat tolerance contribution when inserted singly into a wild-type cell (see Fig. 11). For each of these genes we looked for further evidence to support their substantial heat tolerance contribution. Reassuringly, we indeed find that YCR065W (*HCM1*) that had the highest heat tolerance contribution (23.5%) was reported in another study to confer increases heat tolerance when overexpressed in yeast [23]. For YCR045C (*RRT12*) that had 19.3% heat

tolerance contribution, we preformed a heat-shock expression profile and found an induction of more than 27-fold upon 45 minutes of heat-shock (42^{o}C). We preformed the same method for YCR071C (*IMG2*) that had 18.4% heat tolerance contribution and observed induction of 1.5-fold upon 90 minutes of heat-shock. For these two last genes we also found a study that reports heat sensitivity phenotype upon deletion [24]. Two of the five most heat contributing genes that we found on chromosome III are still uncharacterized ORFs in yeast, yet also they show high responsiveness to heat-shock. Interestingly, YCR016W (19.9% heat tolerance contribution) shows a fast strong repression upon 15 minutes of heat-shock that is followed by a growing induction as the heat persist and YCR102C (18.8% heat tolerance contribution) shows 3-fold induction upon 90 minutes of heat-shock.

Overall, these results indicate that a part of the solution to the heat challenge obtained by duplicating chromosome III was replaced during the extended evolutionary period under heat by solutions based on refined gene-expression alterations on chromosome III (and on other chromosomes, as mentioned below).

Genes on other chromosomes may have also evolved changes in expression level after the elimination of the extra copy of chromosome III, and it is most likely that they, too, must have contributed to the evolution of heat tolerance. The heat-shock proteins [25] (HSPs) that are scattered on 11 different chromosomes provide a particularly interesting example. We analyzed all 14 annotated, verified HSPs in the yeast genome [26] for changes in expression in the *evo39* strain in comparison to its descendants that eliminated the trisomy. Curiously, while the expression levels of these heat-shock genes changed very modestly after 450 generations of *evo39* strain during which the trisomy was fixated, most of these genes showed significant up-regulation in the refined descendants, i.e. after another 1000 generations under heat when the trisomy was eliminated (Fig. 12). Three of the 14 *HSP* genes did not show this trend, including *HSP78*, a mitochondrial heat-shock protein, and *HSP30*, which resides in the eliminated chromosome III. This indicates that the trisomy-based solution to heat does not require a concomitant up-regulation of the heat-shock genes, while the replacement of the trisomy is associated with an enhancement in the evolutionary expression of the heat shock genes. This scenario may thus imply that the duplication of a certain chromosome may

provide the evolutionary time-window needed for the population to search for solutions not only on the duplicated chromosome, but throughout the genome.

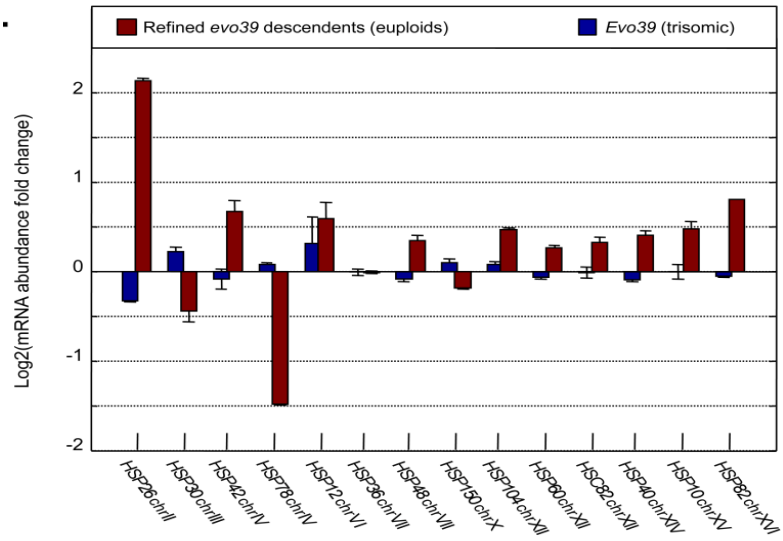

Figure 12. **The refined solution is also comprised of gene expression change of heat-shock genes scattered in other chromosomes.** The refined solution replacing the trisomy is characterized by changes in expression levels of most HSP genes. Log_2 expression ratios over wild-type are shown for all HSPs, for trisomic *evo39* (blue) and for its descendants that eliminated the trisomy (red). Data are presented as mean and s.e.m.

In order to characterize the genetic changes that replaced the trisomy and underlie the gene expression changes described above, we sequenced selected populations from the evolutionary tree (Figure 1) and found that many mutations occurred in stress related genes. Possibly, such trans-activation of stress activators can explain how the trisomy was replaced by coordinated changes in genes expression of heat-related genes, which are scattered throughout the yeast genome.

3.3.6 Elimination of the extra copy of chromosome III at permissive temperature

Another consequence that follows the above stated properties of acquired aneuploidy would be that when the stress is relieved, i.e. when the benefit diminishes and the cost remains, aneuploidy will be selected against [27]. In fact, continued evolution of *evo39* under the permissive temperature (30^{o}C) eliminates the extra copy of chromosome III after 600 generations, and with it, its tolerance to heat (Fig. 13). Reassuringly, the trisomy elimination in all four repetitions attests to its superfluous cost at permissive temperature, when it is no longer beneficial. This experiment also demonstrates the limited durability of aneuploidy-based solutions, as they are quickly eliminated when organisms evolve in the absence of the original stress.

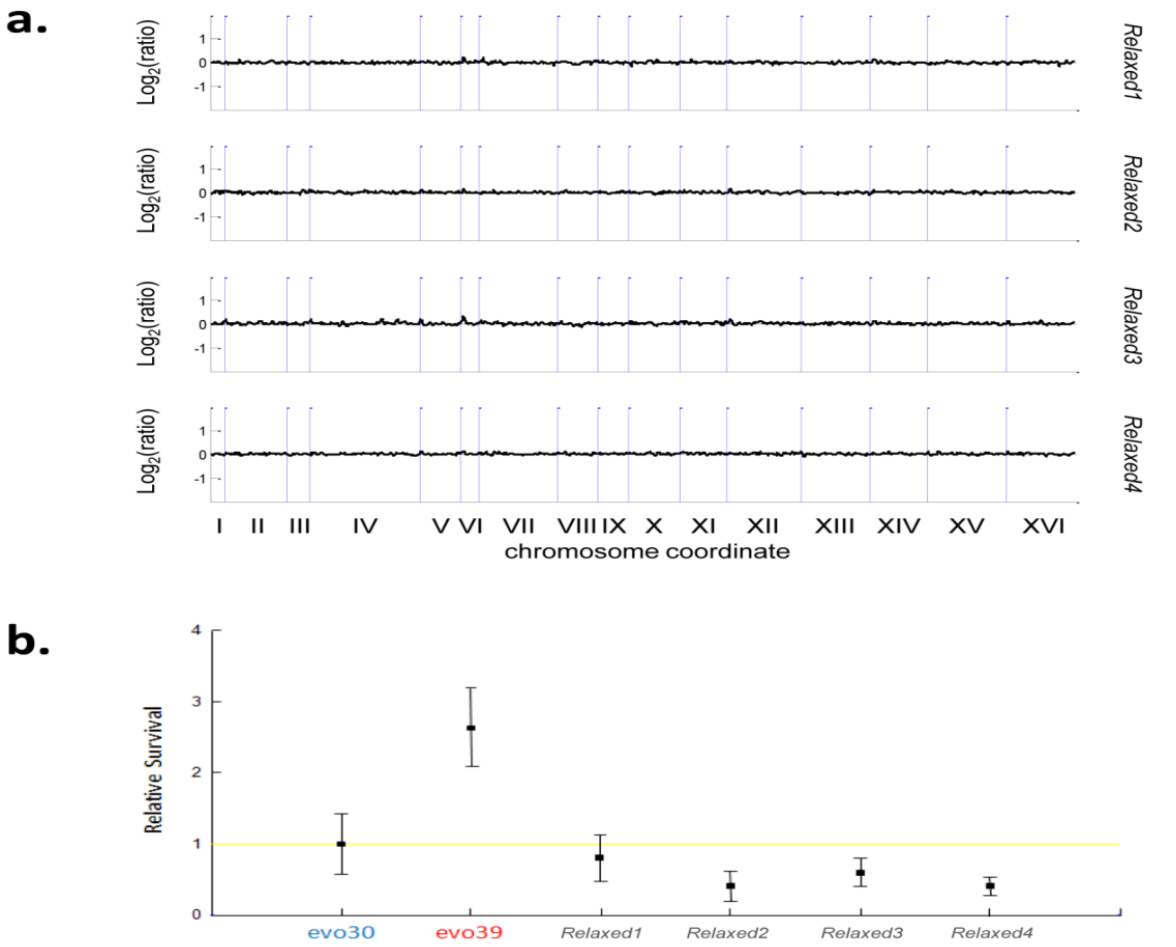

Figure 13 - Chromosome III trisomic *evo39* strain, further evolved at permissive temperature eliminated the trisomy and lost its increased heat tolerance. Four independent repetitions, descendants of *evo39*, were further evolved for 600 generations under 30°C and rich medium (defined as *Relaxed1-4*). **a,** Chromosome III trisomy was eliminated in all four repetitions. Black lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of permissive temperature evolved strain over a diploid wild-type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. **b,** Heat-shock tolerance rates decreased with the elimination of chromosome III trisomy. Shown is the heat-shock survival fold-change of chromosome III trisomic *evo39* and it's for descendants *Relaxed1-4*. The yellow line represents the heat-shock survival of *evo30* that was evolved in parallel to *evo39* but at 30°C and remained euploid. Data are presented as mean and s.e.m.

3.3.7 Evolution to gradually-applied heat avoids aneuploidy-based solutions

To this point, aneuploidy had been observed to evolve in response to an abrupt evolutionary challenge. We next hypothesized that an evolution experiment in which the stress is applied in a gradual manner might not select for chromosomal duplication, since under minor stress increments the considerable cost associated with aneuploidy might surpass the benefit at each increment of the stress. To explore this hypothesis, we evolved the same ancestral strain towards tolerance to heat, yet in increments of one degree per 50 generations (from 30°C up to 39°C). This evolutionary design resembles the "morbidostat" that was recently applied to evolution of drug resistance in bacteria [28]. Curiously, under this regime an extra copy of chromosome III was not gained after 450 generations in any of the four repetitions (termed *Gradual 1-4*) of the experiment (Fig. 14*A*). Nonetheless, the growth of these gradual populations was better at 39°C (and also at 30°C) than that of the four populations (*H1-4*) that acquired trisomy in the same evolutionary time under 39°C (Fig. 14*B*). This experiment indicates that gradual application of a stress alleviates the need to adopt the rapidly available yet highly costly solution of chromosomal duplication.

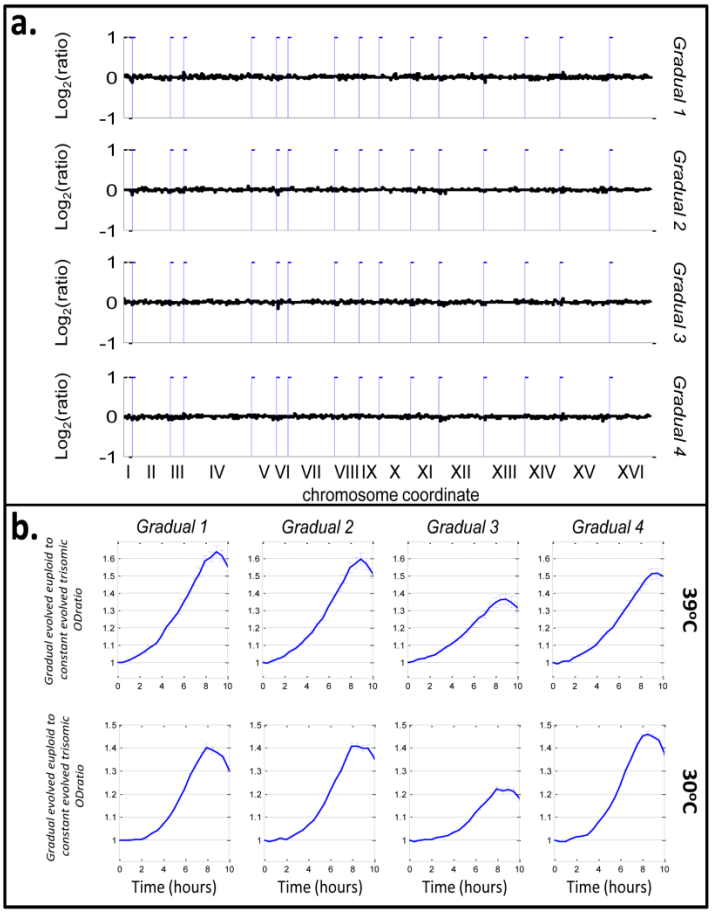

Figure 14 – Cells evolved under gradually applied heat do not adopt aneuploidic adaptations. The lab-evolution experiment in which four independent repetitions were evolved under 39°C and duplicated chromosome III (*H1-4*) was repeated under the same conditions but heat was applied in a gradual manner, in four independent repetitions (*Gradual 1-4*). For these strains evolution started at 30°C and every 50 generations the temperature was raised by 1°C. The total number on generations was 450, identical to the number of generations in the case of *H1-4*. **a,** All *Gradual 1-4* remained euploid, i.e. chromosome III trisomy was not detected in any of the four repetitions. Black lines represent $\log_2$ intensity ratios of mRNA abundance, calculated by a sliding window, of gradual-heat evolved strain over a diploid wild-type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. **b,** Comparing the growth of the euploid *Gradual 1-4* to the growth of the trisomic *H1-4* shows better growth of the gradual evolving strains both at 39°C and at 30°C. Each sub-graph shows the OD ratios of a one of the gradual strains over its non-gradual counterpart, measured during continuous growth at 39°C (upper graphs) and at 30°C (lower graphs).

Looking into the genetic changes that underlie the heat adaptation of the gradually evolved populations, we found two interesting mutations: (1) a mutation in the *HCM1* gene from chromosome III. This gene was one of the genes from chromosome III that retained high expression after the elimination of the trisomy. Strikingly, the *HCM1* gene was found to have the highest heat-tolerance contribution from this group of genes of chromosome III (see Table 1 and Figure 11). (2) an inactivating mutation of the *WHI2* gene that regulates the general stress response in yeast. The inactivating mutation in the form of a premature stop codon possibly lowered the stress response of the evolved cells and allowed them to exhibit high proliferation despite the heat stress that normally halts cellular proliferation.

3.3.8 Acquisition and subsequent elimination of chromosome V aneuploidy during adaptation to high-pH. To examine the generality of the findings obtained from evolution experiments under heat, we also examined evolutionary dynamics under an additional physiological stress of a constant high pH. For this we used an evolved strain from a previous experiment [29] in which a haploid *S.cerevisiae* was evolved under a constant high pH(8.6), using a similar serial transfer protocol. We examined the evolved strain, termed here *evoHigh-pH*, for potential chromosomal duplications. Interestingly, in addition to seven point mutations previously detected in the genome sequence of the strains [29] (Table 2*A*), we revealed that *evoHigh-pH* gained an extra copy of chromosome V (Fig. 15*A*). In order to show a direct contribution of the additional copy of chromosome V to the high pH tolerance, *evoHigh-pH* was crossed with its wild-type ancestor, sporulated, and the meiotic products (spores) were analyzed. Among 40 spores scanned, we identified a pair of spores that carried the exact same subset of mutations (Table 2*B*) and differed only in the copy number of chromosome V (one spore, an euploid with one copy of the chromosome, and the other, a disome with two copies, Fig. 15*A*). Reassuringly, the disomic spore showed better growth at high-pH (Fig. 15*B*). However, when growth was measured at normal pH (6.7), the disomic spore exhibited a reduced growth compared with the euploid spore (Fig. 15*B*). These results demonstrate a causal link between the extra copy of chromosome V and high pH tolerance.

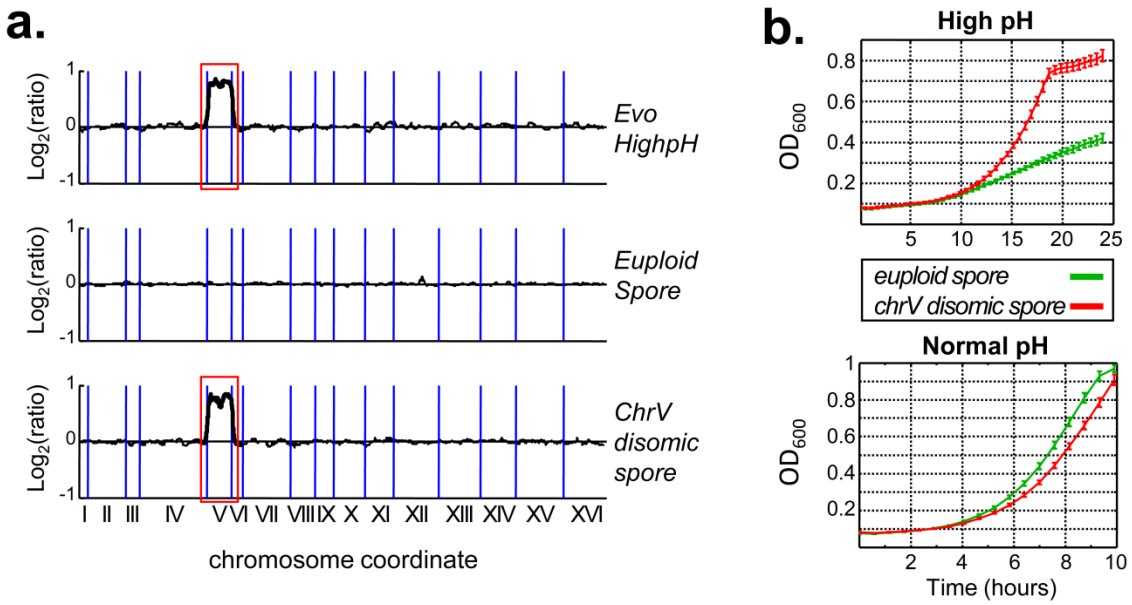

Figure 15. **Evolution on high pH selects for transient duplication of chromosome V which is later eliminated. (A)** Haploid wild-type that evolved under high pH[29] acquired chromosome V disomy (upper sub-graph). We identified two spores obtained from crossing the disomic evolved strain with a haploid wild-type. These spores carry the same mutations subset (Table 2*B*) but differ in the copy number of chromosome V (two lower sub-graphs). Black lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window over a haploid wild-type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. **(B)** An extra copy of chromosome V confers high-pH (8.6) tolerance but causes impaired growth on normal pH (6.7). Growth curves of disomic spore (red) and euploid spore (green) under high-pH (upper) and normal pH (lower).

a. Mutation in EvoHigh-pH:

Gene	Mutation	Function
GTT2/ MMP1	Converging 3'UTRs	Glutathione S-transferase / S-methylmethionine permease.
YFR057w	Base substitution at promoter	Unknown.
ECM21	Nonsense at codon 193	Ubiquitin-ligase adaptor.
NMD4 /YLR363w-a	Base substitution at promoter	Nonsense-mediated mRNA decay / Unknown.
GPI17	S63 to L	GPI-anchor transamidase.
YHR140w/ SPS100	Base substitution at promoter	Unknown / spore wall maturation.
MAC1	C271 to W	Copper-sensing transcription factor.

Table 2 – List of mutations in the high-pH strains. **a,** Mutations found in the sequencing of *evoHigh-pH* obtained from Romano *et al*. (27) **b,** The subset of mutation commonly found in two spores of *evoHigh-pH* – one euploid and another with an extra copy of chromosome V.

b. Mutation in EvoHigh-pH spores (euploid and disomic):

Gene	Mutation	Function
GTT2/ MMP1	Converging 3'UTRs	Glutathione S-transferase/ S-methylmethionine permease.
YFR057w	Base substitution at promoter	Unknown.
GPI17	S63 to L	GPI-anchor transamidase.
MAC1	C271 to W	Copper-sensing transcription factor.

We have further extended the analogy to the heat tolerance experiments to examine potential elimination of the extra copy of chromosome V under two evolutionary tracks: one in which *EvoHigh-pH* continue to evolve at high pH, and another in which the stress was relieved and *EvoHigh-pH* was switched to evolve under normal pH conditions (pH 6.7). In both tracks we detected gradual elimination of the extra copy of chromosome V from the evolving populations (Fig. 16 and Fig. 17). The fact that here too, aneuploidy is reversed even if the stress persists indicates that such large duplications do not typically serve as sustainable evolutionary solutions.

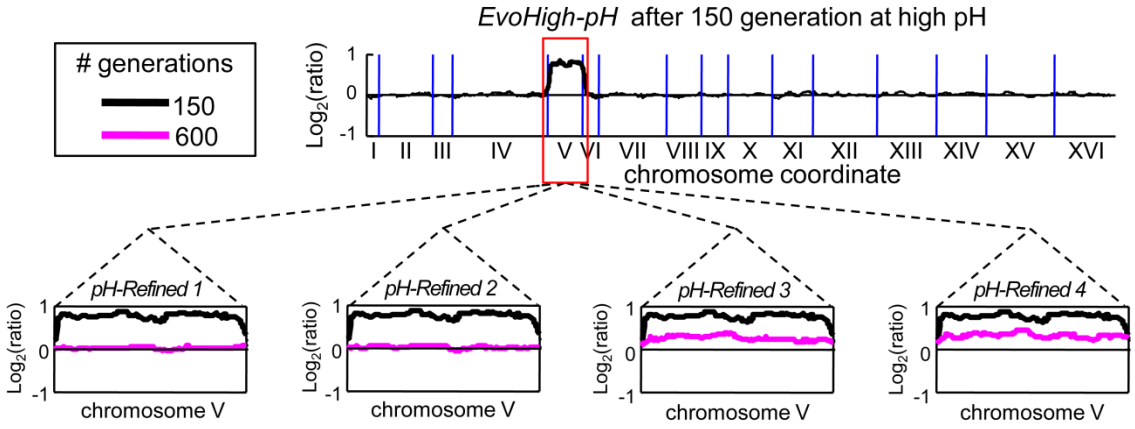

Figure 16. **Chromosome V aneuploidy acquired under high pH is eliminated upon further evolurtion.** Chromosome V disomy, gained by *EvoHigh-pH* after 150 generations at high pH (black lines), is eliminated during further evolution under the same high pH in which it was originally gained. Four independent descendants of *EvoHigh-pH* (*pH Refined1-4*) continued to evolve for 600 generations at high pH (8.6). All evolved populations show elimination of the disomy (magenta lines) with two populations showing complete elimination (upper two) and the other two populations showing the majority of population's cells to eliminate the disomy (lower two). All lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of high-pH evolved strain over a haploid wild-type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes.

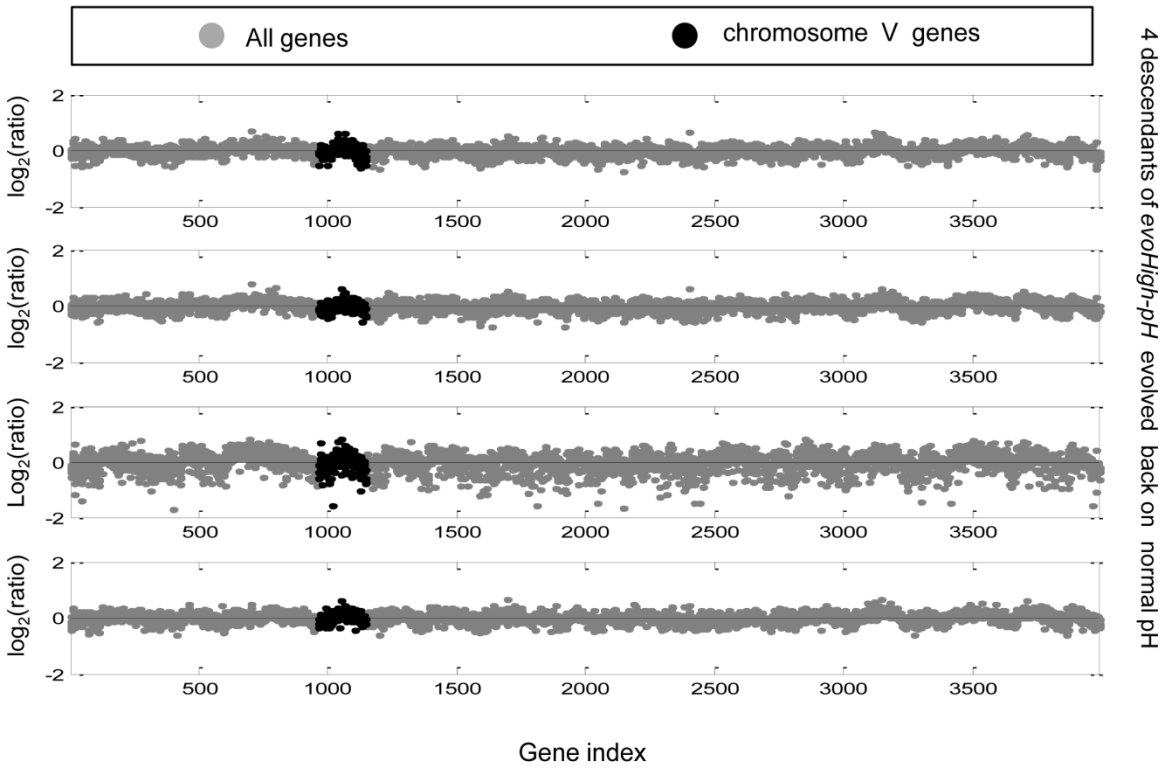

Figure 17 – Chromosome V disomic *evoHigh-pH* strain eliminated the disomy after further evolution at normal pH (6.7). Four independent repetitions, descendants of *evoHigh-pH*, evolved for 280 generations under pH 6.7 were analyzed and chromosome V disomy was eliminated in all repetitions. Dots represent $\log_2$ intensity ratios of DNA copy number over a haploid wild-type, aligned according to chromosomal order where black dots represent genes from chromosome V and grey from all other chromosomes.

3.3.9 Chromosome III genes robust to change in their DNA copy number

Our results indicate that specific aneuploidies can be used as temporary evolutionary intermediates in order to cope with certain stresses. Yet, the ability of cells to utilize chromosome duplication as an evolutionary operation might depend on their capacity to control some of the duplication's adverse affects. It appears that both benefits and costs of chromosome duplications are due to the fact that mRNA and protein levels mostly scale with gene copy number. Nonetheless, robustness for gene dosage was recently shown only for a small selection of the genes in yeast[30]. In accordance, we also found that several genes on chromosome III are buffered from aneuploidy – their mRNA levels remain close to those of the wild-type despite the chromosome duplication. For those genes, one could expect a feedback mechanism that limits the effect of extra chromosome copy, whereby the change in DNA copy number is buffered at the mRNA

level, thus not showing a corresponding elevation in gene expression. How could such buffering work? There are two types of potential mechanisms: according to the first the cell simply lacks the capacity to increase expression of some genes even if their gene copy number has doubled, while in the second scenario a true negative feedback operates on the expression or degradation of these genes. The feedback scenario predicts that genes that are buffered against gene copy number increase will also resist gene copy number decrease, while this is not expected in case of the first potential mechanism. We found that the sets of genes that show buffering in expression after chromosome III duplication were highly overlapping with the set of genes buffered in case of monosomy of chromosome III (Fig. 18), supporting the buffering through the negative feedback scenario. Interestingly, these buffered genes show also low amount of cell-to-cell variation, or "noise" in the wild-type[31] (mean noise level for the chromosome III buffered genes = 15.5% CV, mean noise level for the rest of the genes on chromosome III = 21.3% CV, P-value = 0.019) suggesting that their buffering against noise and against genetic manipulation are essential and may work through the same mechanism. We thus conclude that the utility of chromosome duplication as an evolutionary operation might depend on the capacity of the cell to buffer the dosage effect for some genes that must be retained at wild-type levels.

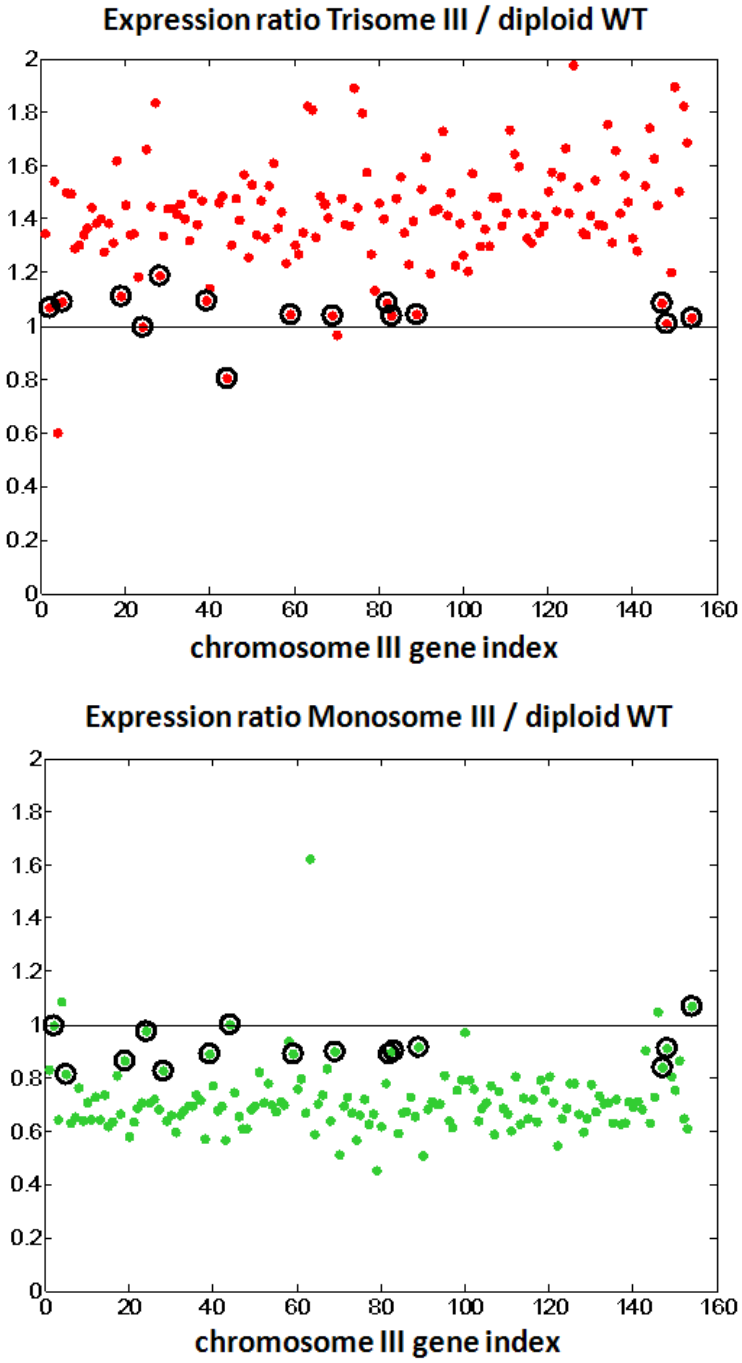

Figure 18. **Expression of genes robust to their copy number.** Expression levels of chromosome III genes in trisomy (upper box) and in monosomy (lower box) both normalized to the diploid wild-type. Genes robust for both trisomy and monosomy are circled in black.

3.4 Discussion

Aneuploidy is a readily available, yet costly, evolutionary solution. Such solutions are thus expected to be followed by refinement steps that would alleviate part of the costs associated with the original solution. We predict that such refined solutions will prove to be more durable than aneuploidy-based solutions due to low reversion rate even after prolonged periods in which the stress is not applied. On the other hand chromosomal duplications may be quickly reversed, leaving minimal imprint on the genome, a potentially desired characteristic for a genomic solution to short-term stresses. Aneuploidy, among other genomic aberrations, is also common in cancer. An interesting possibility is that during the progression of cancer, aneuploidy-based malignancies might be refined and replaced by modifying expression of specific genes. Such dynamics would be consistent with the observation that cancer driver genes are more often characterized by high mRNA levels than by high gene copy numbers [32,33].

This study emphasizes the importance of long-term lab-evolution experiments [34]. Limiting the experiments to only a few hundred generations would have shown the chromosomal duplication, but would have not revealed the massive subsequent aneuploidy elimination towards refinements. On the other hand, in order not to miss rapidly transient solutions, such as the current aneuploidies, one must follow long evolutionary dynamics at relatively high temporal resolution. Future full genome sequencing of the strains in this highly branched lab-evolution tree will further sharpen our understanding of the evolutionary dynamics examined here.

3.5 Methods

Strains & Growth: All evolved strains in this work were based on BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) or BY4743 (*MATa/MATα; his3Δ0/his3Δ0; leu2Δ/leu2Δ0; met15Δ0/MET15; LYS2/lys2Δ0; ura3Δ0/ura3Δ0*). *WTtrisomeIII* and *WTmonosomeIII* were obtained from Saccharomyces Genome Deletion Consortium[35] and found to have aneuploidies of chromosome III [5]. For each of these strains, the original gene deletion was reversed by adding the deleted ORF on a centromeric plasmid [21]. *H1-4*, are evolution strains evolved at 39^{o}C and rich medium. *Evo39* is an abbreviation for one of the two *H1-4* strains that gained only the trisomy of chromosome III after 450 generation and was verified by CGH microarrays to carry an extra copy of chromosome III. *Evo30* is an evolution strain evolved in parallel to *evo39* for 450 generations, but at permissive temperature of 30^{o}C. *Refined 1-4*, are evolution descendants of chromosome III trisomic strains (1-2 of *evo39* and 3-4 of *WTtrisomeIII*) that have been further evolved at 39^{o}C and minimal medium for another 1000 generations and eliminated the extra copy of chromosome III, yet retained the high heat tolerance. *Relaxed1-4*, are evolution descendants of the chromosome III trisomic *evo39* that have been further evolved at 30^{o}C and rich medium for another 600 generations and eliminated the extra copy of chromosome III, and returned back to heat tolerance similar to a diploid wild-type. *Gradual 1-4*, are evolution strains evolved under gradually applied heat for 450 generations and did not gain chromosome III trisomy. *EvoHigh-pH* is a strain obtained from the study of Romano *et al* [29] that have evolved under high pH(8.6).

Evolution experiments: All lab-evolution experiments were carried out by serial dilution. Cells were grown until reaching stationary phase under the relevant condition and then diluted by a factor of 1:120 into fresh media (6.9 generations per dilution). This procedure was repeated daily until significant phenotype change was detected in population growth under the applied condition. In all measurements of evolved populations we used a population sample and not selected clones.

Media: YPD was used as rich medium and SD as minimal. YPD (1L) – 10g yeast extract, 20g peptone, 20g dextrose and DDW. SD (1L) – 6.7g yeast nitrogen base, 20g dextrose, 1.5g amino acids mixed powder and DDW.

Liquid growth measurements: Cultures were grown at the relevant condition and OD_{600} measurements were taken during the growth at 30 minutes intervals until reaching early stationary phase. Qualitative growth comparisons were performed using 96 wells plates in which two strains were divided on the plate in a checker-board manner to cancel out positional effects. For each strain, a growth curve was obtained by averaging over 48 wells.

Heat-shock tolerance measurements: In order to eliminate physiological adaptations (due to prior exposure to heat before the measurements) all cultures were grown for 16 generations on 30°C. Then, cultures in mid-log phase were transferred to 45°C (t_0) for 90 minutes (t_1). At each time point, samples were taken and plated on YPD plates and incubated at 30°C. The survival ratio was calculated by dividing the number of colonies obtained in t_1 by the number of colonies obtained in t_0.

The selection of genes from chromosome III that retain high expression level after the elimination of chromosome III trisomy under heat:

We defined the genes from chromosome III that retained high expression level, despite the trisomy elimination, based on the four trisomic populations that eliminated chromosome III trisomy during 1000 generations under heat. Only genes that maintained average $\log_2$ expression change (compared to w.t.) above 0.46 in at least 3 out of the 4 repetitions were selected. The 0.46 criterion was according to the average expression increase in chromosome III genes observed in the trisomic *evo39* (compared to its diploid ancestor) that was 0.4562 ($\log_2$). This definition led to 23 genes from chromosome III that retained high expression, out of which 17 were available in the MoBY plasmids library. For these genes, gene ontology analysis did not show any significant term compared to all other genes on chromosome III.

Heat tolerance of selected genes from chromosome III: In order to measure the heat tolerance contribution of selected genes from chromosome III when inserted as extra copies into a diploid wild-type we used centromeric plasmids [21]. Heat-shock tolerance was measured for each of these strains compared to a diploid wild type with an empty plasmid. We defined 100% heat tolerance as the tolerance of *WTtrisomeIII* and 0% tolerance as the tolerance of diploid wild type with an empty plasmid. For each of the transformed strains the heat tolerance calculated by dividing over the *WTtrisomeIII* tolerance, after subtraction the tolerance of wild type with an empty plasmid.

General Stress Tolerance: Cultures of diploid WT and *WTtrisomeIII* were grown in rich medium until mid-log phase and then transferred to one of the following conditions: YPD+KCl 1M, YPD at pH=4, YPD+Ethonol 8%, YP +Glycerol 3%, YPD+NaCl 0.5M, YPD+ copper sulfate($CuSO_4$) 6.25mM. OD_{600} measurements were taken at constant intervals.

Microarrays: Affymetrix Yeast Genome 2.0 arrays were used to measure gene expression and to detect aneuploidies. The ability to detect large-scale genomic duplications by expression microarrays was based on previous studies that demonstrate the correspondence between chromosomal duplication and increased expression [1,5]. Agilent CGH microarrays were used to verify karyotype for selected strains (we report aneuploidy of evolved populations only when the aneuploidic part in population is > 95%, see *SI Text*). Taking into consideration that adaptation could also be carried out at the physiological level, rather than encoded at a genetic/epigenetic level, all expression microarrays were preformed following 16 generations of growth at normal temperature of 30^{o}C. This setup allows us to focus on the non-physiological component of adaptation. Genomic DNA and RNA extractions were done using Epicenter Yeast kits. The microarrays data for detection of chromosomal duplications is shown after performing sliding window averaging with a window size of 10 genes from each side. In each point the value represent the average of the values of the current window. All Affymetrix microarrays data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession number GSE40817 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40817).

Determining aneuploidy and other measurements preformed on samples from evolved populations: Along this study, in cases where we analyzed an evolved population we always measured the population and not a single clone. This is in order for the measurements to reflect the mean value for all of the population and not of a potential sub-population from which a single clone could have been taken for measurement. Since we measure a population of cells, in the cases of determining aneuploidy we had to assess the part of population that contains the reported aneuploidy by comparing to microarray of a mono-clonal strain with the same aneuploidy. In all cases where we report aneuploidy the estimated part of the population that is reported to either gain or eliminate a chromosome is at least 95%, as judged by the comparison to reference strain. Nonetheless, in some cases the population was more heterogenic and indeed we note that the trait has not yet been entirely fixated (see for example "Refined 3" and "Refined 4" in the high pH evolution experiments).

Heat tolerance measurements – liquid growth vs. heat-shock survival: In this study we use two assays to compare heat tolerance of different strains: liquid growth and heat-shock survival. The liquid growth curve comparisons in the various conditions are the most suitable way to compare between evolved strains, because evolution itself is preformed via liquid growth under defined conditioned. Thus, our method is based on the notion that the test should be on the same setup in which the evolutionary process took place. Also, when comparing two strains that did not go through evolution it is legitimate to compare them by growth rate analysis. However when we compare a strain that evolved via liquid growth transfer to another strain that did not go through such an evolutionary process (e.g. when comparing *evo39* and *WTtrisomeIII* on heat stress) we must not use the growth rate analysis since during the evolution strains adapt to the liquid growth itself in addition to the adaptation to the high temperature. Thus, in such cases the more reliable way to compare heat tolerance is to measure heat shock survival ratio. In addition, when appropriate each heat survival analysis is also backed up by growth rate comparison in.

3.6 Literature

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4. The evolution of translation machinery to meet codons demand

4.1 Abstract

The translational balance is a key characteristic of the translation process in the cell as the supply, i.e. the expression level of each tRNA type is well adapted to the demand, i.e. the codon-usage of expressed genes. Nonetheless, the translational balance may be broken by changes in expression patterns upon new environmental challenges, e.g. when migrating to a new ecological niche. In such cases, the ways by which the tRNA pool evolves to restore the translational balance are poorly understood. To elucidate the adaptiveness of the translational balance, we interrupted the demand-to-supply balance in yeast by deletion of a tRNA gene that resulted in compromised growth. We followed lab-evolution experiments performed on the deletion strain and observed that the translational balance was rapidly restored. Surprisingly, the adaptation was based on a strategic mutation that changed the anticodon of other tRNA genes to match the anticodon of the deleted tRNA. To further study the evolutionary forces that shape the tRNA pool, we artificially added multiple copies of various tRNAs genes into wild-type yeast. We show that increasing tRNA gene copy number has a deleterious effect by causing proteotoxic stress, yet only in cases of low-copy tRNA families. Thus, we demonstrate how tRNA genes are maintained in a balanced gene family size while utilizing the multiple copies architecture as means to rapidly adapt the tRNA pool to meet a new translational demand.

4.2 Introduction

The translation machinery is fundamental to the function of living cells and thus highly conserved among species. Yet, evidence from codon usage of many species implies for a fine-tuned adaptation of the translation machinery to facilitate rapid expression of highly expressed genes. Therefore a key question in the translation process is the mechanisms by which it can adapt to different conditions. While ribosomal genes do not exhibit appreciable changes in adaptation to environmental conditions, tRNA genes may provide a source of plasticity for adaptive evolution. In this study we ask how evolution of tRNA genes can confer adaptive advantage upon challenging translational requirements.

tRNAs are fundamental to the genetic code by linking anticodons to their corresponding amino acids[1]. Each tRNA gene exists as a gene family with several copies scattered throughout the genome. Importantly, it has been experimentally observed for *Saccharomyces cerevisiae*[2] and *Escherichia coli*[3] that the cellular concentrations of each tRNA type in the cell (the tRNA pool) is correlated with its genomic tRNA copy number[4,5]. Notably, the rate limiting step of polypeptide synthesis is the recruitment of a tRNA that matches the translated codon[6]. Thus, the translation efficiency of a coding sequence is governed by the codon usage of the translated mRNA[7] and the extent to which its codon usage is adapted to the cellular tRNA pool[8,9].

Codon usage bias, the non-random use of synonymous codons that better match the tRNA pool, was found to be most extreme in highly expressed genes. Largely, such genes exhibit codon usage bias toward "optimal" codons[10], whose corresponding tRNA gene-copy number is high[10,11]. The evolutionary force that acts to maintain high translation efficiency for highly expressed genes was coined *translational selection*[9]. It was previously suggested that translational selection acts both on the codon usage and on the tRNA pool. On the one hand, there is a selective pressure to increase the frequency of preferred codons in highly-expressed genes. On the other hand, changes in the tRNA pool may also occur, e.g. duplication of tRNA genes for which high codon demand exists. Thus, codon frequencies and tRNA copy numbers co-evolve toward a supply vs. demand balance that facilitate proper protein production[12,13].

Evolutionary changes to the tRNA pool were appreciated mainly by bioinformatics studies. Sequence analyses of divergent genomes have demonstrated that both

sequence and the copy number of tRNA genes may change during evolution[12,14–16]. Yet, it is unclear whether the observed variations in the tRNA pool are a consequence of an unbalanced translational demand and whether they are utilized as an adaptive mechanism to achieve translational balance. Besides, the fitness effects of an unbalanced translational demand and its potential role in shaping the tRNA pool are vague due to lack of experimental evidence.

Furthermore, in the light of translational selection, the tendency of organisms to maintain some of their tRNAs in low copy number is unclear as it results in non-optimal codons. It has been proposed in several studies that non-optimal codons may play a role in governing the process of co-translational folding, by slowing translation to facilitate proper folding, e.g. between domain boundaries[17–20]. Yet, the contribution of non-optimal codons to proper protein folding was mainly observed for individual genes[21–26]. Furthermore, the extent and importance of this phenomenon to the global folding state of the proteome remains elusive. Thus, the evolutionary constrains on copy number changes in tRNA genes families are not well understood. Specifically, it is not clear if translational selection acts only to optimize codons, or also to maintain a low copy number of other tRNA genes.

To elucidate the importance of restoring the translational balance, we utilized experimental evolution to follow the evolutionary process that acts to restore such balance. We genetically perturbed the tRNA pool of the budding yeast *S.cerevisiae*. In this yeast, the genetic code is decoded by 42 different tRNA families that are present in its genome in a total of 274 tRNA genes[27]. Each tRNA family ranges from 1 to 16 copies, with six tRNA families consist only of one copy. We perturbed the translational balance by a deletion of a singleton tRNA gene, which decodes a relatively high used codon (21% of arginine codons in *S.cerevisiae*, which is the second most used arginine codon). This deletion interrupted the demand-to-supply balance in the cell and compromised growth. Lab-evolution experiments performed on this strain demonstrated that the balance was rapidly restored by mutations in other tRNA genes that compensated for the tRNA deletion.

Additionally, in order to shed light on the constrains that shape tRNA gene filmily sizes, we artificially over-expressed singleton tRNAs, rather than deleting them. We found that when a low-copy tRNAs were over-expressed the protein quality control machinery was challenged due to increased folding stress. Hence, we demonstrate the key role of the tRNA pool in the adaptiveness of the translation machinery.

4.3 Results

4.3.1 Deletion of singleton tRNA gene breaks the translational balance

To demonstrate the importance of the balance between codon-usage and the cellular tRNA pool we obtained a yeast strain in which the single copy of the arginine tRNA gene, *tR(CCU)J*, was deleted (termed $\Delta tRNA^{Arg}_{CCU}$). Consequently, the deletion strain cannot translate the arginine codon AGG with a full-match tRNA and presumably relies on wobble interaction with another arginine tRNA, $tRNA^{Arg}_{UCU}$. This shortage in supply cannot match the demand for translation of AGG codons, which encompasses ~21% of the arginine codons in the yeast genome and ~16% of the arginine codons in the yeast transcriptome under standard lab conditions[13]. Indeed, the $\Delta tRNA^{Arg}_{CCU}$ strain showed a severe growth defect compared to the wild-type strain (Fig. 1) indicating the effect on the tRNA pool ability to meet translational demand of the trascriptome. The severe growth defect also demonstrates the limited capacity of wobble interactions to efficiently translate AGG codons via $tRNA^{Arg}_{UCU}$, which is found in the genome in 11 copies.

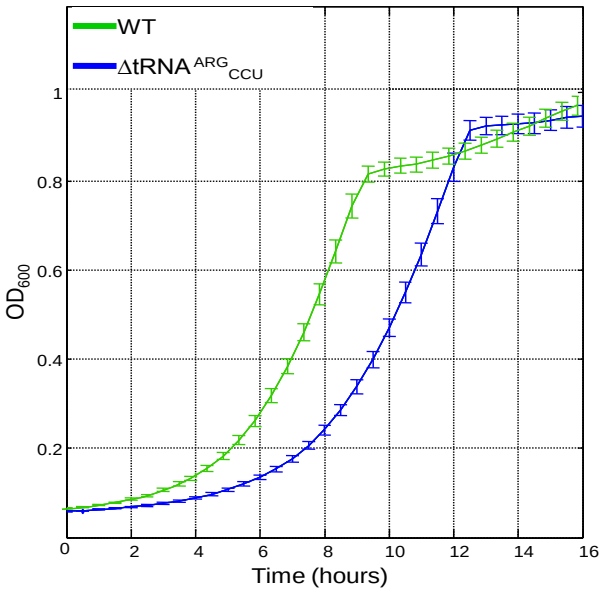

Figure 1 – The growth defect associated with deletion of the singleton $tRNA^{Arg}_{CCU}$
Growth curve measurements of WT (green) and of $\Delta tRNA^{Arg}_{CCU}$ (blue) are shown in optical density (OD) values over time during continuous growth at 30°C.

4.3.2 tRNA pool can rapidly evolve to meet translational demands

In order to learn how cells can evolutionarily adapt to translational imbalances, we performed lab-evolution experiments on $\Delta tRNA^{Arg}_{CCU}$ via serial dilutions[28]. The deletion strain was grown under optimal conditions and diluted into fresh medium by a factor of 120, i.e. ~7 generations per day. Every 50 generations, growth of the evolving population was compared to both wild-type and the ancestor $\Delta tRNA^{Arg}_{CCU}$ strains. Strikingly, after 200 generations a full recovery of $\Delta tRNA^{Arg}_{CCU}$ growth defect was observed as the growth of the evolved population was indistinguishable from that of the wild-type (Fig. 2).

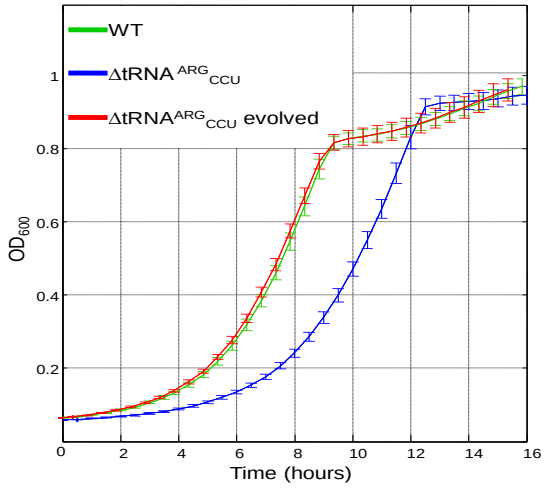

Figure 2 – Short lab-evolution cured the deletion phenotype of $\Delta tRNA^{Arg}_{CCU}$
Growth curve measurements of WT (green), $\Delta tRNA^{Arg}_{CCU}$ (blue) and the evolved deletion (red) are shown in OD values over time during continuous growth at 30°C.

A similar dynamics was observed in all four independent evolutionary lines of repeated experiments. In search for potential genetic adaptations underlying this rapid recovery, we first looked for genetic alterations in other arginine tRNAs. We found a single point mutation in another arginine tRNA gene which codes for $tRNA^{Arg}_{UCU}$. This mutation changed the anticodon triplet of $tRNA^{Arg}_{UCU}$ from UCU to CCU (i.e. U→C transition). Consequently, the evolved $tRNA^{Arg}_{UCU}$ matches perfectly the AGG codon (Fig. 3).

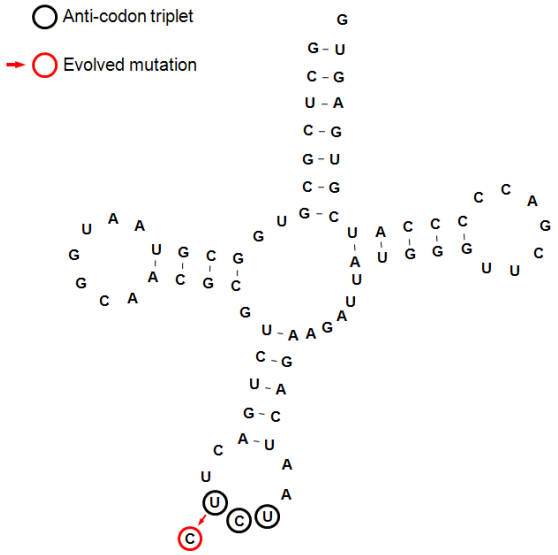

Figure 3 – During the lab-evolution of ΔtRNA$^{Arg}_{CCU}$, tRNA$^{Arg}_{UCU}$ mutated its anticodon to CCU. The secondary structure of tRNA$^{Arg}_{UCU}$ is drawn with the UCU anti-codon nucleotides marked with black circles. The red circle indicates the mutation that occurred during the lab-evolution experiments, i.e. U→C transition.

Unlike the singleton tRNA$^{Arg}_{CCU}$ there are 11 copies of tRNA$^{Arg}_{UCU}$ in the yeast genome. Although each of the four independent lab-evolution experiments showed the exact same solution, i.e. a mutation in the anticodon of a tRNA$^{Arg}_{UCU}$ gene, three different copies of this gene were changed in the four repetitions (i.e. one of the 11 copies of tRNA$^{Arg}_{UCU}$ mutated in two repetitions, see supplementary text). In order to confirm that a single point mutation in the anticodon of tRNA$^{Arg}_{UCU}$ is sufficient to eliminate the growth defect of ΔtRNA$^{Arg}_{CCU}$, we artificially inserted the U→C mutation into a ΔtRNA$^{Arg}_{CCU}$ background. The mutation was inserted into one of the 11 copies of the tRNA$^{Arg}_{UCU}$ genes, a copy that was spontaneously mutated in one of the evolution lines, which is located on chromosome XI. Indeed, the artificially mutated strain, termed here *Mut*Δ*tRNA*$^{Arg}_{UCU}$, showed a full recovery of the deletion adverse phenotype (Fig. 4). This indicates that the U→C mutation in the anticodon is sufficient for the full recovery of the tRNA$^{Arg}_{CCU}$ deletion phenotype, even if additional mutations may have fixed in the genome of the evolved strains.

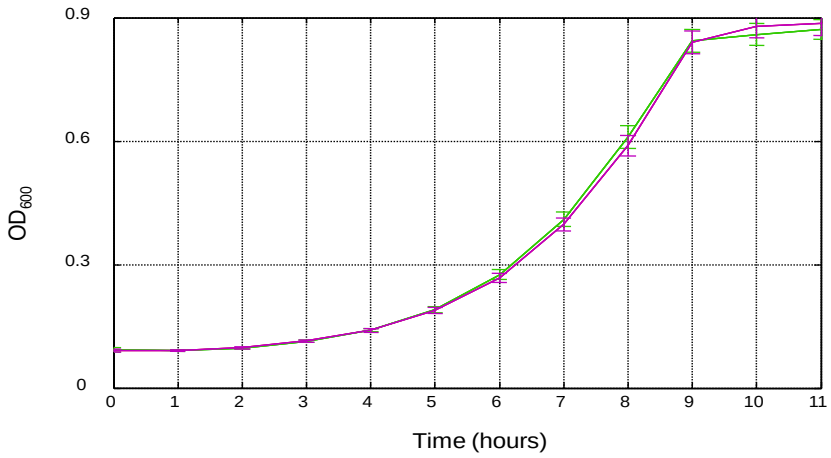

Figure 4 – *Mut*Δ*tRNA*$^{Arg}_{UCU}$ exhibits similar growth as WT
A rescue experiments in which the U→C mutation was introduced to ΔtRNA$^{Arg}_{CCU}$ cells (termed *Mut*Δ*tRNA*$^{Arg}_{UCU}$) was performed. Growth curve measurements of WT (green) and of *Mut*Δ*tRNA*$^{Arg}_{UCU}$ (magenta) are shown in OD values over time during continuous growth at 30°C.

4.3.3 Mutated tRNA$^{Arg}_{UCU}$ is fully functional despite sequence dissimilarities with respect to the deleted tRNA$^{Arg}_{CCU}$

The two arginine tRNA genes: tRNA$^{Arg}_{UCU}$ and tRNA$^{Arg}_{CCU}$ differ in 21 out of their 72 nucleotides (including the third anticodon position). Thus, the evolutionary solution that occurred in our experiments created a "hybrid" tRNA with a CCU anticodon while the rest of the tRNA sequence (termed here as the "tRNA body") remained as tRNA$^{Arg}_{UCU}$. Therefore, the body of the new hybrid tRNA differs in another 20 positions from the original tRNA$^{Arg}_{CCU}$ (Fig. 5).

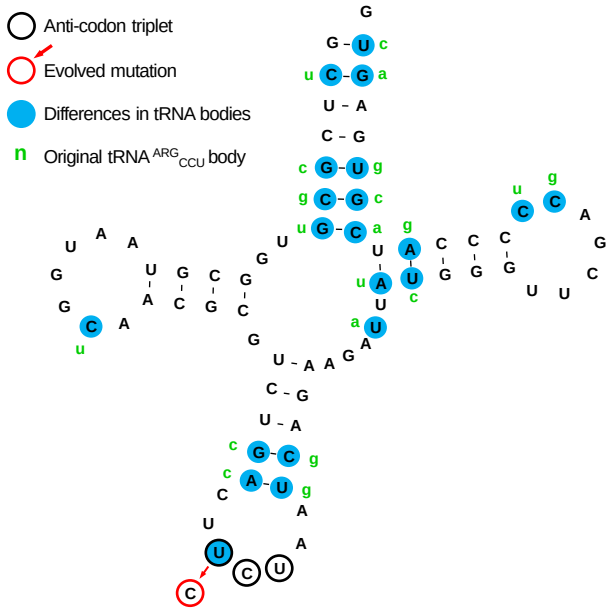

Figure 5 – Overview of the hybrid tRNA sequence created during the lab-evolution
The secondary structure of the hybrid tRNA is drawn. This hybrid is composed of the body of $tRNA^{Arg}_{UCU}$ with a CCU anti-codon. The anti-codon triplet is marked with black circles. The evolved mutation is marked with a red circle. All 20 nucleotide differences between $tRNA^{Arg}_{UCU}$ and $tRNA^{Arg}_{CCU}$ are marked with blue circles, next to which- in green letters- the nucleotide of $tRNA^{Arg}_{CCU}$ is written.

In general, the copies of tRNA gene family tend to be highly similar in sequence[27] and all tRNA families of the same amino acid are loaded by the same tRNA amino-acyl transferase. In particular, the sequence of the 11 copies of $tRNA^{Arg}_{UCU}$ is 100% identical. A sequence similarity of that extent could suggest that the unique sequence of each tRNA body has a functional role in addition to that of the anticodon[29]. Therefore, it is surprising that the hybrid tRNA performed just as well as the deleted $tRNA^{Arg}_{CCU}$ in terms of cell growth, despite the sequence differences in their bodies. Thus, we raised the hypothesis that challenging growth conditions may expose an inferiority of the hybrid tRNA, as was demonstrated more generally in the yeast deletion library[30,31]. To test this notion, we compared the rescued strain, *Mut*Δ$tRNA^{Arg}_{CCU}$, that carries the hybrid tRNA to the wild-type, under an array of unfavorable conditions. Surprisingly, under all checked conditions, there was no significant growth difference between the two strains (Fig. 6). Hence, the hybrid tRNA provides a direct *in-vivo* indication that the bodies of tRNAs that code for the

same amino-acid are interchangeable, since in terms of effect on growth the anticodon is the predominant feature of the tRNA.

These results raised an alternative hypothesis (currently tested in our lab by Idan Frumkin) that the sequence identity among all copies of $tRNA^{Arg}_{UCU}$ is neutral and the result of massive gene conversion events among the tRNA genes.

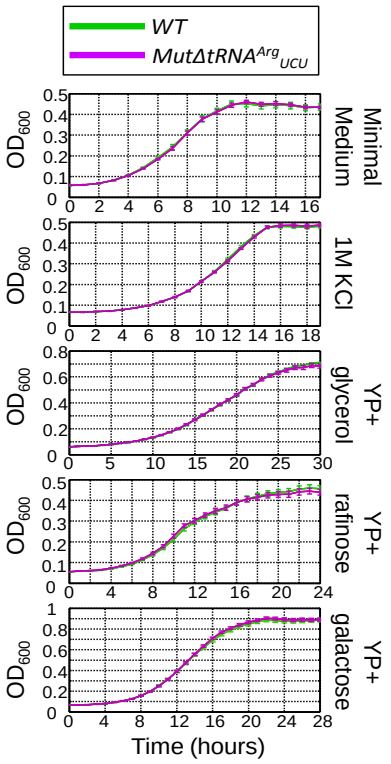

Figure 6 – The growth of *Mut*Δ*tRNA*$^{Arg}_{UCU}$ compared to WT under different conditions
Growth curve measurements of WT (green) and of *Mut*Δ*tRNA*$^{Arg}_{UCU}$ (magenta) are shown in OD values over time during continuous growth. Under all stresses tested (minimal medium, 1M KCl, YP+glycerol, TP+rafinose and YP+galactose) the rescued strain demonstrated similar growth as the WT strain.

To examine the generality of our observation that the anticodon identity, and not the tRNA body, confers the tRNA essential functionality, we again perturbed the tRNA pool in a wild-type strain by deletion of an entire serine tRNA family. Here, the supply of $tRNA^{Ser}_{GCU}$ was eliminated by deletion of all four identical genes of this family located on chromosomes *IV, VI, XII* and *X*. A complete deletion of this gene family is lethal, indicating that the $tRNA^{Ser}_{GCU}$ is essential in *S.cerevisiae*. To validate that the lethality is indeed due to the $tRNA^{Ser}_{GCU}$ genes deletion and not due to an unintentional perturbation of other putative genetic features in the vicinity of the deleted $tRNA^{Ser}_{GCU}$ genes, we introduced a plasmid with the $tRNA^{Ser}_{GCU}$ gene.

Indeed, the deletion strain was rescued by supplementation of a plasmid carrying the $tRNA^{Ser}_{GCU}$ gene (Fig. 7).

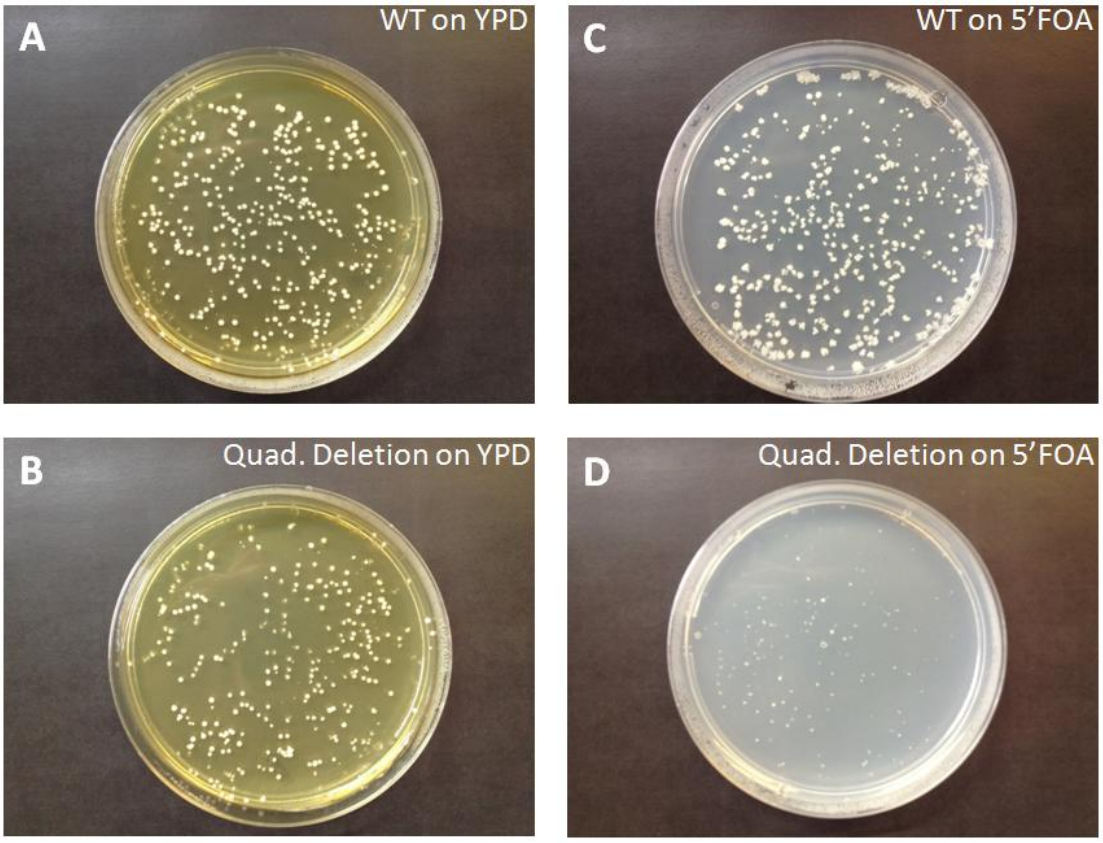

Figure 7 – The quadruple deletion of tRNA$^{Ser}_{GCU}$ is lethal
Plate of the WT(control) and tRNA$^{Ser}_{GCU}$ quadruple deletion, both supplemented with plasmid containing tRNA$^{Ser}_{GCU}$ and Ura selection, were grown on a non-selective medium (A and B respectively). After colonies appeared, these plates were replicated into medium that negatively select for the presence of the Ura selection. The selective medium only allows the growth of cells that lost the plasmid, thus provide evidence that the tRNA$^{Ser}_{GCU}$ quadruple deletion cannot be viable without a plasmid containing tRNA$^{Ser}_{GCU.}$

Then, we hypothesized that similarly to tRNA$^{Arg}_{CCU}$, other hybrid serine tRNAs that carry a GCU anticodon can prevent the observed lethality regardless of their tRNA body sequence. Therefore, rather than inserting a plasmid with the original tRNA$^{Ser}_{GCU}$ gene, we complemented the tRNA$^{Ser}_{GCU}$ family deletion strain with a plasmid containing a hybrid serine tRNA with GCU anticodon but with an alternative body of a different serine tRNA, tRNA$^{Ser}_{CGA,}$ that differs in 22 positions from the deleted serine tRNA family. Indeed, the strain carrying the hybrid tRNA is viable on the background of the tRNA$^{Ser}_{GCU}$ family deletion. Therefore, we conclude that the identity of the anticodon is essential for the function of the tRNA$^{Ser}_{GCU}$ genes family. Yet, this essential function can be performed with hybrid serine tRNAs as long as the anticodon is GCU.

4.3.4 Adding multiple copies of the rare tRNA$^{Arg}_{CCU}$ gene is deleterious to the cell

The ability of tRNA$^{Arg}_{UCU}$ genes to rapidly mutate their anticodon and consequently function like tRNA$^{Arg}_{CCU}$ brings up the question about the functional constraints during evolution that left some families with more members while others with fewer. In particular, the tendency of different yeast species to maintain tRNA$^{Arg}_{CCU}$ at a low copy number implies that selection maintains tRNA$^{Arg}_{CCU}$ at a low copy number (Fig. 8).

Yeast Specie	# of tRNAs with CCU anticodon	AGG usage in ARG codons (%)	# of tRNAs with UCU anticodon	AGA usage in ARG codons (%)
Saccharomyces cerevisiae	1	21	11	48
Candida albicans	1	8	5	56
Candida glabrata	1	20	9	52
Aspergillus nidulans	2	11	2	13
Debaryomyces hansenii	1	17	10	56
Encephalitozoon cuniculi	1	43	1	36
Eremothecium gossypii	1	14	6	16
Kluyveromyces lactis	1	18	7	51
Schizosaccharomyces pombe	1	11	2	23
Yarrowia lipolytica	1	5	4	17

Figure 8 – Various yeast species tend to keep tRNA$^{Arg}_{CCU}$ in a single copy
All examined yeast species, except for *Aspergillus nidulans*, keep a single copy of tRNA$^{Arg}_{CCU}$ compared to tRNA$^{Arg}_{UCU}$ which is mostly found in multiple copies.

Such translational selection force can prevent the transformation of UCU anticodons to CCUs in arginine tRNA genes. Indeed, introduction of a multi-copy plasmid containing a tRNA$^{Arg}_{CCU}$ gene to a wild-type strain (termed here *WTmultiCCU*) resulted in a substantial growth reduction compared to wild type cells carrying a similar multi-copy plasmid that does not contain any tRNA gene (termed *WTmultiControl*). To control for the effect of multiple copies of arginine tRNA genes, we also created a strain with a similar multi-copy plasmid that contains tRNA$^{Arg}_{UCU}$, termed here *WTmultiUCU*. Comparing the growth of *WTmultiCCU* and *WTmultiUCU* revealed that *WTmultiCCU* has significantly reduced growth compared to *WTmultiUCU*, which exhibit a growth profile much closer to that of *WTmultiControl*

(Fig. 9). These data are in line with the evolutionary tendency to keep low-copy number of $tRNA^{Arg}_{CCU}$ and suggest that high copy number of such rare tRNA genes is deleterious to the cell.

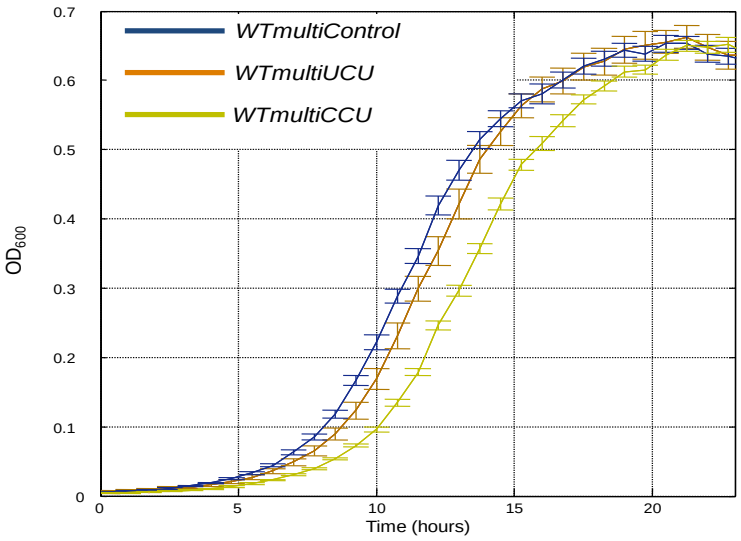

Figure 9 – *WTmultiCCU* suffers from growth defect compared to *WTmultiUCU*
Growth curve measurements of *WTmultiControl* (blue), *WTmultiUCU* (brown) and *WTmultiCCU* (khaki) are shown in OD values over time during continuous growth. The *WTmultiCCU* strain with a high copy number plasmid harboring $tRNA^{Arg}_{CCU}$ demonstrates a slower growth compared to cells with an empty plasmid or with $tRNA^{Arg}_{UCU}$.

To demonstrate the generality of the findings that adding multiple copies of rare tRNA genes is deleterious compared to abundant tRNA genes, we employed the same assays in two other cases. First, we examined two serine tRNAs, the singleton $tRNA^{Ser}_{CGA}$ and $tRNA^{Ser}_{AGA}$ which is found in the genome in 11 copies (Fig. 10). In the second case, we focused in two glutamine tRNAs, the singleton $tRNA^{Gln}_{CUG}$ and $tRNA^{Gln}_{UUG}$ which is found in the genome in 9 copies (Fig. 11). In both cases, we observed that wild-type strain supplemented with multiple copies of the singleton tRNA exhibit impaired growth compared to the same strain supplemented with the abundant tRNA of the same amino acid.

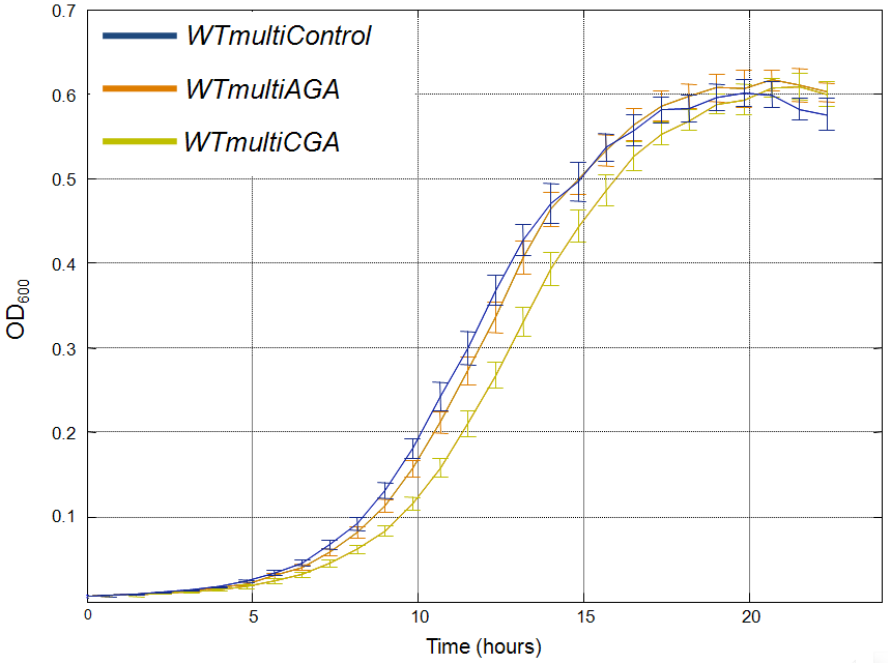

Figure 10 – *WTmultiCGA* suffers from growth defect compared to *WTmultiAGA*
Growth curve measurements of *WTmultiControl* (blue), *WTmultiAGA* (brown) and *WTmultiCGA* (khaki) are shown in OD values over time during continuous growth. The *WTmultiCGA* strain with a high copy number plasmid harboring $tRNA^{Ser}_{CGA}$ demonstrates a slower growth compared to cells with an empty plasmid or with $tRNA^{Ser}_{AGA}$.

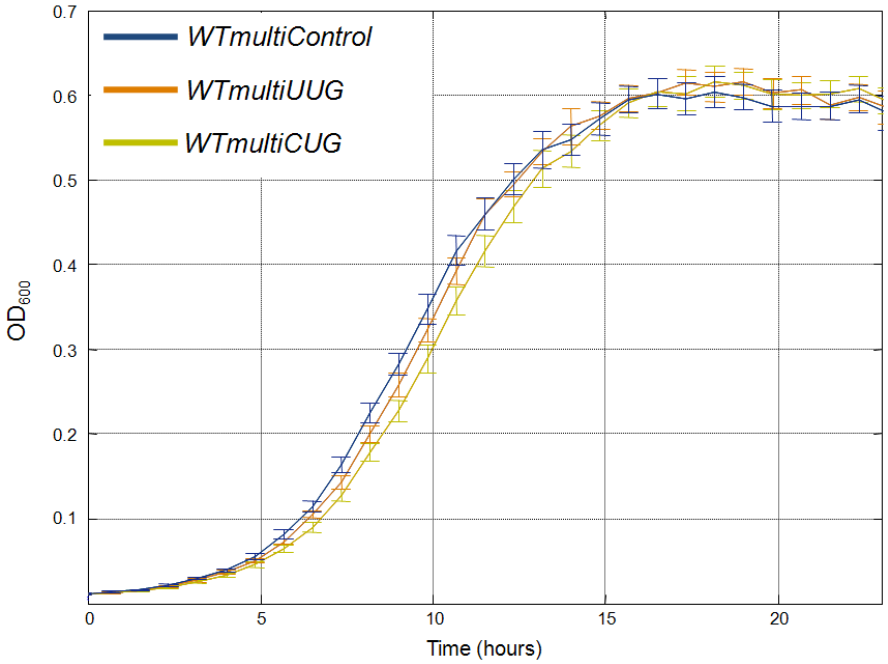

Figure 11 – *WTmultiCUG* suffers from growth defect compared to *WTmultiUUG*
Growth curve measurements of *WTmultiControl* (blue), *WTmultiUUG* (brown) and *WTmultiCUG* (khaki) are shown in OD values over time during continuous growth. The *WTmultiCUG* strain with a high copy number plasmid harboring $tRNA^{Gln}_{CUG}$ demonstrates a slower growth compared to cells with an empty plasmid or with $tRNA^{Gln}_{UUG}$.

Since family size changes may occur in a stepwise manner, we also examined the effect of adding a low copy number plasmids carrying either $tRNA^{Arg}_{CCU}$ or $tRNA^{Arg}_{CCU}$. Cells with the $tRNA^{Arg}_{CCU}$ plasmid showed a modest growth defect compared to cells with $tRNA^{Arg}_{UCU}$ plasmid, yet only under heat of 39°C (Fig. 12).

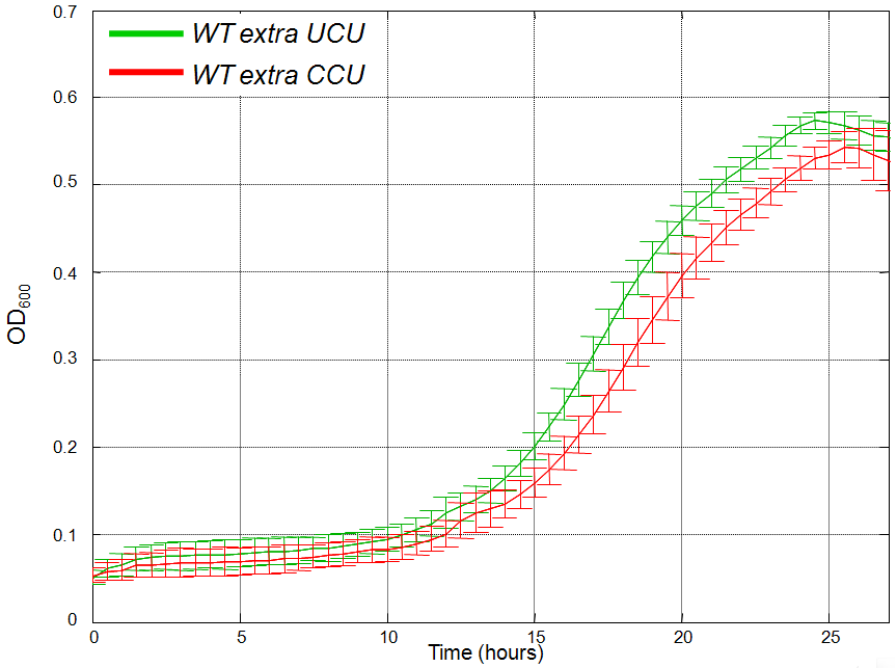

Figure 12 – Addition of low copy number plasmid of $tRNA^{Arg}_{CCU}$ compromise growth under heat compared to addition of $tRNA^{Arg}_{UCU}$.
Growth curve measurements of wild-type cells supplemented with low copy number plasmid of $tRNA^{Arg}_{UCU}$ (green) and low copy number plasmid of $tRNA^{Arg}_{CCU}$ (red). Dates are shown in OD values over time during continuous growth under heat (39°C).

4.3.5 Multiple copies of the rare $tRNA^{Arg}_{CCU}$ induce proteotoxic stress

There are several potential mechanisms that can cause the growth defect exhibited by multiple copies of rare tRNA genes, as shown most substantially in the *WTmultiCCU* strain: 1) Mis-incorporation of arginine into non-arginine codons. 2) Mis-loading of wrong amino acids on arginine tRNA molecules which results in wrong translation of the genetic code. 3) Low concentration of certain tRNAs can be essential for proper folding of specific domains during the synthesis of a new protein[17,19,32,33]. While these potential errors are not mutually exclusive and can each contribute to the observed growth defect, they all directly affect protein folding and might induce proteotoxic stress. To examine the possibility that the growth defect associated with multiple copies of $tRNA^{Arg}_{CCU}$ is indeed associated with proteotoxic stress, we utilized a previously published method that allows examination of the load on the protein quality control machinery of the cell [34] (see methods). In this assay, we introduced cells with a plasmid that contains the human gene von-Hippel-Lindau (VHL) fused to

a florescent tag (mCherry). When the fluorescently tagged VHL appears as a punctum phenotype (Fig. 13A), and not in the cytosol (Fig. 13B), this indicates high levels of misfolded proteins and a saturated protein quality control machinery. We transformed the VHL-mCherry plasmid to each of the multi-copy tRNA strains, *WTmultiCCU*, *WTmultiUCU* and *WTmultiControl* and monitored the level of proteotoxic stress by quantifying the number of puncta in each population. The fold change in puncta number was then deduced by normalization to the *WTmultiControl* population. We found that while *WTmultiUCU* exhibited similar puncta levels as the *WTmultiControl*, the *WTmultiCCU* exhibited a 3-fold increase in puncta levels (Fig. 13C). These findings suggest that high copy number of the rare tRNA gene, $tRNA^{Arg}_{CCU}$, but not an already abundant tRNA, $tRNA^{Arg}_{CCU}$, results in elevated levels of proteotoxic stress.

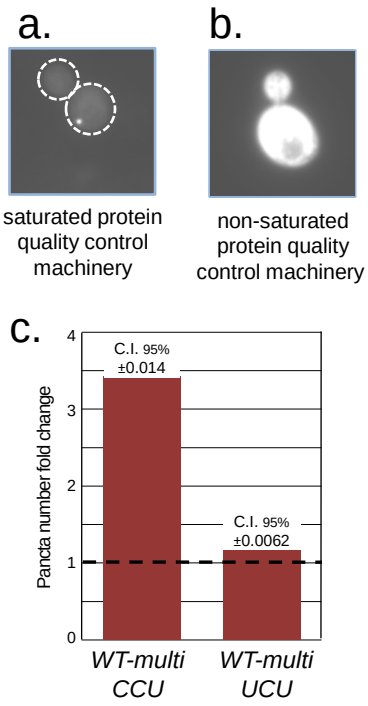

Figure 13 – *WTmultiCCU* demonstrates higher levels of misfolded proteins compared to *WTmultiUCU*. (a) A *WTmultiCCU* cell in which the mCherry-VHL proteins appear with a punctum phenotype when the protein quality control machinery is saturated with misfolded proteins. **(b)** A *WTmultiUCU* cell in which the quality control machinery is not occupied with other proteins, mCherry-VHL is localized to the cytosol of the cell. **(c)** *WTmultiCCU*, *WTmultiUCU* and *WTmultiControl* were transformed with a mCherry coding plasmid and visualized under the microscope. 1000 cells per strain were counted for either cytosolic or punctum localization of the VHL protein. The fold change in puncta number was then deduced by normalization to the *WTmultiControl* population.

4.4 Discussion

Genomic duplications, deletions and anticodon mutations shape tRNA gene families, yet the evolutionary scenarios that trigger changes in the tRNA pool was not thoroughly explored. In our evolution experiments, a translational imbalance was induced by a tRNA gene deletion, which compromised growth and led to tRNA pool adaptations. Organisms may experience equivalent imbalances also when their gene expression changes due to environmental conditions or upon migrating to a new ecological niche.

Upon environmental changes, the cell's expression pattern can change and a set of genes that was lowly expressed in the former environment are highly expressed in the new one[13]. Such changes in the transcriptome alter the codon usage and may cause translational imbalance. In order to maintain proper protein production, the tRNA pool has to accommodate this new translational demand, i.e. to restore the translational balance. In our work we demonstrate how anticodon mutations provide a rapid means to alter the tRNA pool. We propose that during evolution, the adaptation of the translation machinery to expression changes can be met by anticodon shifting of tRNA gene copies more readily than by duplications and deletions. This ability of the tRNA pool, i.e. the supply, to rapidly adapt can be mainly attributed to the gene-families architecture of the tRNA pool. Since many copies of the same tRNA gene resides in the genome, the needed mutation can be found considerably faster and the effect for shifting one copy out of a multi-copy tRNA family is considerably small. We suggest that in terms of adaptation to new conditions, the tRNA genes are the "plastic" part of the translation machinery. Therefore, only on a much longer evolutionary time-scale, codon usage of ORFs will change for further fine-tuning of the translational balance.

Interestingly, the hybrid tRNAs that emerged in our lab-evolution experiments replaced the deleted tRNA despite different sequence of their tRNA bodies, which differ in 20 positions. If tRNAs bodies are practically interchangeable in terms of the effect of their function on the fitness, what can explain the high sequence similarity observed between tRNA gene copies of the same family? It is possible that the tRNA body sequence is indeed important under specific conditions that were not examined in this work, and that purifying selection maintained sequence identity within tRNA families. Yet, there is also a possibility that the sequence similarity is due to concerted

evolution that maintains sequence identity by frequent recombination events among copies of the same tRNA family. This option implies that the high conservation observed within tRNA gene families is not due functionality reasons, but rather the result of a more neutral evolutionary process that is less sensitive to the fitness of the tRNA sequence.

Furthermore, the ability of $tRNA^{Arg}_{UCU}$ genes to rapidly mutate their anticodon and consequently decode like $tRNA^{Arg}_{CCU}$ brings up a question regarding the evolutionary constrains that determined copy number of tRNA families. If a single point mutation in one of the $tRNA^{Arg}_{UCU}$ copies leads it to perform like a tRNA from a different family, what were the evolutionary constraints that left some families with more members while others with fewer? Is there purifying selection to maintain "non-optimal" codons? To examine potential adaptive functions of tRNA family sizes, we tested the consequences of increasing the sizes of several tRNA families. We found that keeping low-copy tRNA families is adaptive, as increasing their size result in proteotoxic stress due to problems in protein folding.

Most of the published works thus far have tested how modified codon usage of specific proteins influences their proper folding. In contrast, we took a different approach and monitor the global proteotoxic level in the cell while modifying the tRNA pool. The proteotoxic stress observed in *WTmultiCCU* can result from various reasons. First, over-expression of $tRNA^{Arg}_{CCU}$ may mis-incorporate arginine into non-arginine codons. Second, other aminoacyl tRNA synthetases may aminoacylate a wrong amino acid to the highly expressed tRNA. Mis-loading will result in the incorporation of a different amino acid where arginine is needed. Both of these reasons should be relevant not only for the over-expression of $tRNA^{Arg}_{CCU}$ but also for the over-expression of $tRNA^{Arg}_{UCU}$. However, cells with a highly expressed $tRNA^{Arg}_{CCU}$ suffer from a greater growth defect and a severe proteotoxic stress than their equivalent $tRNA^{Arg}_{UCU}$ cells. The third, and maybe most intriguing hypothesis, suggests that the phenotypes observed for over expression of $tRNA^{Arg}_{CCU}$ are due to the increase in translation speed of the AGG codon. Since slow translation of low-frequency codons was shown on a single gene basis to influence correct folding of proteins[17,19,35], it is possible that keeping certain tRNAs at low concentrations may be essential for proper folding of specific domains during the synthesis of a protein. Indeed, this hypothesis explains why over expressing $tRNA^{Arg}_{UCU}$ is less harmful to the cell, as the translation speed of this tRNA is already high. We demonstrated that

over expression of $tRNA^{Arg}_{CCU}$ challenges the protein quality control machinery and generates a folding stress in the cell. These observations propose that selection may actively maintain tRNA genes at low copy numbers via the protein folding problems that are caused once the levels of a rare tRNA increase.

In conclusion, we propose that the tRNA pool is the plastic part of the translation machinery that allows it to adapt to changing environments. We demonstrate how mutations in the anticodon of tRNA genes are rapidly selected in order to meet the translational demand and to restore translational balance. Furthermore, we provide evidence for the maintenance of low-copy tRNA families by purifying selection and elucidate the evolutionary constrains that shape the tRNA pool in living cells.

4.5 Methods

Strains & Growth: All Saccharomyces cerevisiae strains were created on the genetic background of BY4741 (MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0).The creation of the strains Δ*tR(UCU)*, Δ*rad51* and Δ*rad52* was performed via transformation of a G418 resistance marker to the genes' loci by homologous recombination. During all experiments, cells were grown at 30^{o}c in either YPD(rich medium) or SD (poor medium). When needed, Galactose was supplemented to the medium to induce expression of a gene. YPD or SD-Ura were used for the lab-evolution experiments.

Plasmid construction& Restriction Free Cloning: All plasmids were cloned using the Restriction-Free cloning methodology[36]. The gene tR(UCU) was cloned into a high copy 2μ pRS426 plasmid[37] with a ranging number of up- and down-stream bps. See text for full explanation on the plasmid construction.

Lab-Evolution Experiments: All laboratory evolution experiments were carried out by serial dilution. The lab-evolution experiments were performed in 24 wells plate format (each well contained 1.2ml of culture). Cells were grown until reaching stationary phase under the relevant condition and were diluted by a factor of either 1:120 (6.9 generations per dilution) into fresh media once a day.

Liquid Growth Measurements: Deep stationary (48h) cultures were diluted into fresh medium and were grown at the relevant condition. OD_{600} measurements were taken every 45 minutes until reaching stationary phase. Qualitative growth comparisons were performed using 96 wells plates in which two strains were divided on the plate in a checkerboard manner to cancel out geographical effects. For each strain, a growth curve was obtained by averaging over 48 wells.

Measurements for Saturation of the Protein Quality Control Machinery: We utilized a previously published method that allows examination of the protein quality control of the cell[34]. This assay provides an indication for the protein unfolding stress in cells by assessing the load on the protein quality control machinery. In this assay, cells were introduced with a high copy number plasmid that contains the human gene von Hippel-Lindau (VHL) fused to a florescent tag (mCherry). VHL is a naturally unstructured protein whose proper folding in human cells depends on a complex formation with two other proteins, Elongin B and C.

Expressing VHL in yeast cells which lack VHL's complex partners, leads to misfolding of the translated proteins. Under normal conditions, the misfolded VHL proteins are handled by the cell's quality control machinery. When the quality control machinery is not saturated, the fluorescently tagged VHL appears in the cytosol. However, under stress, in which the quality control machinery is fully occupied, misfolded proteins in the cytosol are processed into dedicated inclusions (JUNQ and IPOD) and form punctum structures. Hence, a punctum phenotype of the VHL-mCherry construct is an indication that cells suffer from high protein misfolding levels and saturated protein quality control machinery. Selected strains were transformed with the plasmid and selected on SD-URA plates. Overnight cultures, were diluted (1:10) and visualized in the mid-log growth phase using an Olympus IX71 microscope controlled by Delta Vision software. Next, hundreds of cells (~1000) from each strain were counted for the number of cells with the puncta-phenotype and the results were normalized to the control strain.

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5. Concluding Remarks

The focal point of this study was the role of genomic duplication in adaptation and shaping the genome. Upon an environmental challenge, there is a set of genes, whose upregulation can result in higher survivability. Remarkably, when stepwise accumulation of mutations, in this set of critical genes, cannot be obtained in a timely manner a more rapid tool of genome evolution is utilized. Our results suggest that spontaneous duplications of chromosome (or chromosomal segments), resulted by chromosomal missegregation during cell cycle, provide the population with a much needed means to survive harsh and abrupt stresses. The small portion of cells that carry such duplications has much lower fitness under permissive conditions and thus such cells do not normally dominate the population. However, particular duplications confer a fitness advantage under specific selective pressures. Despite being effective as a first evolutionary line of defense that stabilizes population survivability under stress, more efficient solutions accumulate and further improve the fitness under the stress. This process gets to a point where the initial duplication becomes redundant, in the background of the new mutations, and therefore eliminated (presumably here too by chromosomal missegregation during cell cycle).

The details of this evolutionary process in which mutations fixate on top of the aneuploidy and eventually render the aneuploidy unnecessary remained to be studied. It is not clear if the transient aneuploidies observed in this study are merely local-maxima in the fitness landscape that can be avoided. Moreover, it is also possible that if we could exclude aneuploidic cells from the evolved population it would eventually result in a better adaptation to the stress.

Transient aneuploidy might also be relevant to malignancy processes, as more than 90% of solid cancer tumors exhibit aneuploidy. Malignancy as a result of a tumor suppressor gene (TSG) inactivation can occur if both alleles of a TSG are inactivated by sequence mutation. In addition it has been proposed that inactivation of one allele can be followed by elimination of the other allele by whole chromosome loss (Nowak et. al. 2006). Yet, since monosomy has the potential of inhibiting cell proliferation rates, I propose an alternative mechanism to inactivate both alleles of a TSG via acquiring and elimination of aneuploidy. Possibly, after the inactivation of the first TSG allele, the chromosome that carries the mutated allele can be duplicated by acquired aneuploidy. This chromosomal duplication will result in two copies of the

inactivated allele and yet another copy of the active TSG. From this point, elimination of the chromosomal copy that carries the active TSG gene can initiate malignancy. This hypothetical mechanism can accelerate malignancy since a single, relatively slow, step of TSG inactivation is replaced by two relatively fast steps of chromosomal missegregation. Obviously, this can accelerate malignancy only for cells in which the rate of two chromosomal missegregation events is higher than the rate of one inactivation event by sequence mutation.

The use of genomic duplications as a design principle in the evolution of tRNA gene families emphasizes the advantages of multiple-copy architecture. First, duplications of tRNA genes facilitate efficient protein synthesis as highly expressed genes tend to prefer codons of high tRNA family size. In addition, we suggest that the multiple-copies architecture allows the tRNA pool to rapidly change in order to alleviate translational imbalances. In our evolution experiments, a translational imbalance was induced due to a tRNA gene deletion, yet many organisms probably experience such imbalances due to environmental changes or upon migrating to a new niche. Upon environmental changes the expression pattern may change and a set of genes that were lowly expressed in the former environment may be highly expressed in the new environment. For an efficient protein synthesis, the tRNA pool has to accommodate the high expression of the newly up-regulated genes, i.e. to restore the translational balance. We hypothesize that during evolution, the adaptation of the translation machinery to expression changes can be first met by anticodon mutations of tRNA gene copies as well as by duplications and deletions of other copies. We suggest that only on a much longer evolutionary time-scale, codon usage of ORFs will change for further fine-tuning. Here we claim that the tRNA genes are the "plastic" part of the translation machinery in terms of adaptation to new conditions. An interesting implication of our hypothesis would be to aim for a specific condition for which one can try and predict a set of few mutations in several tRNA gene copies that would result in better fitness under the specified condition.

6. Declarations

This thesis summarizes my independent research, yet some parts are the result of the following fruitful collaborations:

1) The ancestral strain that was used in the yeast evolution under high pH and the spore analysis on this strain, was done in the Lab of Martin Kupiec at the Tel-Aviv University, by Gal Romano.

2) The yeast strain $\Delta tRNA^{Arg}_{CCU}$ was created by Zohar Bloom from our lab.

3) Mutations scans in the tRNA genes were performed by Idan Frumkin from our lab, and proteotoxic stress assays were run mutually with Idan Frumkin and Zohar Bloom.

4) The different serine tRNA strains and plasmid were created in the lab of Jef D. Boeke by Qinghua Feng.

7. Acknowledgments

I would like to thank my mentor, Prof. Yitzhak Pilpel, for nurturing me and provide a great inspiration for my scientific journey.

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8. Appendix

8.1 Yona et. al. *PNAS* 2012:

Chromosomal duplication is a transient evolutionary solution to stress

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Aneuploidy, an abnormal number of chromosomes, is a widespread phenomenon found in unicellulars such as yeast, as well as in plants and in mammalians, especially in cancer. Aneuploidy is a genome-scale aberration that imposes a severe burden on the cell, yet under stressful conditions specific aneuploidies confer a selective advantage. This dual nature of aneuploidy raises the question of whether it can serve as a stable and sustainable evolutionary adaptation. To clarify this, we conducted a set of laboratory evolution experiments in yeast and followed the long-term dynamics of aneuploidy under diverse conditions. Here we show that chromosomal duplications are first acquired as a crude solution to stress, yet only as transient solutions that are eliminated and replaced by more efficient solutions obtained at the individual gene level. These transient dynamics of aneuploidy were repeatedly observed in our laboratory evolution experiments; chromosomal duplications gained under stress were eliminated not only when the stress was relieved, but even if it persisted. Furthermore, when stress was applied gradually rather than abruptly, alternative solutions appear to have emerged, but not aneuploidy. Our findings indicate that chromosomal duplication is a first evolutionary line of defense, that retains survivability under strong and abrupt selective pressures, yet it merely serves as a "quick fix," whereas more refined and sustainable solutions take over. Thus, in the perspective of genome evolution trajectory, aneuploidy is a useful yet short-lived intermediate that facilitates further adaptation.

evolutionary dynamics | environmental stress | heat tolerance | pH tolerance

Adaptation to stressful conditions often requires modified expression of certain genes that can generally be obtained by genome sequence changes. In addition, structural rearrangements of the genome, and in particular chromosomal duplications, that lead to aneuploidy, may offer a simple means to boost expression level (1, 2). Indeed, cancer cells often exercise massive genomic duplications, particularly of regions that harbor growth-promoting genes (3). Unicellular organisms, too, can duplicate chromosomes that contain genes needed at a given condition (4–9). The high prevalence of chromosomal duplications, especially under stress (10), accounts for the frequent acquisition of aneuploidies on a short evolutionary timescale (11). Chromosomal duplication may indeed offer the advantage of simultaneous elevation of a large set of genes, some of which may be beneficial under a particular selective pressure. Whole genome duplications, too, can offer selective advantage under specific conditions (12), yet genome analysis has suggested that they also survive only under specific conditions (13). Indeed, whole genome and chromosomal duplication constitute crude solutions with significant overheads on the cell that are in part associated with increased copies of DNA, RNA, and proteins (1, 2). Duplication of particular chromosomes (i.e., aneuploidy) creates, in addition, a stoichiometric imbalance between gene products (14, 15) and promotes further genome destabilizing events (16, 17). Thus, it appears that aneuploidy is concurrently advantageous and highly costly (18, 19). Therefore, it is not clear under what conditions and to what extent organisms will adopt this solution. Here we offer a resolution of the dual nature of aneuploidy, in the form of dynamic and prolonged laboratory evolution experiments in yeast. We show that aneuploidy that is rapidly gained under stress is a transient solution that is replaced by focal, refined, and sustainable solutions that require more time to evolve.

Results

Acquisition and Subsequent Elimination of Chromosome III Trisomy During Adaptation to Heat. Toward evolving stress-tolerant yeast strains, we applied the well-established methodology of laboratory evolution through serial dilution (20). In this procedure, populations of yeast cells are grown under a certain condition and are diluted daily into fresh medium, still under the same condition. Our laboratory evolution experiments all together create a highly branched evolutionary scheme (Fig. 1). We started with diploid *Saccharomyces cerevisiae* cells that were grown in four independent repetitions in rich medium under a constant heat stress of 39 °C. After an evolution period of 450 generations, all populations were examined, and duplication of chromosome III (trisomy) was detected in all four repetitions (Fig. 2). Two additional segmental duplications occurred in two repetitions on chromosomes IV and XII. We focused on the two populations in which only chromosome III was duplicated (Fig. S1), as they provide a useful model for single acquired aneuploidy, and further evolved them under the same heat stress. Strikingly, we observed elimination of chromosome III trisomy within 2,350 generations (Fig. 2). We then turned to carefully study the dynamics of evolutionary gain and loss of aneuploidies under various growth conditions in which we vary temperature, nutrient availability, and pH.

Causal Link Between Chromosome III Copy Number and Heat Tolerance. We began by determining whether the trisomy was selected for its direct contribution to heat tolerance or whether it was merely hitchhiking on another beneficial mutation and thus was lost further along the evolution under heat. To that end, strains that carry chromosome III aneuploidies (without evolution under heat) were obtained and measured for their heat tolerance (*Materials and Methods*). One strain is a diploid with a trisomy of chromosome III, termed here *WTtrisomeIII* and the other is a diploid with a monosomy of chromosome III, termed *WTmonosomeIII*. Interestingly, *WTtrisomeIII* showed elevated heat

Author contributions: A.H.Y., Y.P., and O.D. conceived and designed the project; A.H.Y., Y.S.M., R.H.H., and G.H.R. performed research; M.K. designed experiments and contributed strains; All authors analyzed data; A.H.Y., Y.P., and O.D. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE40817).

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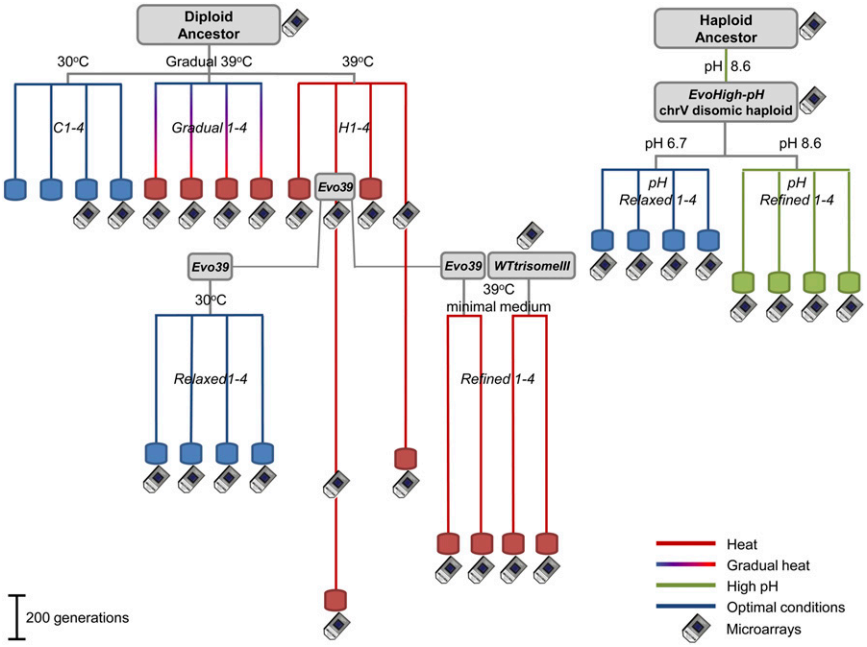

Fig. 1. Laboratory evolution (Lab-evolution) tree describing the experimental outline. Each evolution experiment starts with an ancestor strain (gray box) that was subjected to certain growth conditions: high temperature, 39 °C (red); permissive temperature, 30 °C (blue); gradually increasing temperature, from 30 °C to 39 °C (gradient line); and high pH 8.6 (green). Parallel lines splitting from the same branch represent independent repetitions and their length is in scale with the number of generations under the specified condition. *Evo39* strain was taken after 450 generations on high temperature (39 °C) as an ancestor for another two evolutionary branches (*Refined 1–4* and *Relaxed 1–4*). Microarray icons represent points during the evolution tree in which such measurement was performed.

tolerance at a level that is similar to that of the trisomic strains that evolved under heat. On the other hand, *WTmonosomeIII* exhibited lower heat tolerance than the diploid wild type (Fig. 3*A*). These findings show a clear quantitative correspondence between chromosome III copy number and heat tolerance. To confirm that the extra chromosome is the predominant genetic change underlying the heat tolerance, tetrad analysis was performed both on a strain that evolved at 39 °C for 450 generations and became trisomic (*H2* after 450 generations, abbreviated as *evo39*) and on *WTtrisomeIII*. These two trisomic strains were subjected to meiosis and the heat tolerance of the haploid progeny (spores) was measured. The results showed that all euploid spores (normal number of chromosomes) have heat tolerance similar to a haploid, whereas all disomic spores, which carry two copies of chromosome III, have a marked increase in heat tolerance (Fig. 3*B*). We further examined whether chromosome III trisomy confers advantage under other stress conditions by measuring the tolerance of *WTtrisomeIII* to a battery of other stresses. We found that duplication of chromosome III decreased the tolerance to all of the other stresses tested (Fig. 3*C*). Therefore, increased heat tolerance cannot be attributed to a general stress tolerance conferred by chromosome III trisomy. Together, these results establish a causal link between the relative number of chromosome III copies and heat tolerance and may explain why this chromosomal duplication was repeatedly fixated in all laboratory evolution experimental lines under heat.

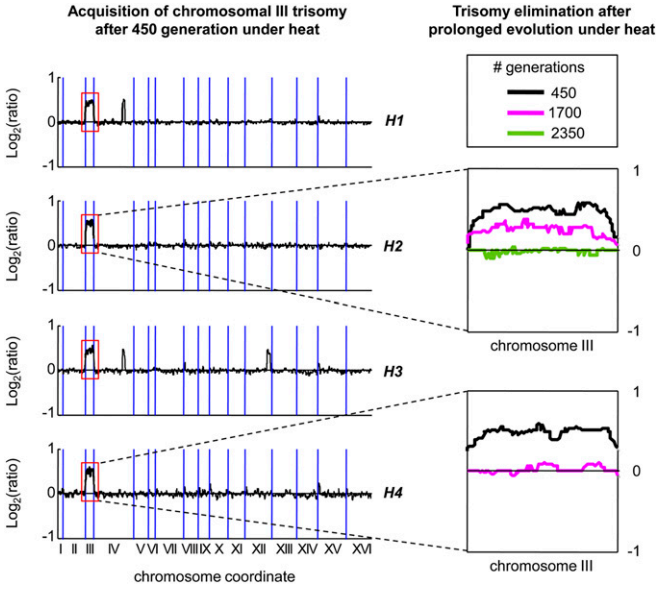

Fig. 2. Aneuploidy appears and subsequently was eliminated during evolutionary adaptation to heat. Four independent repetitions (*H1–4*) that evolved for 450 generations in rich medium and heat (39 °C) show chromosomal duplications (black lines). Notably, duplication of chromosome III occurred in all four repetitions. *H2* and *H4*, which carry no large-scale duplication other than chromosome III trisomy, were further evolved under the same conditions and after 1,700 generations (magenta lines) and 2,350 generations (green line), the trisomy was eliminated. All lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of heat-evolved strain over a diploid wild type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes.

Cost Associated with Chromosome III Trisomy. An additional copy of chromosome III thus clearly contributes to heat tolerance, yet its elimination later on raises the hypothesis that the substantial burden associated with chromosomal duplications (1) prevents the trisomy from serving as a sustainable solution to the stress. Therefore, a next goal was to assess the costs associated with the chromosome III trisomy and to characterize the role of these costs in the trisomy elimination. We reasoned that such costs can be measured when neutralizing the benefit, i.e., when heat is not applied. For that reason, the cost of chromosome III trisomy was measured in terms of growth defect by comparing *WTtrisomeIII* to a diploid wild type, at the permissive temperature (30 °C). A considerable cost of the trisomy was detected in rich medium and a further cost increase was detected in minimal medium (Fig. 3*D* and Fig. S2).

Optimized Adaptations Replace the Aneuploidy-Based Solution. Due to the increased cost of the trisomy in minimal medium, we hypothesized that during prolonged evolution in minimal medium and heat, the trisomy would be eliminated more rapidly compared with rich medium and heat. Following this hypothesis, an additional evolution experiment was carried out. We started with two ancestral

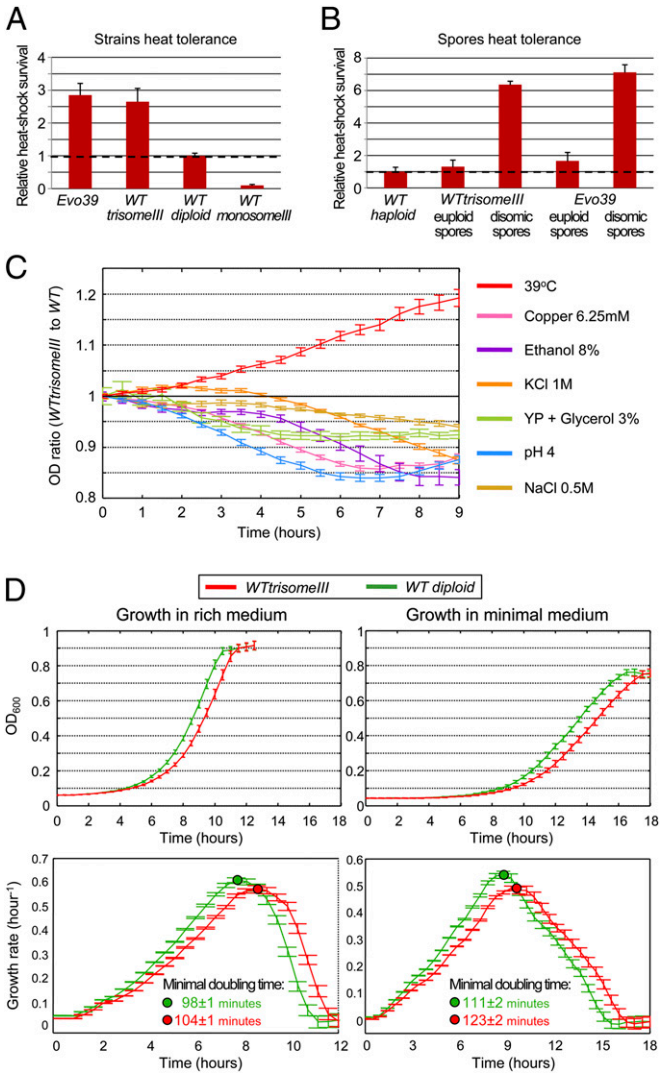

Fig. 3. An extra copy of chromosome III is beneficial under heat, yet it is maladaptive under other conditions. (*A*) Heat-shock tolerance rates are proportional to the copy number of chromosome III. Heat-shock survival fold change of chromosome III aneuploidic strains compared with a diploid wild type is shown (dashed line). (*B*) The extra copy of chromosome III is the predominant genetic trait responsible for the increased heat tolerance. Heat-shock survival fold change of spores from *WTtrisomeIII* and *evo39*, compared with a haploid wild type (dashed line), presented separately for euploid spores and for chromosome III disomic spores (P value $< 8 \times 10^{-5}$; for spore karyotype, see Fig. S7). (*C*) The growth advantage conferred by chromosome III trisomy under heat (red line) cannot be attributed to a general stress tolerance. Colored lines represent OD ratios of *WTtrisomeIII* over a diploid wild type during continuous growth under various stresses (*Materials and Methods*). (*D*) The cost of chromosome III trisomy at permissive temperature (30 °C) is increased on minimal medium compared with rich medium. Growth curve measurements of *WTtrisomeIII* (red) and of a diploid wild type (green) are shown in OD values over time during continuous growth at 30 °C (*Upper*), in rich medium (*Left*) and in minimal medium (*Right*). (*Lower*) Growth rate analyses, derived from the OD values, and the differences in minimal doubling time between *WTtrisomeIII* and *WT*. Data are presented as mean and SEM.

strains that carry chromosome III trisomy, namely *evo39* and *WTtrisomeIII*. Interestingly, after 1,000 generations under heat and in minimal medium, all four repetitions (termed *Refined 1–4*), from both ancestral strains, eliminated the extra copy of chromosome III (Fig. S3). These evolutionarily refined strains, which eliminated the cost associated with the trisomy, exhibit improved growth both at the permissive and the high temperatures compared with their trisomic ancestors (Fig. 4*A*). The rapid elimination in minimal medium compared to rich medium shows that the high cost of the trisomy accelerates the evolutionary dynamics of its elimination.

Focusing on these refined evolutionary resultants, it is expected that the original contribution of trisomy to heat tolerance was replaced by alternative solutions. We hypothesize that such refined solutions could take over the trisomy by conferring heat tolerance, while avoiding much of the cost associated with aneuploidy. To characterize the refined solutions, we examined the gene expression of the newly evolved refined strains from the different repetitions. A subset of 17 genes on chromosome III was found to retain elevated expression despite the elimination of the extra chromosome copy (Fig. 4*B*). Next, we checked whether these genes can contribute to heat tolerance when introduced to a wild-type strain, one at a time. To this end, a diploid wild-type strain was transformed with centromeric plasmids (21), each containing one of these genes. As a negative control, 22 random genes from chromosome III that did not retain elevated expression were inserted in the same manner. Reassuringly, most of the genes that retained high expression demonstrated an increased heat tolerance in the transformed wild-type cells (Table S1), with the highest contribution being as high as 23.5% of the heat tolerance of *WTtrisomeIII* (defined here as 100% heat tolerance) (Fig. 4*C*; for heat tolerance functional analysis, see *SI Text*). In contrast, none of the genes from the negative control set had a considerable effect on the wild-type heat tolerance (mean −2.4% ± 2.6%, Table S1). Note that the contribution of the individual genes adds up to more than 100% (defined as the tolerance of the trisomic strain). This probably reflects the fact that the trisomic level of heat tolerance includes the considerable cost of aneuploidy and may also suggest a negative epistasis between the contributions of the individual genes (22). Overall, these results indicate that a part of the solution to the heat challenge, obtained by duplicating chromosome III, was replaced during the extended evolution period under heat by solutions based on refined gene expression changes on chromosome III (and on other chromosomes, as mentioned below).

Genes on other chromosomes may have also evolved changes in expression level after the elimination of the extra copy of chromosome III, and it is most likely that they, too, must have contributed to the evolution of heat tolerance. A particularly interesting example are the heat-shock proteins (23) (HSPs) that are scattered on 11 different chromosomes. We analyzed all of the 14 annotated verified HSPs in the yeast genome (24) for changes in expression in the *evo39* strain compared with its descendants that eliminated the trisomy. Curiously, whereas the expression levels of these heat-shock genes changed very modestly after 450 generations of *evo39* strain, during which the trisomy was fixated, most of these genes showed significant up-regulation in their refined descendants, i.e., after another 1,000 generations under heat, when the trisomy was eliminated (Fig. 4*D*). Three of the 14 *HSP* genes did not show this trend, including *HSP78*, a gene coding for mitochondrial heat-shock protein and *HSP30*, which resides in the eliminated chromosome III. This indicates that the trisomy-based solution to heat does not require a concomitant up-regulation of the heat-shock genes, whereas the replacement of the trisomy is associated with an enhancement in the evolutionary expression of the heat-shock genes. This scenario may thus imply that the duplication of a certain chromosome may provide the evolutionary time window needed for the population to search for solutions not only on the duplicated chromosome but throughout the genome.

Elimination of the Extra Copy of Chromosome III at Permissive Temperature. Another consequence that follows the above-stated properties of acquired aneuploidy would be that when the stress is relieved, i.e., when the benefit diminishes and the cost remains, aneuploidy will be selected against (25). In fact, continued evolution of *evo39* under permissive temperature (30 °C) eliminates the extra copy of chromosome III after 600 generations, and with it, its tolerance to heat (Fig. S4). Reassuringly, the trisomy elimination

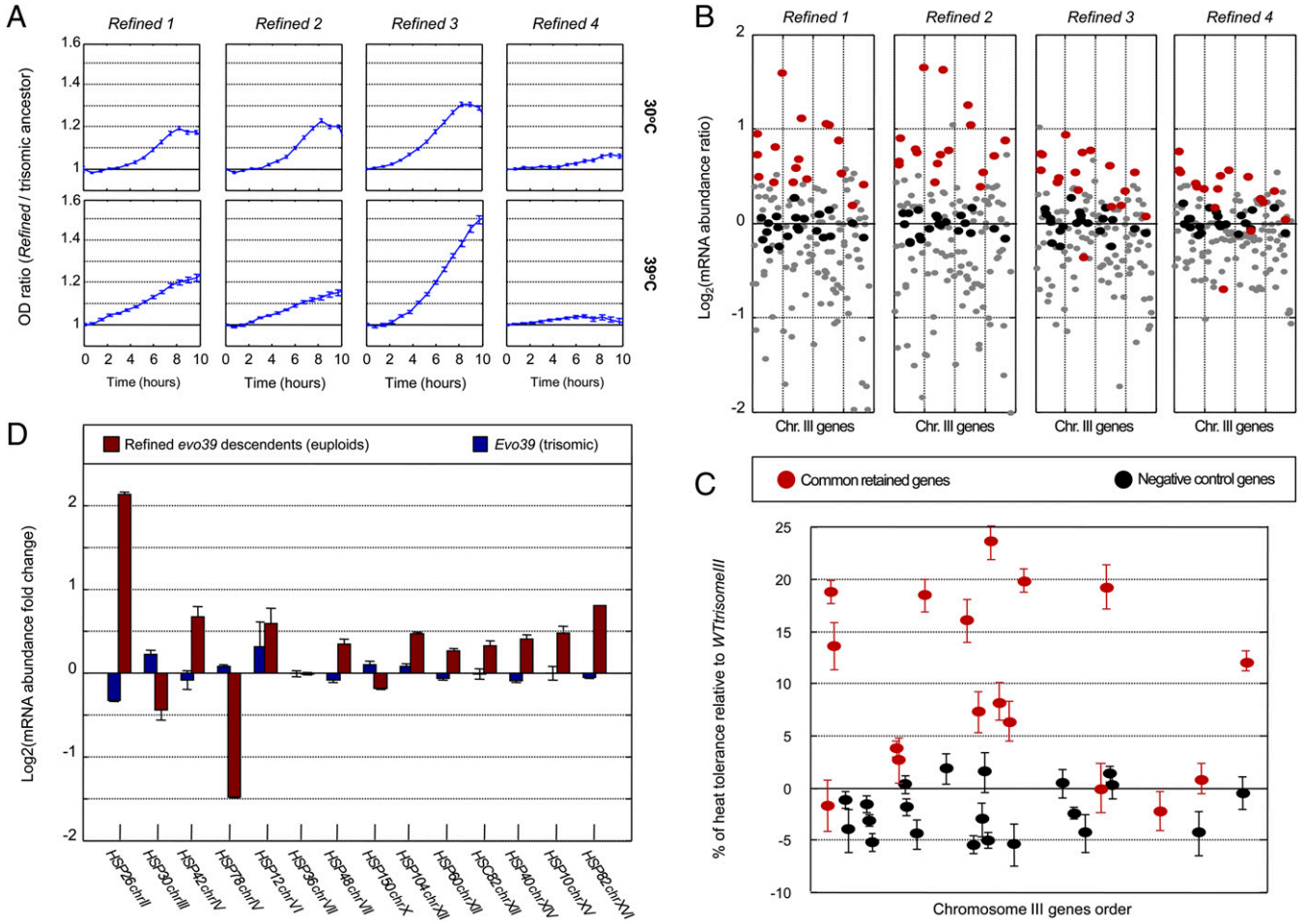

Fig. 4. The aneuploidy-based adaptation was evolutionarily eliminated and replaced by more economical solutions based on refined gene expression adaptations. (*A*) Descendants of chromosome III trisomic ancestors that were further evolved under heat and lost the trisomy (*Refined 1–4*) show improved growth under heat, yet with less cost compared with their trisomic ancestors. Each subgraph shows the OD ratios of a refined descendant over its trisomic ancestor measured during continuous growth at 30 °C (*Upper*) and at 39 °C (*Lower*). (*B*) Despite elimination of the trisomy in *Refined 1–4*, a group of genes from chromosome III retained high expression levels. Dots represent $\log_2$ ratios of mRNA abundance of chromosome III genes over a diploid wild type. Genes that retain high expression (in at least three of the four refined evolutions; for details see *SI Text*) are marked in red, and from the majority of genes that went back to wild-type–like expression (gray dots) a control group was selected and marked in black (used in *C*). These retained genes were also up-regulated in *H2* and *H4*, which also eliminated the trisomy (χ^2 *P* values 4×10^{-5} and 3×10^{-2}). (*C*) The group of genes that retain high expression levels, after the elimination of the trisomy, confers increased heat tolerance when introduced into wild type. Each of the highly expressed genes (red) and the negative control genes (black) was inserted into the diploid wild type, on a centromeric plasmid, and heat-shock tolerance was compared with the heat tolerance of *WTtrisomeIII* (*t* test *P* value $< 5 \times 10^{-7}$; *Materials and Methods*). (*D*) The refined solution replacing the trisomy is characterized by changes in expression levels of most *HSP* genes. Log_2 expression ratios over wild type are shown for all HSPs, for trisomic *evo39* (blue) and its descendants that eliminated the trisomy (red). Data are presented as mean and SEM.

in all four repetitions attests to its superfluous cost at permissive temperature, when it is no longer beneficial. This experiment also demonstrates the limited durability of aneuploidy-based solutions, as they are quickly eliminated when organisms evolve in the absence of the original stress.

Evolution to Gradually Applied Heat Avoids Aneuploidy-Based Solutions. To this point, aneuploidy had been observed to evolve in response to an abrupt evolutionary challenge. We next hypothesized that an evolution experiment in which the stress is applied in a gradual manner might not select for chromosomal duplication, because under minor stress increments, the considerable cost associated with aneuploidy might surpass the benefit at each increment of the stress. To explore this hypothesis, we evolved the same ancestral strain toward tolerance to heat, but in increments of 1 °C every 50 generations (from 30 °C up to 39 °C). This evolutionary design resembles the "morbidostat" that was recently applied to evolution of drug resistance in bacteria (26). Curiously, under this regime an extra copy of chromosome III was not gained after 450 generations in any of the four repetitions (termed *Gradual 1–4*) of the experiment (Fig. S5*A*). Nonetheless, the growth of these gradual populations was better at 39 °C (and also at 30 °C) than that of the four populations (*H1–4*) that acquired trisomy in the same evolutionary time under constant 39 °C (Fig. S5*B*). This experiment indicates that gradual application of a stress alleviates the need to adopt the rapidly available yet highly costly solution of chromosomal duplication.

Acquisition and Subsequent Elimination of Chromosome V Aneuploidy During Adaptation to High pH. To examine the generality of the findings obtained from evolution experiments under heat, we also examined evolutionary dynamics under an additional physiological stress of high pH. For this experiment, we used an evolved strain from a previous experiment (27) in which a haploid *S. cerevisiae* was evolved under high pH (8.6), using a similar serial transfer protocol. We examined the evolved strain, termed here *evoHigh-pH*, for potential chromosomal duplications. Interestingly, in addition to

seven point mutations previously detected in the genome sequence of the strains (27) (Table S2), we revealed that *evoHigh-pH* gained an extra copy of chromosome V (Fig. 5*A*). To show a direct contribution of the additional copy of chromosome V to the high pH tolerance, *evoHigh-pH* was crossed with its wild-type ancestor, sporulated and the meiotic products (spores) were analyzed. Among 40 spores scanned, we identified a pair of spores that carried the exact same subset of mutations (Table S2) and differed only in the copy number of chromosome V (one spore, an euploid with one copy of the chromosome, and the other, a disome with two copies; Fig. 5*A*). Reassuringly, the disomic spore showed better growth at high pH (Fig. 5*B*). However, when growth was measured at normal pH (6.7), the disomic spore exhibited a reduced growth compared with the euploid spore (Fig. 5*B*). These results demonstrate a causal link between the extra copy of chromosome V and high pH tolerance.

We have further extended the analogy to the heat tolerance experiments to examine potential elimination of the extra copy of chromosome V under two evolutionary tracks: one in which *EvoHigh-pH* continues to evolve at high pH and another in which the stress was relieved and *EvoHigh-pH* was switched to evolve under normal pH conditions (pH 6.7). In both tracks we detected gradual elimination of the extra copy of chromosome V from the evolving populations (Fig. 5*C* and Fig. S6). The fact that here too, aneuploidy is reversed even if the stress persists indicates that such large duplications do not typically serve as sustainable evolutionary solutions.

Discussion

Aneuploidy is a readily available, yet costly, evolutionary solution. Such solutions are thus expected to be followed by refinement steps that would alleviate part of the costs associated with the original solution. We predict that such refined solutions will prove to be more durable than aneuploidy-based solutions due to low reversion rate even after prolonged periods in which the stress is not applied. On the other hand, chromosomal duplications may be quickly reversed, leaving minimal imprint on the genome, a potentially desired characteristic for a genomic solution to short-term stresses. Aneuploidy, among other genomic aberrations, is common in cancer too. An interesting possibility is that during the progression of cancer, aneuploidy-based malignancies might be refined and replaced by modifying expression of specific genes. Such dynamics would be consistent with the observation that cancer driver genes are more often characterized by high mRNA levels than by high gene copy numbers (28, 29).

This study emphasizes the importance of long-term laboratory evolution experiments (30). Limiting the experiments to only a few hundred generations would have shown the chromosomal duplication but would have not revealed the massive subsequent aneuploidy elimination toward refinements. On the other hand, in order not to miss fast transient solutions, such as the current aneuploidies, one must follow long evolutionary dynamics at relatively high temporal resolution. Future full genome sequencing of the strains in this highly branched laboratory evolution tree will further sharpen our understanding of the evolutionary dynamics examined here.

Materials and Methods

Strains. All evolved strains in this work were based on *BY4741* (*MATa*; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0) or *BY4743* (*MATa/MATα*; *his3*Δ1/*his3*Δ1; *leu2*Δ/*leu2*Δ0; *met15*Δ0/*MET15*; *LYS2/lys2*Δ0; *ura3*Δ0/*ura3*Δ0). *WTtrisomeIII* and *WTmonosomeIII* were obtained from the *Saccharomyces* Genome Deletion Consortium (31) and found to have aneuploidies of chromosome III (5) (for our verification of aneuploidy of these strains, see Figs. S8 and S9). For each of these strains, the original gene deletion was reversed by adding the deleted ORF on a centromeric plasmid (21). *H1–4* are evolution strains evolved at 39 °C and in rich medium. Evo39 is an abbreviation for one of the two *H1–4* strains that gained only the trisomy of chromosome III after 450 generations (*H2* in Fig. 1) and was verified by comparative genomic hybridization (CGH) microarrays to carry an extra copy of chromosome III (Fig. S1).

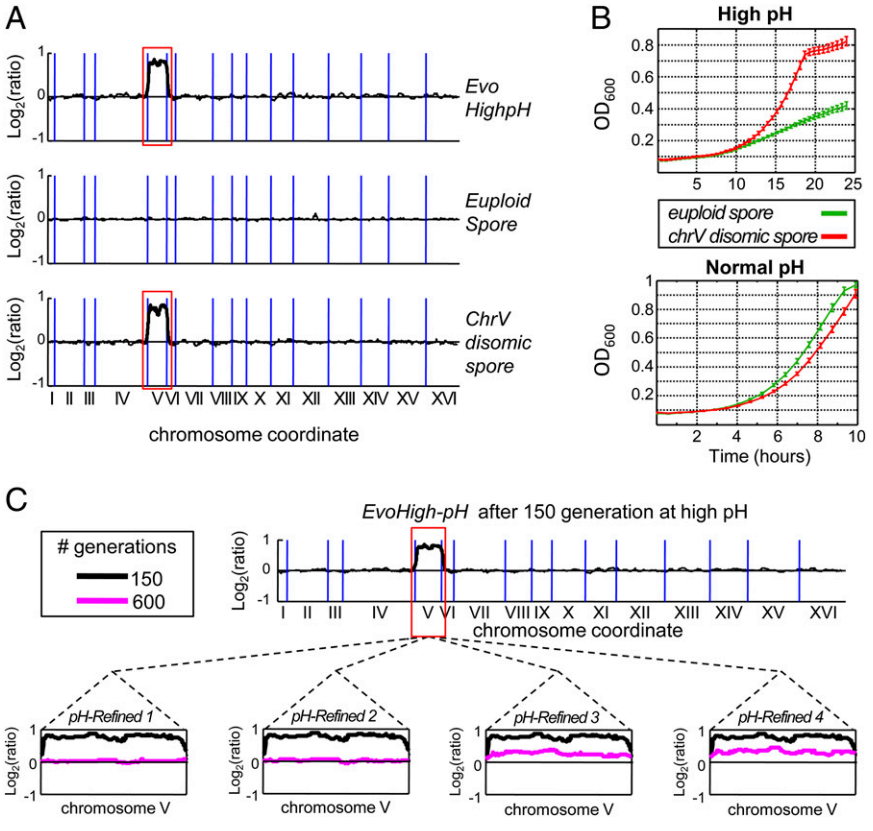

Fig. 5. Evolution on high pH selects for transient duplication of chromosome V, which is later eliminated. (*A*) Haploid wild type that evolved under high pH (27) acquired chromosome V disomy (*Top* subgraph). We identified two spores obtained from crossing the disomic evolved strain with a haploid wild type. These spores carry the same mutation subset (Table S2) but differ in the copy number of chromosome V (*Middle* and *Bottom* subgraphs). Black lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window over a haploid wild type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. (*B*) An extra copy of chromosome V confers high-pH (8.6) tolerance but causes impaired growth on normal pH (6.7). Growth curves of disomic spore (red) and euploid spore (green) under high pH (*Upper*) and normal pH (*Lower*). (*C*) Chromosome V disomy, gained by *EvoHigh-pH* after 150 generations at high pH (black lines), is eliminated during further evolution under the same high pH in which it was originally gained. Four independent descendants of *EvoHigh-pH* (*pH Refined 1–4*) continued to evolve for 600 generations at high pH (8.6). All evolved populations show elimination of the disomy (magenta lines) with two populations showing complete elimination (*Left* two) and the other two populations showing the majority of the population's cells eliminate the disomy (*Right* two). All lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of the high pH evolved strain over a haploid wild type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes.

Evo30 is an evolution strain evolved in parallel to *evo39* for 450 generations, but at the permissive temperature of 30 °C. *Refined 1–4* are evolution descendants of chromosome III trisomic strains (1 and 2 of *evo39* and 3 and 4 of *WTtrisomeIII*) that have been further evolved at 39 °C and in minimal medium for another 1,000 generations, with the extra copy of chromosome III eliminated, yet retaining high heat tolerance. *Relaxed 1–4* are evolution descendants of the chromosome III trisomic *evo39* that have been further evolved at 30 °C and in rich medium for another 600 generations, with the extra copy of chromosome III eliminated, and heat tolerance returned back, similar to a diploid wild type. *Gradual 1–4* are evolution strains evolved under gradually applied heat for 450 generations and without gaining chromosome III trisomy. *EvoHigh-pH* is a strain obtained from the study of Romano et al. (27) that has evolved under high pH (8.6). *pH Relaxed 1–4* are evolution descendants of *EvoHigh-pH* that have been further evolved at normal pH (6.7) for 280 generations. *pH Refined 1–4* are evolution descendants of *EvoHigh-pH* that have been further evolved at high pH (8.6) for 600 generations.

Evolution Experiments. All laboratory evolution experiments were carried out by serial dilution. Cells were grown until reaching stationary phase under the relevant condition and then diluted by a factor of 1:120 into fresh media (6.9 generations per dilution). This procedure was repeated daily until significant phenotype change was detected in population growth under the applied condition. In all measurements of evolved populations, we used a population sample and not selected clones (*SI Text*).

Media. YPD (Yeast Extract Peptone Dextrose) [(1 L) 10 g yeast extract, 20 g peptone, 20 g dextrose, and DDW (Double distilled water)] was used as rich medium and SD (Synthetic Defined) [(1 L) 6.7 g yeast nitrogen base, 20 g dextrose, 1.5 g amino acids mixed powder, and DDW] was used as minimal medium.

Liquid Growth Measurements. Cultures were grown at the relevant condition and OD_{600} measurements were taken during growth at 30-min intervals until reaching early stationary phase. Qualitative growth comparisons were performed using 96-well plates in which two strains were divided on the plate in a checkerboard manner to cancel out positional effects. For each strain, a growth curve was obtained by averaging over 48 wells.

Heat-Shock Tolerance Measurements. To eliminate physiological adaptations (due to prior exposure to heat before the measurements) all cultures were grown for 16 generations on 30 °C. Then, cultures in midlog phase were transferred to 45 °C (t_0) for 90 min (t_1). At each time point, samples were taken and plated on YPD plates and incubated at 30 °C. The survival ratio was calculated by dividing the number of colonies obtained in t_1 by the number of colonies obtained in t_0.

Heat Tolerance of Selected Genes from Chromosome III. To measure the heat tolerance contribution of selected genes from chromosome III when inserted as extra copies into a diploid wild type, we used centromeric plasmids (21). Heat-shock tolerance was measured for each of these strains compared with a diploid wild type with an empty plasmid. We defined 100% heat tolerance as the tolerance of *WTtrisomeIII* and 0% tolerance as the tolerance of diploid wild type with an empty plasmid. For each of the transformed strains, heat tolerance was calculated by dividing over the *WTtrisomeIII* tolerance, after subtracting the tolerance of wild type with an empty plasmid.

General Stress Tolerance. Cultures of diploid WT and *WTtrisomeIII* were grown in rich medium until midlog phase and then transferred to one of the following conditions: YPD + KCl 1 M, YPD at pH = 4, YPD + ethanol 8% (vol/vol), YP + glycerol 3% (vol/vol), YPD + NaCl 0.5 M, or YPD + copper sulfate ($CuSO_4$) 6.25 mM. OD_{600} measurements were taken at constant intervals.

Microarrays. Affymetrix Yeast Genome 2.0 arrays were used to measure gene expression and to detect aneuploidies. The ability to detect large-scale genomic duplications by expression microarrays was based on previous studies that demonstrate the correspondence between chromosomal duplication and increased expression (1, 5). Agilent CGH microarrays were used to verify karyotype for selected strains (we report aneuploidy of evolved populations only when the aneuploidic part in population is >95%; see *SI Text*). Taking into consideration that adaptation could also be carried out at the physiological level, rather than encoded at a genetic/epigenetic level, all expression microarrays were preformed following 16 generations of growth at the normal temperature of 30 °C. This setup allows us to focus on the non-physiological component of adaptation. Genomic DNA and RNA extractions were performed using Epicenter Yeast kits. The microarray data for detection of chromosomal duplications are shown after performing sliding window averaging with a window size of 10 genes from each side. At each point the value represents the average of the values of the current window. All Affymetrix microarray data have been deposited in the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus and are accessible through GEO accession no. GSE40817 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40817).

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Supporting Information

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SI Text

Determining Aneuploidy and Other Measurements Performed on Samples from Evolved Populations. Throughout this study, in cases where we analyzed an evolved population, we always measured the population and not a single clone. This is so that the measurements reflect the mean value for all of the population and not of a potential subpopulation from which a single clone could have been taken for measurement. Because we measure a population of cells, in the cases of determining aneuploidy we had to assess the part of the population that contains the reported aneuploidy by comparing it to a microarray of a monoclonal strain with the same aneuploidy. In all cases where we report aneuploidy, the estimated part of the population that is reported to either gain or eliminate a chromosome is at least 95%, as judged by the comparison with the reference strain. Nonetheless, in some cases, the population was more heterogenic and indeed we note that the trait has not yet been entirely fixated (see for example Fig. 5*C*, Refined 3 and 4).

Heat Tolerance Measurements: Liquid Growth vs. Heat-Shock Survival. In this study, we use two assays to compare heat tolerance of different strains: liquid growth and heat-shock survival. The liquid growth curve comparisons in the various conditions are the most suitable way to compare evolved strains, because evolution itself is performed via liquid growth under defined conditioned. Thus, our method is based on the notion that the test should be in the same setup in which the evolutionary process took place. Also, when comparing two strains that did not go through evolution, it is legitimate to compare them by growth rate analysis (Fig. 3*D*). However, when we compare a strain that evolved via liquid growth transfer to another strain that did not go through such an evolutionary process (e.g., when comparing *evo39* and *WTtrisomeIII* on heat stress), we must not use the growth rate analysis because during the evolution, strains adapt to the liquid growth itself in addition to the adaptation to the high temperature. Thus, in such cases the more reliable way to compare heat tolerance is to measure heat-shock survival ratio (as done in Fig. 3*A*). In addition, when appropriate, each heat survival analysis in Fig. 3*A* is also backed up by growth rate comparison in Fig. 3*D* and in Fig. S2.

Selection of Genes from Chromosome III That Retain High Expression Level After the Elimination of Chromosome III Trisomy Under Heat. We defined the genes from chromosome III that retained high expression level, despite the trisomy elimination, based on the four trisomic populations that eliminated chromosome III trisomy during 1,000 generations under heat (*Refined 1–4*). Only genes that maintained average $\log_2$ expression change (compared with WT) above 0.46 in at least three of the four repetitions were selected. The 0.46 criterion is based on the average expression increase in chromosome III genes observed in the trisomic *evo39* (compared with its diploid ancestor), which was 0.4562 ($\log_2$). This definition led to 23 genes from chromosome III that retained high expression, of which 17 were available in the Molecular Barcoded Yeast plasmid library (1). These genes were examined in Fig. 3. For these genes, gene ontology analysis did not show any significant term compared with all other genes on chromosome III.

Heat Tolerance Functional Analysis of Genes from Chromosome III That Retain High Expression Level After Elimination of Chromosome III Trisomy Under Heat. We have focused on the genes with the most substantial heat tolerance contribution when inserted singly into a wild-type cell (Fig. 4*C*). For each of these genes, we looked for further evidence to support their substantial heat tolerance contribution. Reassuringly, we indeed find that YCR065W (*HCM1*), which had the highest heat tolerance contribution (23.5%), was reported in a study to confer increased heat tolerance when overexpressed in yeast (2). For YCR045C (*RRT12*), which had 19.3% heat tolerance contribution, we preformed a heat-shock expression profile (mRNA abundance) and found an induction of more than 27-fold upon 45 min of heat shock (42 °C). We performed the same method for YCR071C (*IMG2*), which had 18.4% heat tolerance contribution and observed induction of 1.5-fold upon 90 min of heat shock. For these last two genes, we also found a study that reports heat sensitivity phenotype upon deletion (3). Two of the five most-heat-contributing genes that we found on chromosome III are still uncharacterized ORFs in yeast, yet they also show high responsiveness to heat shock. Interestingly, YCR016W (19.9% heat tolerance contribution) shows a fast, strong repression upon 15 min of heat shock, which is followed by a growing induction as the heat persists, and YCR102C (18.8% heat tolerance contribution) shows a 3-fold induction upon 90 min of heat shock.

1. Ho CH, et al. (2009) A molecular barcoded yeast ORF library enables mode-of-action analysis of bioactive compounds. *Nat Biotechnol* 27(4):369–377.
2. Rodriguez-Colman MJ, et al. (2010) The forkhead transcription factor Hcm1 promotes mitochondrial biogenesis and stress resistance in yeast. *J Biol Chem* 285(47): 37092–37101.
3. Sinha H, et al. (2008) Sequential elimination of major-effect contributors identifies additional quantitative trait loci conditioning high-temperature growth in yeast. *Genetics* 180(3):1661–1670.

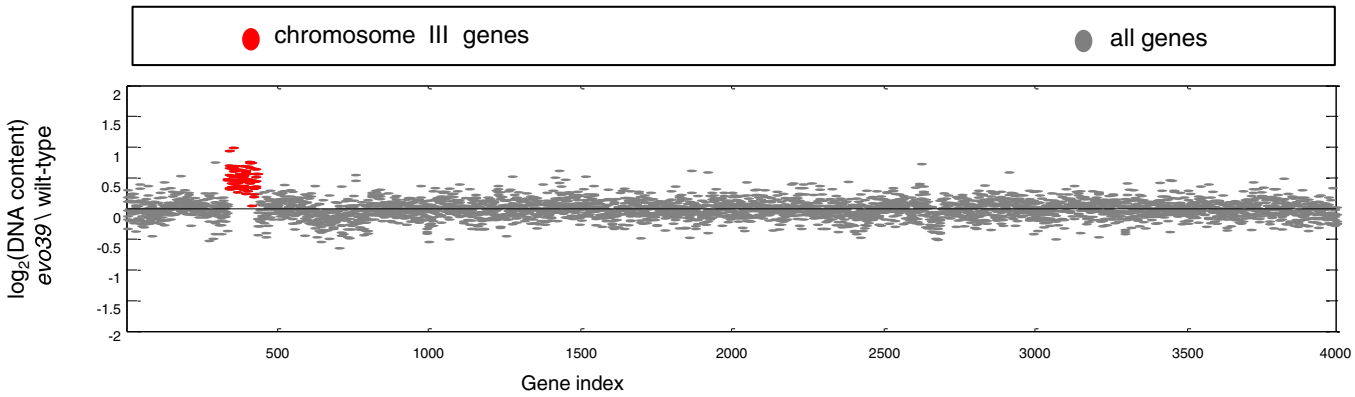

Fig. S1. Verification of chromosome III trisomy in *evo39*. Shown here is the elevated DNA content level of the segment that spans chromosome III. Dots represent $\log_2$ intensity ratios of DNA copy number, measured by DNA hybridization microarrays, of *evo39* over a diploid wild type, aligned according to chromosomal order where red dots represent genes from chromosome III and gray from all other chromosomes.

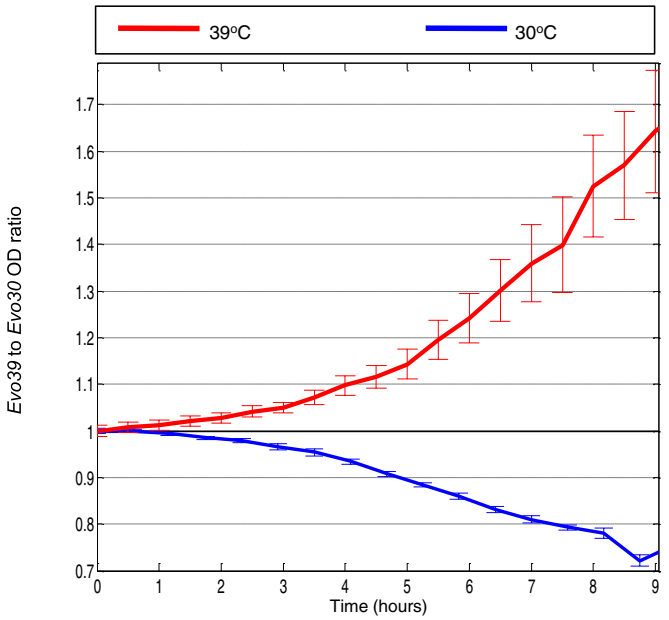

Fig. S2. The benefit and cost of chromosome III trisomy. *Evo39*, a strain that evolved 450 generations under heat and gained an extra copy of chromosome III, is growing better under heat than *evo30*, a strain that evolved at permissive temperature for the same number of generations and remained euploid. The results are reversed when measuring growth at 30 °C; when heat is not applied, the extra copy of chromosome III decreases the growth of *evo39* compared with the euploid *evo30*. Values represent OD ratios of *evo39* over *evo30* measured during continuous growth at 39 °C (red) and at 30 °C (blue). Data are presented as mean and SEM.

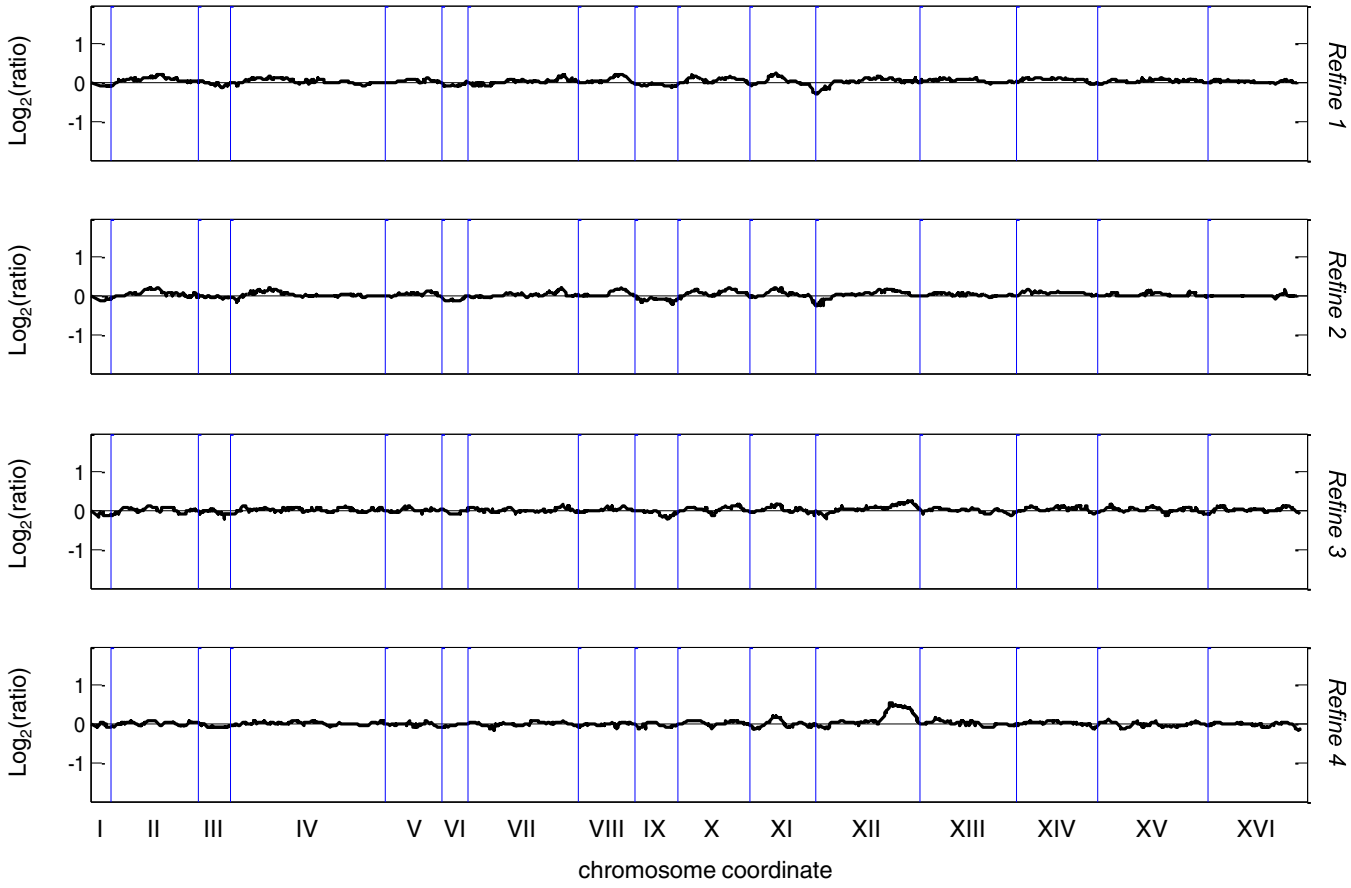

Fig. S3. Chromosome III trisomic strains, further evolved under heat, eliminated the trisomy. Four independent repetitions, descendants of *evo39* (*Refined 1* and *2*) and *WTtrisomeIII* (*Refined 3* and *4*), were further evolved for 1,000 generations under heat (39 °C) and minimal medium. All lines represent $\log_2$ intensity ratios of mRNA abundance, calculated by a sliding window of heat-evolved strain over a diploid wild type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. Whereas chromosome III trisomy was eliminated in all four repetitions, in *Refined 4* it appears that a subpopulation also duplicated a segment in chromosome XII. Interestingly, *Refined 4* shows the lowest improvement under heat and the lowest cost reduction compared with the other *Refined 1–3* (Fig. 4*A*).

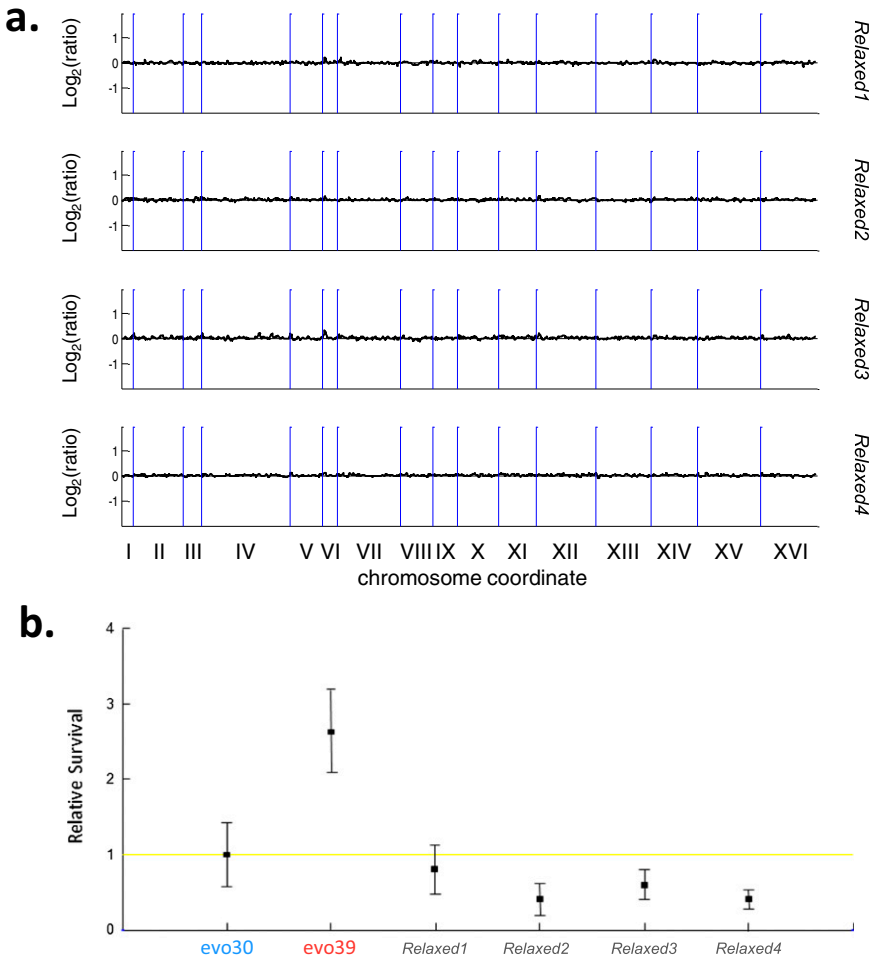

Fig. S4. Chromosome III trisomic *evo39* strain, further evolved at permissive temperature eliminated the trisomy and lost its increased heat tolerance. Four independent repetitions, descendants of *evo39*, were further evolved for 600 generations under 30 °C and rich medium (defined as *Relaxed 1–4*). (*A*) Chromosome III trisomy was eliminated in all four repetitions. Black lines represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of permissive temperature-evolved strain over a diploid wild type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. (*B*) Heat-shock tolerance rates decreased with the elimination of chromosome III trisomy. Shown is the heat-shock survival fold change of chromosome III trisomic *evo39* and its four descendants *Relaxed 1–4*. Yellow line represents the heat-shock survival of *evo30* that was evolved in parallel to *evo39* but at 30 °C and it remained euploid. Data are presented as mean and SEM.

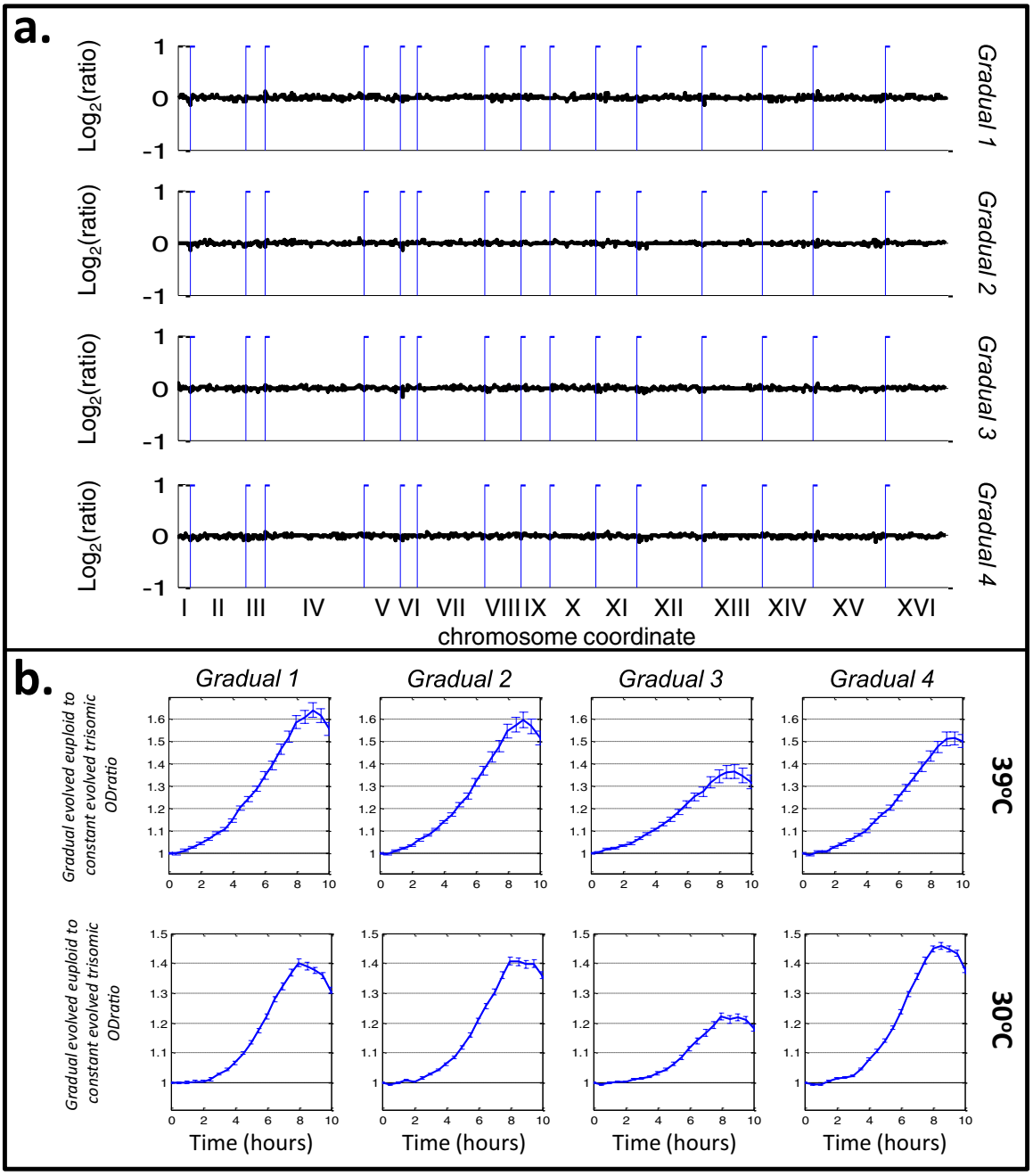

Fig. S5. Cells evolved under gradually applied heat do not adopt aneuploidic solutions. The laboratory evolution experiment in which four independent repetitions were evolved under 39 °C and duplicated chromosome III (*H1–4*) was repeated under the same conditions but heat was applied in a gradual manner, in four independent repetitions (*Gradual 1–4*). For these strains evolution started at 30 °C and every 50 generations the temperature was raised by 1 °C. Total number of generations was 450, identical to the number of generations in the case of *H1–4*. (*A*) All *Gradual 1–4* remained euploid, i.e., chromosome III trisomy was not detected in any of the four repetitions. Black lines represent $\log_2$ intensity ratios of mRNA abundance, calculated by a sliding window, of gradual heat-evolved strain over a diploid wild type, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes. (*B*) Comparing the growth of the euploid *Gradual 1–4* to the growth of the trisomic *H1–4* shows better growth of the gradual evolving strains both at 39 °C and at 30 °C. Each subgraph shows the OD ratios of one of the gradual strains over its nongradual counterpart, measured during continuous growth at 39 °C (*Upper*) and at 30 °C (*Lower*). Data are presented as mean and SEM.

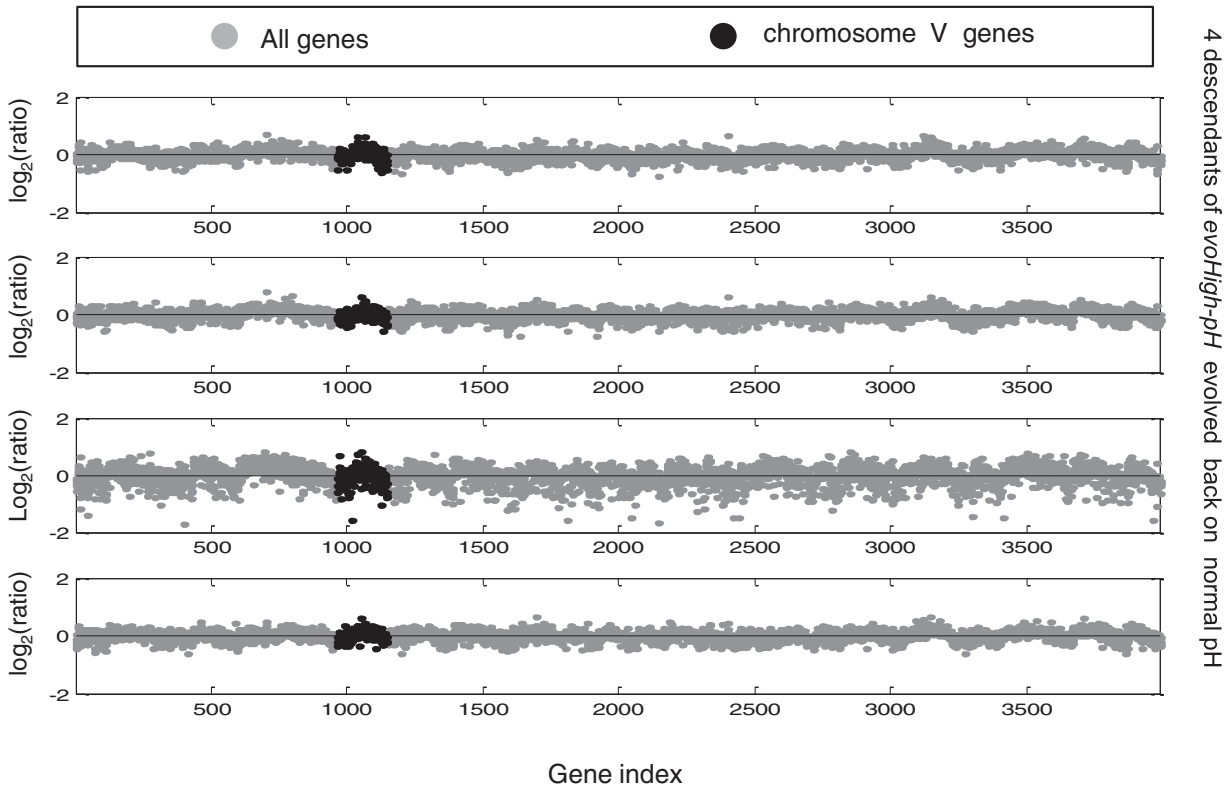

Fig. S6. Chromosome V disomic *evoHigh-pH* strain eliminated the disomy after further evolution at normal pH (6.7). Four independent repetitions, descendants of *evoHigh-pH*, evolved for 280 generations under pH 6.7 were analyzed and chromosome V disomy was eliminated in all repetitions. Dots represent $\log_2$ intensity ratios of DNA copy number over a haploid wild type, aligned according to chromosomal order where black dots represent genes from chromosome V and gray from all other chromosomes.

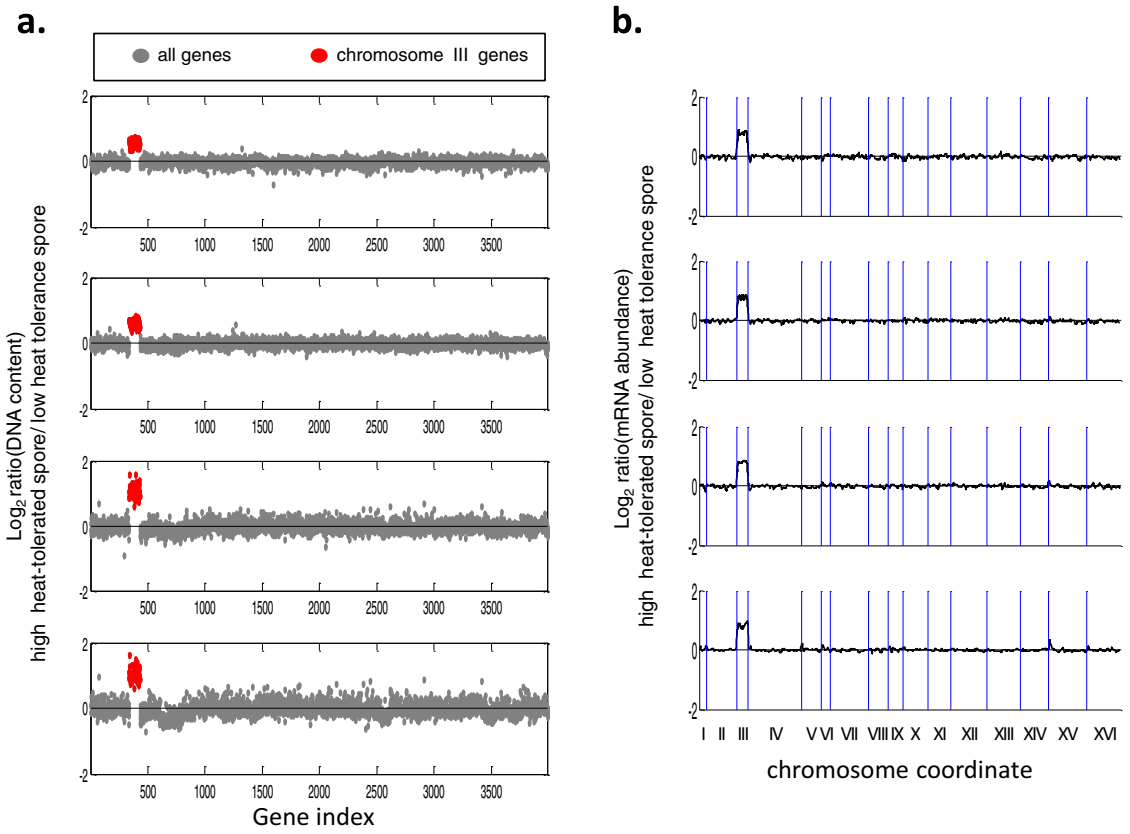

Fig. S7. Determination of extra chromosome III segregation in *evo39* and *WTtrisomeIII* spores. (*A*) *WTtrisomeIII* was subjected to tetrad analysis and the heat tolerance of eight spores from two tetrads was measured. Spores were divided into pairs according to their heat tolerance; each pair consists of one spore that showed increased heat tolerance and one spore with a heat tolerance level similar to that of a haploid wild type. Dots represent $\log_2$ intensity ratios of DNA copy number of increased heat tolerance spore over wild-type–like heat tolerance spore, aligned according to chromosomal order, where red dots represent genes from chromosome III and gray from all other chromosomes. (*B*) *Evo39* was subjected to tetrad analysis and the heat tolerance of eight spores from two tetrads was measured. Spores were divided into pairs according to their heat tolerance; each pair consists of one spore that showed increased heat tolerance and one spore with a heat tolerance level similar to that of the wild type. Dots represent $\log_2$ intensity ratios of mRNA abundance calculated by a sliding window of increased heat tolerance spore over wild-type–like heat tolerance spore, aligned according to chromosomal order where blue vertical lines differentiate between chromosomes.

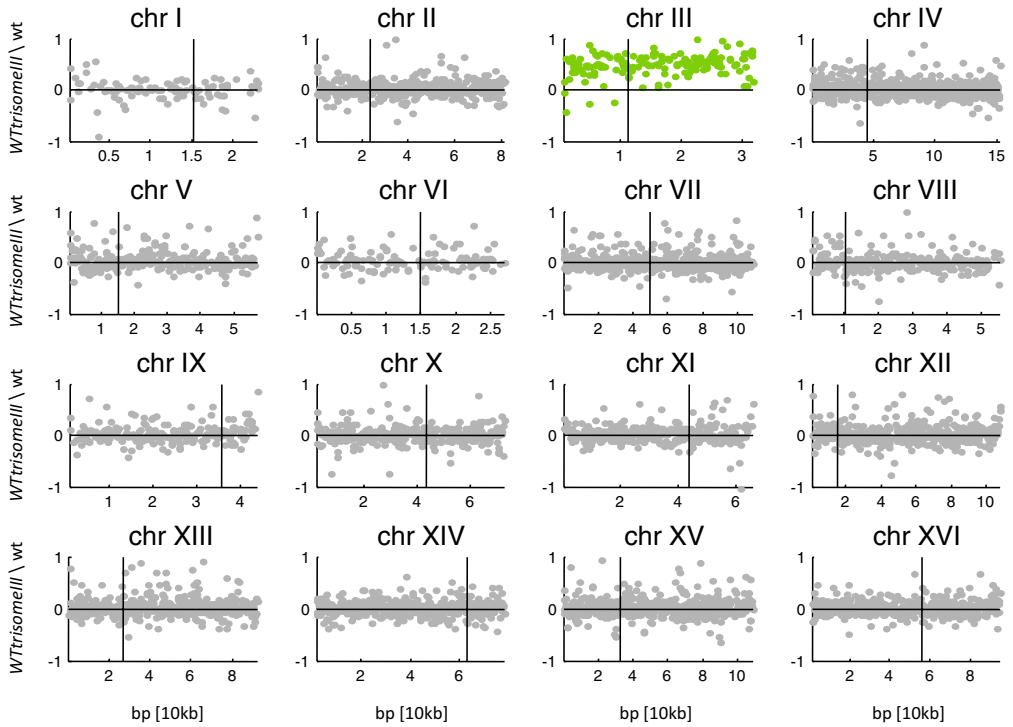

Fig. S8. Verification of *WTtrisomeIII* strain to be trisomic for chromosome III. Dots represent $\log_2$ intensity ratios of *WTtrisomeIII* to a diploid WT from expression microarray, for each of the 16 chromosomes and aligned according to chromosomal order. Green dots represent genes from chromosome III and gray from all other chromosomes. The vertical line within each chromosome represents the centromere position. Whereas in all chromosomes the average ration is around zero (indication for the identical chromosome copy number), for chromosome III there is ~50% higher expression, an indication of three copies of chromosome III compared with two copies in the WT.

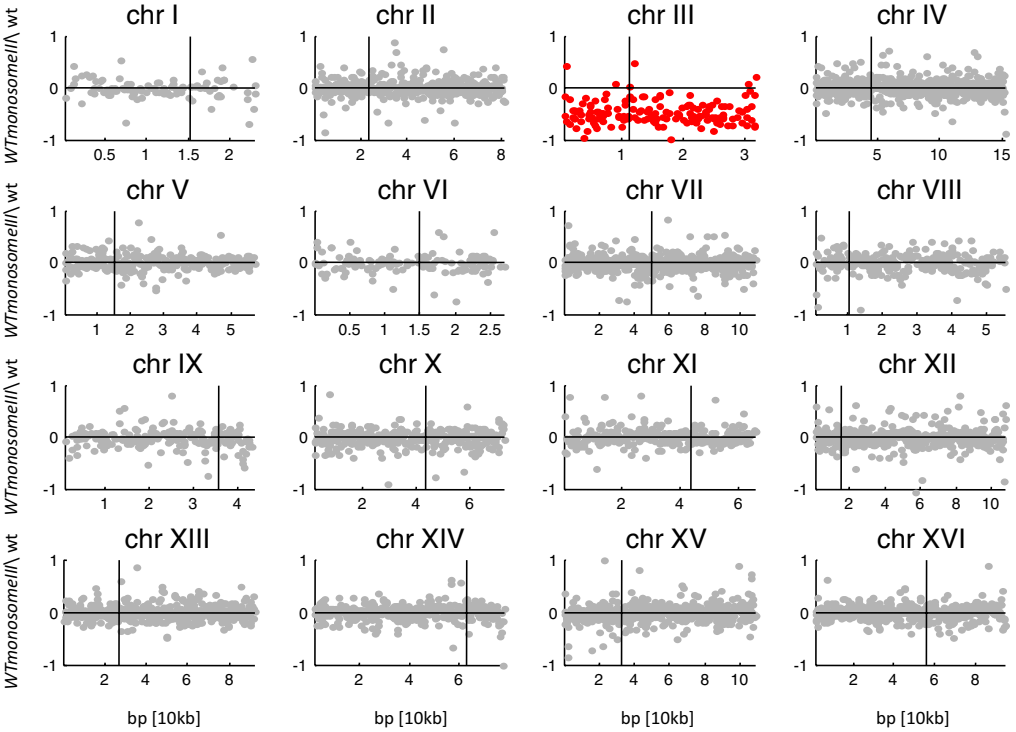

Fig. S9. Verification of *WTmonosomeIII* strain to be monosomic for chromosome III. Dots represent $\log_2$ intensity ratios of *WTmonosomeIII* to a diploid WT from expression microarray for each of the 16 chromosomes and aligned according to chromosomal order. Red dots represent genes from chromosome III and gray from all other chromosomes. The vertical line within each chromosome represents the centromere position. Whereas in all chromosomes the average ration is around zero (indication for the identical chromosome copy number), for chromosome III there is ~50% expression relative to the WT, an indication of one copy of chromosome III compared with two copies in the WT.

Table S1. Heat tolerance contribution of genes from chromosome III introduced separately into a diploid wild type

ORF	Heat tolerance contribution, %	Gene name
Genes that retain elevated expression after the elimination of chromosome III trisomy in *Refined 1–4*		
YCR065W	23.5	HCM1
YCR016W	19.9	Uncharacterized
YCR045C	19.3	RRT12
YCR102C	18.8	Uncharacterized
YCR071C	18.4	IMG2
YCL005W-A	16.0	VMA9
YCL059C	13.6	KRR1
YCL035C	10.6	GRX1
YCR007C	8.3	Uncharacterized
YCL001W	7.2	RER1
YCL063W	6.4	VAC17
YCL036W	3.7	GFD2
YCR003W	2.6	MRPL32
YCR087C-A	0.9	LUG1
YCR043C	0	Uncharacterized
YCL061C	−1.7	MRC1
YCL026C-B	−2.2	HBN1
Genes that returned to wild-type expression level after the elimination of chromosome III trisomy in *Refined 1–4*		
YCL056C	−1.2	PEX34
YCL055W	−4.1	KAR4
YCL048W	−1.6	SPS22
YCL047C	−3.1	POF1
YCL045C	0.2	EMC1
YCL033C	0.3	MXR2
YCL032W	−1.9	STE50
YCL029C	−4.5	BIK1
YCL016C	1.8	DCC1
YCL004W	−0.5	PGS1
YCL001W-A	−3.1	Uncharacterized
YCL001W-B	1.5	Uncharacterized
YCR002C	−2.4	CDC10
YCR010C	−6.3	ADY2
YCR012W	−5.5	PGK1
YCR027C	0.4	RHB1
YCR031C	−5.0	RPS14A
YCR035C	−4.4	RRP43
YCR046C	1.3	IMG1
YCR047C	−5.2	BUD23
YCR086W	−4.4	CSM1
YCR101C	−5.4	Uncharacterized

See *SI Text* for heat tolerance functional analysis of these genes. Heat tolerance was measured by survival after 90-min exposure to 45 °C (*Materials and Methods*). The contribution of each gene was calculated by subtracting the tolerance of the wild type with an empty plasmid and then dividing by the tolerance of *WTtrisomeIII* (to define wild-type contribution as 0% and trisomeIII as 100%).

Table S2. List of mutations in high-pH strains

Gene	Mutation	Function
Mutation in EvoHigh-pH*		
GTT2/ MMP1	Converging 3′UTRs	GST/S-methylmethionine permease
YFR057w	Base substitution at promoter	Unknown
ECM21	Nonsense at codon 193	Ubiquitin-ligase adaptor
NMD4 /YLR363w-a	Base substitution at promoter	Nonsense-mediated mRNA decay/unknown
GPI17	S63 to L	GPI-anchor transamidase
YHR140w/ SPS100	Base substitution at promoter	Unknown/spore wall maturation
MAC1	C271 to W	Copper-sensing transcription factor
Mutation in *evoHigh-pH* spores (euploid and disomic)†		
GTT2/ MMP1	Converging 3′UTRs	GST/ S-methylmethionine permease
YFR057w	Base substitution at promoter	Unknown
GPI17	S63 to L	GPI-anchor transamidase
MAC1	C271 to W	Copper-sensing transcription factor

*Mutations found in the sequencing of *evoHigh-pH* obtained from Romano et al. (1).
†Subset of mutations commonly found in two spores of *evoHigh-pH*: one euploid and another with an extra copy of chromosome V.

1. Romano GH, et al. (2010) Different sets of QTLs influence fitness variation in yeast. *Mol Syst Biol* 6:346.