Segmental copy number amplifications are stable in the absence of selection

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Abstract

Copy number variants (CNVs) are duplications and deletions of DNA sequences that contribute to genetic variation between individuals and underlie rapid adaptive evolution. Increases in gene copy number can confer a strong selective advantage in some circumstances, but CNVs can also incur fitness costs. Prior research has shown that experimental evolution of *Saccharomyces cerevisiae* in nutrient-limited chemostats recurrently selects for amplifications of nutrient transporter genes, including *GAP1*, *MEP2,* and *PUT4* in glutamine, ammonium and proline limited conditions, respectively. However, the fate of these CNVs upon return of the organism to a non-selective environment, is unknown. To investigate the fitness costs and stability of CNVs upon reversal of the original selection pressure, we studied 15 unique CNV lineages that had been selected in different nitrogen-limited chemostats, including both segmental amplifications and whole-chromosome aneuploidies. We studied the stability of CNVs using a fluorescent CNV reporter system during propagation in nutrient-rich media using serial dilution of batch cultures for 110-220 generations. We found that only one-third of CNV lineages repeatedly underwent loss of CNVs and reversion to a single-copy genotype. All lineages containing aneuploidies showed rapid reversion dynamics, whereas lineages containing segmental amplifications were remarkably stable – only one of 11 strains reverted to a single copy. Pairwise competitive fitness assays revealed strong fitness defects associated with reverting CNVs in the nutrient-rich environment but minimal fitness defects in lineages that did not revert. Reversion of CNVs led to increased fitness. Using simulation-based inference to estimate reversion rates and fitness effects we find that negative selection is the primary driver of CNV loss although aneuploid reversion rates are high with estimates of 10^{-5} to 10^{-3}. Whole-genome sequencing of clones isolated from evolved populations revealed that reversion of both aneuploids and a segmental amplification left no evidence of prior existence of the CNV, making the genomes of revertants indistinguishable from the single-copy ancestor. Our findings provide novel evidence of the low fitness cost and high stability of most segmental CNVs upon removal of the selection pressure, and that gene amplifications with large fitness costs are readily reversible, highlighting the significance of CNVs for genome evolution as well as rapid and reversible adaptation to transient selection pressures.

Introduction

Understanding the determinants and dynamics of adaptation is a fundamental goal of evolutionary biology. Genetic differences within a species, acted upon by forces of natural selection, are a major driver of adaptive evolution. One prevalent source of natural genetic variation within species are copy number variants (CNVs) – duplications or deletions of DNA sequences [Iskow et al. 2012, Clop et al. 2012, Żmieńko et al. 2014, Zarrei et al. 2015]. CNVs are a distinct class of genetic variation which, unlike single nucleotide variations, typically involve large genomic regions, ranging from a few base pairs (bp) to an entire chromosome, and thus can affect multiple genes [Connolly et al. 2014]. Environmental conditions can lead to selection pressures that act upon CNVs within a population, selecting for those that are best adapted to that condition.

CNVs cause rapid phenotypic diversification by changing the amount of DNA template, thus modifying mRNA and protein levels. This can confer benefits that help the organism survive in a particular environment– such CNVs are rapidly selected for and often attain fixation in the population [Kondrashov 2012, Myhre et al. 2013, Robinson et al. 2023]. For example, CNVs have been found to underlie drug resistance and enhanced nutrient transport in microbes, as well as defence mechanisms against diseases in plants [Elde et al. 2012, Greenblum et al. 2015, Iantorno et al. 2017, Dolatabadian et al. 2017, Cowell et al. 2018]. CNVs can also aid in the survival and proliferation of cancer cells, through amplification of oncogenes, heightened adaptability, and faster resistance to anti-cancer treatments [Rutledge et al. 2016, Ben-David and Amon 2020, Lukow et al. 2021]. In addition to short-term effects, CNVs also mediate long-term evolution through neofunctionalisation and subfunctionalisation of functionally redundant gene copies [Ohno 1970, Innan and Kondrashov 2010, Freeling et al. 2015]. On the other hand, CNVs can also confer negative impacts e.g., duplications or deletions of key genes are involved in a wide range of human disorders such as haemophilia, Alzheimer's disease, autism and schizophrenia. [Zhang et al. 2009]. Notably, the same CNV can potentially be beneficial, neutral, or deleterious depending on the relationship between its functional effect and the environment. Although there have been multiple studies of the dynamics, mechanisms, and effects of CNVs in selective conditions, the stability and maintenance of CNVs in populations once the selective pressure has been removed remains poorly understood.

CNVs are often associated with both fitness costs and fitness benefits [Tang and Amon 2013, Gerstein and Berman 2015]. CNVs are advantageous, and subject to positive selection, only when the benefits outweigh the costs in a particular environment. Typically, CNVs follow the 'driver-hitchhiker' model [Smith and Haigh 1974, Golzio and Katsanis 2013], wherein one of the amplified genes (driver) confers a fitness benefit, but other genes included in the amplified genomic region (hitchhikers) are neutral or confer fitness costs. These costs can be due to different reasons. First, the dosage burden model posits that the fitness cost is proportional to the elevation in unnecessary gene expression resulting from the CNV [Dekel and Alon 2005, Makanae et al. 2013, Bonney et al. 2015]. The fitness costs of unnecessary gene expression is evident in several studies, e.g., sterility is positively selected for in yeast during asexual growth due to elimination of unnecessary gene expression [Payen et al. 2016, Rice & McLysaght 2017] and experimental overexpression of individual genes shows clear evidence for deleterious effects [Robinson et al. 2021]. Second, the dosage imbalance model posits that fitness costs are derived from

stoichiometric imbalances of dosage-sensitive genes [Wagner 2005, Veitia et al. 2008, Rice and McLysaght 2017]. Third, there may also be fitness costs due to stress associated with the higher levels of DNA replication, transcription, and translation that result from CNVs. Although prior research has shed light on the fitness benefits associated with the emergence and selection of CNVs, we do not know how the trade-offs between fitness costs and benefits influence their stability in the absence of the original selection conditions under which they arose.

Long-term experimental evolution allows the study of the evolutionary dynamics under controlled, replicated selection conditions [Lenski et al. 1991, Good et al. 2017]. An integral component of cell growth regulation is acquisition of essential nutrients from the environment through specialized nutrient transporters. When cells are grown continuously in a nutrient-limited environment in a chemostat [Gresham and Dunham 2014, Gresham and Hong 2015], *de novo* CNVs containing nutrient transporter genes are positively selected, as increased abundance of transporter proteins likely allows increased uptake of the limited nutrient [Spealman et al., 2025]. This evolutionary outcome has been shown in multiple studies and species: *Saccharomyces cerevisiae* under limitation of glucose, phosphate, sulfur, and nitrogen [Brown et al. 1998, Dunham et al. 2002, Gresham et al. 2008, Kao and Sherlock 2008, Hong and Gresham 2014, Payen et al. 2016], *Escherichia coli* limited for lactose [Horiuchi et al., 1963, Zhong et al. 2004], and *Salmonella typhimurium* in different carbon source limitations [Sonti and Roth, 1989]. Previous studies have shown that evolution in glutamine-limited chemostats repeatedly selects for *de novo* amplifications of *GAP1*, a general amino acid permease [Lauer et al. 2018, Avecilla et al. 2023, Chuong et al. 2025]. *GAP1* CNVs are also found in natural populations, such as in the nectar yeast *Metschnikowia reukaufii*, where multiple copies of *GAP1* are beneficial when amino acids are limited [Dhami et al. 2016]. Selection for CNVs containing nitrogen transporters is substrate specific. *MEP2* is a high-affinity ammonium transporter that is amplified under ammonium limitation, whereas *PUT4* is a proline transporter amplified by proline-limited selection [Gresham et al. 2010, Hong & Gresham 2014, Hong et al. 2018]. Although multiple studies have demonstrated the recurrent emergence and fixation of CNVs in nutrient-limited chemostats, it is unknown whether acquired CNVs are maintained or lost when the organism is returned to nutrient-replete conditions.

Quantifying the frequency of CNVs in heterogeneous populations using molecular methods poses a considerable challenge and is limited in throughput and resolution. Previously, we developed a method to visualise CNVs in evolving populations using a constitutively expressed fluorescent reporter gene inserted adjacent to the gene of interest [Lauer et al. 2018]. Since CNVs span large genomic regions, the gene of interest and the reporter are typically amplified or deleted together, allowing estimation of the frequency of CNVs on the basis of fluorescence levels [Lauer et al. 2018, Spealman et al. 2023].

In this study, we used budding yeast lineages that previously acquired nutrient transporter-associated genomic amplifications in chemostat evolution experiments, as a system to study the fitness costs and stability of CNVs in absence of the original selection pressure. Using a combination of long-term experimental evolution using serial batch culture in nutrient-rich media, and fitness assays of CNV and revertant lineages, we find that a majority of CNVs do not lead to significant fitness defects compared to the ancestral strain. As a result, most segmental CNVs are stable over hundreds of generations, despite not conferring a fitness benefit in the absence of the original selection pressure. By contrast, aneuploidies exhibit significant fitness costs and instability and therefore are rapidly lost in the absence of selection.

The dynamics of aneuploidy loss differs between chromosomes; we find that chromosome XIV aneuploidy is lost much faster and ubiquitously whereas loss of chromosome XI aneuploidy is much slower and variable between populations. This difference, despite having the same copy number and similar chromosome sizes, is consistent with fitness costs of aneuploids depending on the specific hitchhiker genes on the chromosome. Although it appears to be rare for CNVs to incur strong fitness costs, those that do are recurrently lost in the absence of selection, with the genome undergoing reversion to a single-copy state. Remarkably, once the extra gene copies in the amplified region are lost, there is no remnant trace of the prior presence of CNVs, revealing the ephemeral nature of this class of genetic variation.

Results

We studied 15 unique haploid lineages of *Saccharomyces cerevisiae* to investigate the fate of adaptive gene amplifications upon relaxing the original selection pressure of nutrient limitation in chemostats (**Figure 1A**). Nine strains (*G_1* - *G_9*) had acquired segmental CNVs containing the *GAP1* gene on chromosome XI in glutamine-limited chemostats. Strain *M_10* has a segmental amplification of *MEP2* on chromosome XIV, which arose during selection in a chemostat fluctuating between glutamine- and ammonium-limitation. Strain *P_11* acquired a segmental amplification containing *PUT4* on chromosome XV during selection in a chemostat fluctuating between glutamine- and proline-limitation. Strains *GM_12* - *GM_15* had acquired whole-chromosome duplications, or aneuploidies, of both chromosomes XI and XIV during fluctuating glutamine- and ammonium-limitation. These 15 CNV lineages were chosen to survey a range of different features including the driver gene under selection, copy number increase, size of the CNV, molecular mechanism underlying CNV formation, and number of elapsed generations of selection before the lineage was isolated (**Figure 1B** and **Supplemental Table 1**). CNVs vary between a copy number of 2 - 4, and span 6 kb to the entire chromosome. The segmental CNVs (*G_1* - *P_11*) were formed by either non-allelic homologous recombination (NAHR), origin-dependent inverted repeat amplification (ODIRA), or transposon insertion followed by NAHR. Aneuploidies were formed by missegregation of the entire chromosome XI, XIV or both. All strains contained CNV reporters that enabled single cell estimation of copy number at the specific using flow cytometry. *GAP1* CNV containing lineages contain an mCitrine reporter adjacent to the *GAP1* locus. Additionally, the ancestors for strains *M_10* and *GM_12* - *GM_15*, and for *P_11,* have an mCherry reporter next to *MEP2* or *PUT4*, respectively. Additionally, strains *G_4* - *GM_15* were obtained from lineage tracking experiments and therefore contain unique barcode sequences enabling sequence-based verification of parental lineages and their descendants (**Table S1**).

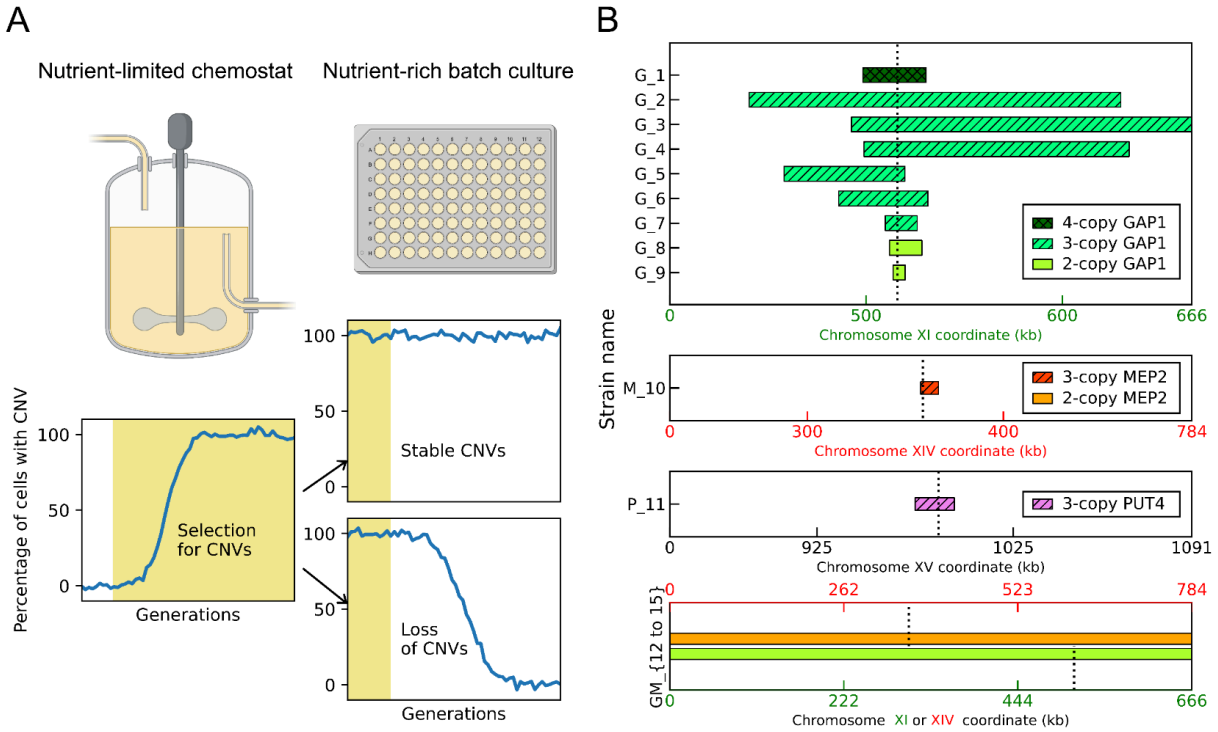

Figure 1. Experimental design. A) The two possible fates of adaptive copy number amplifications acquired during nutrient-limited selection in chemostats (yellow shaded background). In the absence of the original nutrient-limitation selection pressure due to propagation in nutrient-rich media using 96-well serial batch transfer, if fitness costs are comparable to the benefits, CNVs will be stably maintained (upper panel) whereas if fitness defects of CNVs outweigh their benefits, they will be purged from the population through positive selection for revertants (lower panel). **B)** Schematic representation of the 15 unique CNV lineages used in this study. Our naming convention incorporates three parameters. The gene under selection during amplification is indicated by the letter: *G* (*GAP1* on chromosome XI), *M* (*MEP2* on chromosome XIV), *P* (*PUT4* on chromosome XV) or *GM* (both *GAP1* and *MEP2*). Strains of each type are arranged in descending order of copy number with respect to the reference genome (shown by color and shading) and within each copy number, by decreasing size of the amplified region (indicated by length on the x-axis). Positions of the genes (*GAP1, MEP2, PUT4*) are indicated by dotted lines.

Segmental amplifications confer minimal fitness costs in the absence of selection

We sought to test whether adaptive CNVs confer a fitness cost in absence of selective pressure. Therefore, we estimated the fitness of all CNV strains in nutrient-rich media via pairwise fitness competitions against the ancestor (**Figure 2A**). We measured the relative abundance of each CNV strain every 3-4 generations using a flow cytometer and performed linear regression to estimate the per generation growth rate difference (**methods**). We performed all assays in triplicate using a minimum of six time points to maximize our ability to detect small fitness differences.. Interestingly, among the 11 segmental CNVs, only two (*G_4* and *P_11*) have significantly negative fitness effects (p-value < 0.05) compared with the ancestor in rich media. Surprisingly, two strains containing segmental CNVs showed increased fitness suggesting pleiotropic effects. By contrast, all four double aneuploid (*GM_12* - *GM_15*) strains showed significantly negative fitness defects (**Figure 2B**).

We tested whether the fitness cost of segmental CNVs depends on the amount of additional DNA in its genome, calculated as the size of the amplified region multiplied by its copy number. We found no

significant correlation between the relative fitness of a CNV strain and the number of extra base pairs (**Figure 2C**). Together, these results suggest that it is rare for segmental CNVs to confer fitness costs in absence of selection, and that fitness defects are not due to the burden of excess DNA.

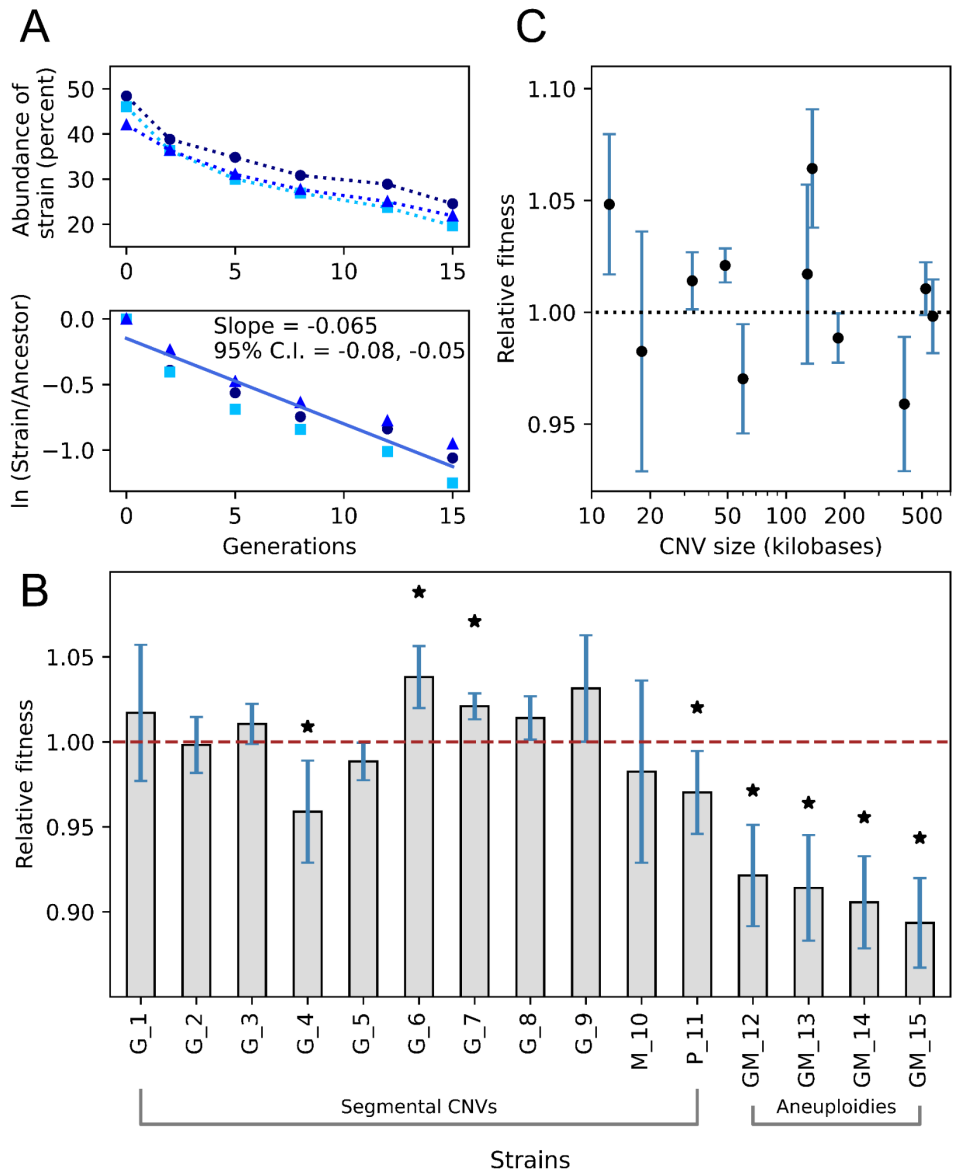

Figure 2. Fitness effects of CNVs. A) Pairwise competitive fitness assays were performed in nutrient-rich media by co-culturing each CNV strain with the ancestral single-copy strain and quantifying their relative abundance (upper panel). Relative fitness was calculated by regressing the natural log of the ratio of the CNV strain and the reference strain against the number of generations for 3 replicate experiments per strain (lower panel). **B)** Fitness of all 15 CNV lineages relative to the single-copy ancestor (whose fitness is 1 by definition, marked by a dashed brown line). Each bar shows the median fitness of each strain, with error bars indicating 95% confidence intervals from the standard error of the regression using 3 replicates. Strains with fitness significantly different from the ancestor are marked with an asterisk. **C)** For each segmental amplification (*G_1* - *P_11*), relative fitness was compared to the number of extra base pairs of DNA, which was calculated by multiplying the size of the amplified region by its copy

number. We found no significant correlation between fitness cost and DNA content (Pearson correlation coefficient = -0.25, 95% confidence intervals: -0.52, 0.03).

Segmental amplifications are typically stable in the absence of selection

To investigate CNV stability upon removing nutrient-limited chemostat selection, we performed long-term experimental evolution in nutrient-rich media using serial transfer batch culture. We performed evolution experiments in two sets: 1) strains *G_1* - *G_3* were propagated for 110 generations, and then 2) strains *G_4* - *GM_15* were propagated for 220 generations. We studied 3-4 independent populations per strain. In addition, we simultaneously evolved 5 control strains: (i) S288c unmodified by any reporter gene, acting as a zero-copy control; (ii) mCitrine_1copy, containing a single copy of the mCitrine reporter at the neutral *HO* locus; (iii) mCitrine_2copy, with the reporter at two neutral loci– *HO* and the dubious ORF *YLR123C*; (iv) mCherry_1copy, with a single copy of the reporter replacing the region from the beginning of locus *YLR122C* to the end of locus *YLR123C*; and (v) mCherry_2copy, with the reporter at the *YLR122-123C* and *HO* loci. We confirmed that there was no change in fluorescence in any of these control strains throughout the experiment (**Figure 3A**).

Using real time tracking of CNV dynamics we found that 11 of the 12 segmental CNVs were maintained at greater than 95% frequency over 110 (**Figure 3A**) to 220 (**Figure 3B**) generations. This indicates that most segmental amplifications that are positively selected in nutrient-limited chemostats are stably maintained upon relaxation of the selective pressure. We find this to be the case for segmental CNVs on chromosome XI, XIV or XV, suggesting that these results are independent of locus and chromosome.

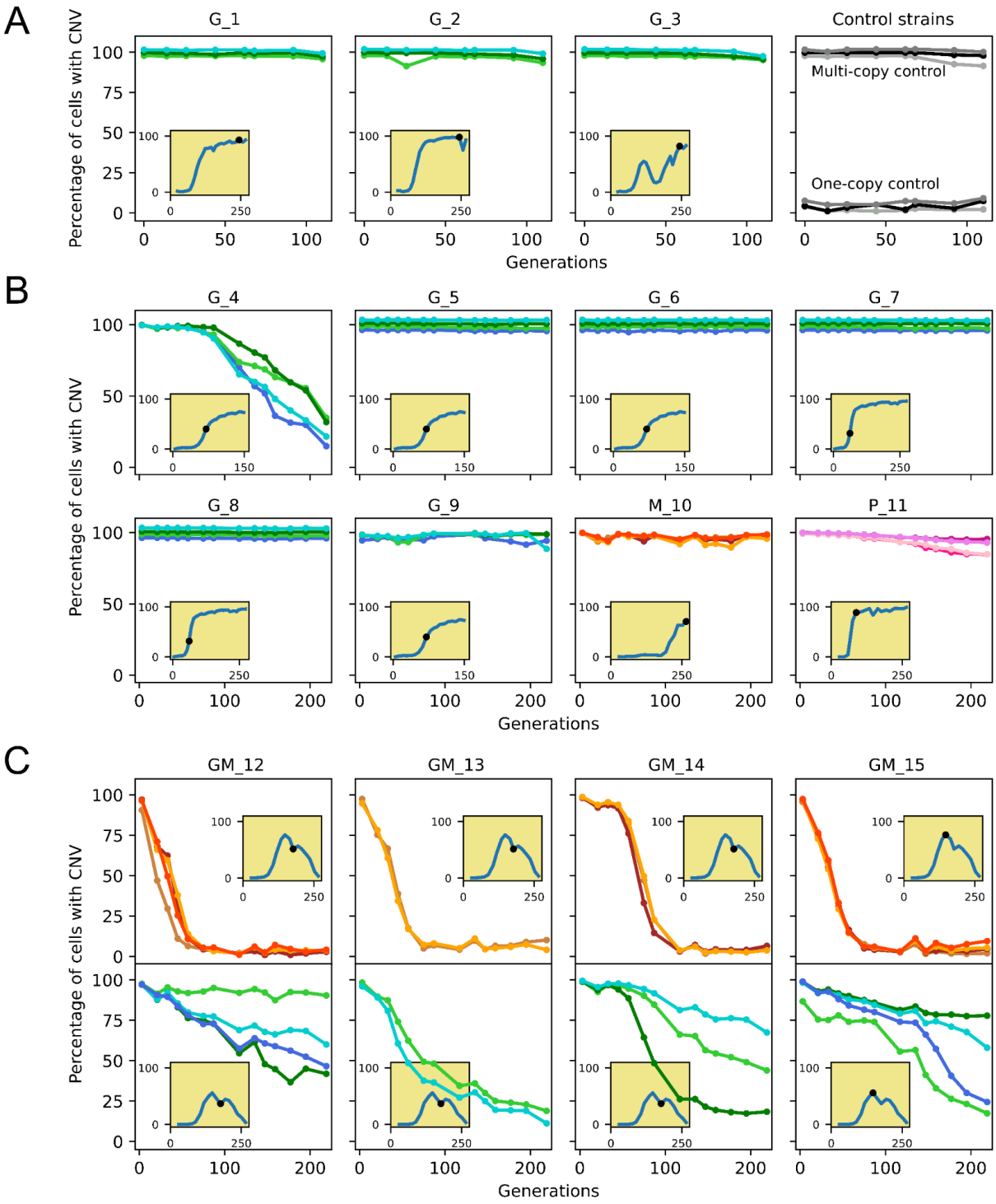

Figure 3. CNV dynamics during long-term experimental evolution in nutrient-rich batch cultures. We quantified the percentage of CNV-containing cells in each population using a fluorescent reporter gene adjacent to each gene of interest. CNVs containing *GAP1*, *MEP2* and *PUT4* are shown in green, orange and pink, respectively, with different shades indicating independent populations of each strain. Insets show CNV dynamics for each strain's parental population in which it acquired a gene amplification during nutrient-limited chemostat selection. The timepoint at which the CNV strain was isolated is marked with a dot. **A)** Strains *G_1* - *G_3* were evolved for 110 generations. **B)** Strains *G_4* - *P_11* were evolved for 220 generations. For all strains except *G_4* and *P_11*, data

points for all replicates overlap, and therefore have been slightly shifted for better visualisation. **C)** Dynamics for both *MEP2* and *GAP1* for double aneuploid strains *GM_12* - *GM_15*.

Fitness costs predict CNV reversion in the absence of selection

Aneuploidies were lost far more rapidly and consistently than segmental CNVs. Of the 12 segmental amplifications, only one (*G_4*) showed evidence for reversion, which was observed in all independent populations. By contrast, all aneuploid strains (*GM_12* - *GM_15*) exhibited some degree of CNV loss in all but one of the independent populations. In these strains, the chromosome XIV aneuploidy was rapidly lost in all 13 populations whereas the chromosome XI aneuploidy showed variable evolutionary dynamics– some populations lost the chromosome XI faster than the segmental amplification in *G_4*, whereas others maintained it stably for all 220 generations (**Figure 3C**).

Notably, the five strains that underwent recurrent CNV loss in independent populations (*G_4* and *GM_12* - *GM_15*) were the only ones among our 15 CNV lineages that had large fitness defects in rich media (**Figure 2C**). One additional strain, *P_11*, had a small fitness defect compared to the aforementioned five strains and showed evidence for a small degree of CNV loss in 2 of 3 populations (**Figure 3B**). Our results indicate that fitness costs are predictive of the fate of adaptive CNVs in a non-selective environment.

CNV reversion and selection dynamics differ between chromosomes

To quantify the dynamics of CNV loss, we defined three parameters: (i) early phase: the generation at which 25% of cells in a population lost the CNV (ii) middle phase: the generation by which 50% of cells lost the CNV (iii) and late phase: the generation by which 75% of cells lost the CNV (**Figure 4A**). We calculated the median number of generations needed for CNV loss, and performed paired t-tests to compare CNV types in the early phase (**Figure 4B**), middle phase (**Figure 4C**) and late phase (**Figure 4D**).

The median generation to loss was highest in the chromosome XI segmental amplification, followed by the chromosome XI aneuploidy and then the chromosome XIV aneuploidy, which had the fastest CNV loss. Statistical comparison confirmed that dynamics of CNV loss vary between chromosome XI and chromosome XIV. However, there was no significant difference between the segmental CNV on chromosome XI and the aneuploidy of chromosome XI (**Figure 4**). This was in spite of these two types of CNVs differing in (i) size of the amplified genomic region (135 kb vs 666 kb, respectively), and hence, the number of amplified genes; and (ii) copy number relative to the ancestor (3 versus 2).

Double aneuploid strains *GM_12* - *GM_15* enable direct comparison of the stability of two different whole-chromosome duplications in the same cell. First, we observed that the extra chromosome XIV was lost very consistently, with all 13 populations across 4 strains showing very similar dynamics, whereas the evolutionary dynamics of the extra chromosome XI were highly variable, both within and across these strains. For instance, at the end of 220 generations, the percentage of cells that lost the extra chromosome XIV ranged between 90-98% across all 13 populations; for chromosome XI, this was between 9-89% for the same populations (**Figure 3C**). Second, the chromosome XIV aneuploidy was lost much faster than the chromosome XI aneuploidy: the median time to early, middle and late phases for populations that lost

the CNVs was 18, 39 and 51 generations for chromosome XIV, versus 80, 143 and 149 generations for chromosome XI (**Figure 4**). Finally, analysis of clones from evolved populations after 159 and 201 generations did not identify any lineages that had lost only the extra copy of chromosome XI but not of chromosome XIV. These results indicate that the fitness costs and stability of aneuploidies differ between chromosomes.

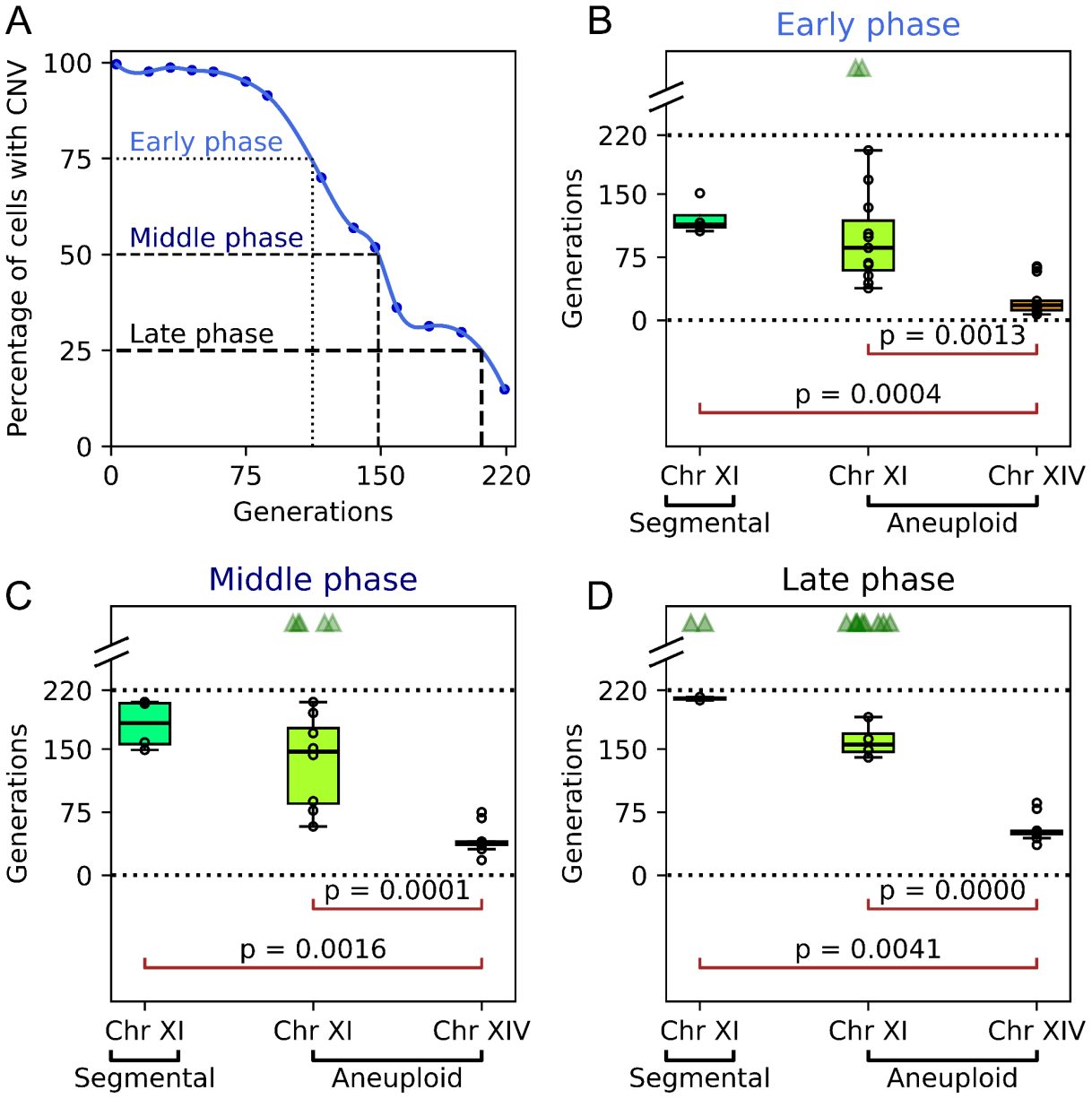

Figure 4. Quantification of CNV loss dynamics. A) We define three phases– early, middle and late, corresponding to loss of CNVs in 25%, 50% and 75% cells of each population. **B)** Number of generations to CNV loss and pairwise t-tests between CNV types in the early phase. **C)** Same as B, in the middle phase. **D)** Same as B, in the late phase. In each phase, CNVs which were lost (*G_4*, *GM_12* - *GM_15*) were grouped by type to perform paired t-tests. Groups with significant differences are indicated by p-values. Populations which did not lose the requisite percentage of CNV-containing cells for each phase were excluded from the barplot (but not from the t-test) and are indicated by jittered triangles at the top. Dotted lines indicate the start and end points of the evolution experiment (0

and 220 generations). For each phase, there were significant differences in the dynamics for chromosome XIV aneuploidy (784 kb, 2 copies) versus both the chromosome XI aneuploidy (666 kb, 2 copies) and the segmental amplification of chromosome XI (135 kb, 3 copies), but not between the two kinds of chromosome XI CNVs.

Evolutionary model and simulation-based inference

The dynamics of CNV loss are determined by the rate of reversion and the associated fitness effect. To infer the rate of reversion, we modelled the CNV loss dynamics of the five strains (*G_4, GM_12 - GM_15*) using a Wright-Fisher model and neural network simulation-based inference (nnSBI) (**Figure 5A**). For all strains, the collective posterior predictions of CNV loss dynamics qualitatively match the empirical data (**Figure 5B**). In addition, the overall fitness estimates for these five strains are very similar to results from competition assays, further validating our approach. The reversion rate estimates of both *GAP1* and *MEP2* CNVs varies between 10^{-5} to 10^{-3} (**supplementary Figure S1**). The fitness estimates of *MEP2* CNV relative to the wild type (0.922-0.949), were significantly lower than those of *GAP1* CNVs (0.971-0.998), suggesting that *MEP2* reversion is under stronger positive selection (**supplementary Figure S1**).

The individual posterior predictions further support our choice of an evolutionary model without epistasis (**supplementary Figure S2**). We also found that using an evolutionary model with epistasis and the same inferred parameters, while assuming independent reversion rates and fitness effects, leads to similar posterior predictions (see supplementary information for details). Finally, we used the evolutionary model to predict the outcome of short- and long-term experiments in a scenario where reversion is effectively neutral. In this case, we predict the decrease in CNV frequency to be extremely slow, requiring about 22,000 generations before the revertant allele is fixed in the population (**supplementary Figure S3**).

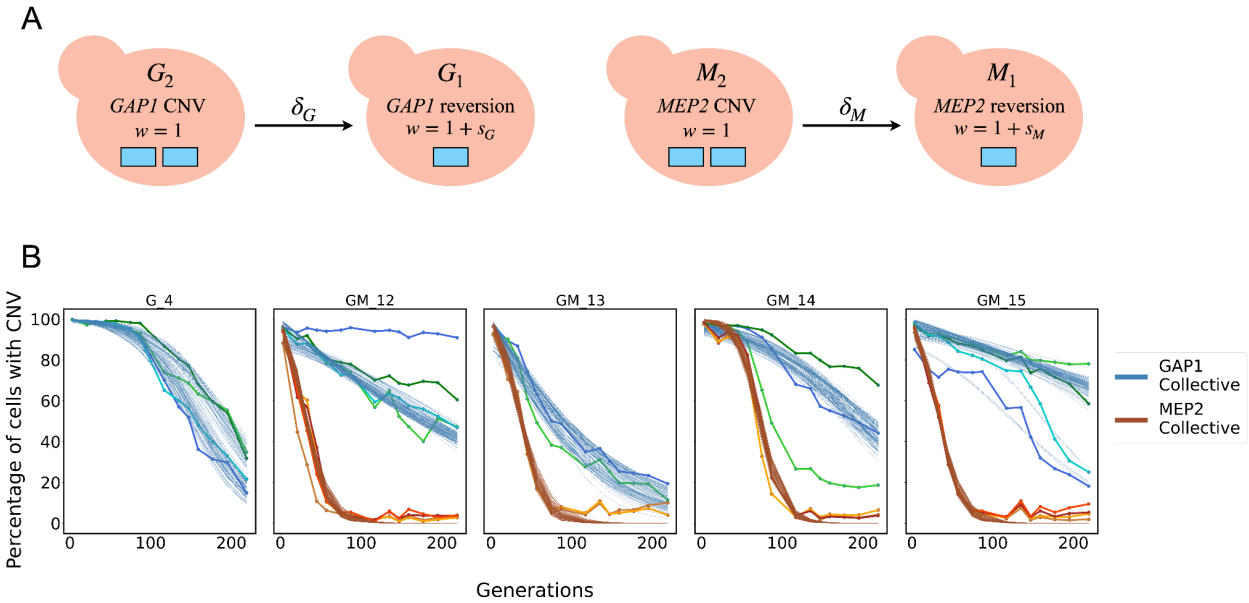

Figure 5. A Wright-Fisher evolutionary model of allele-tracking and collective posterior predictive checks using nnSBI. A) CNV reversions are assumed to be independent, with a selection coefficient of s_i and reversion rate of δ_i for gene i. Cell labels refer to a gene (G - *GAP1* or M - *MEP2*) and its copy number (2 or 1) as an allele. **B)**

Model simulations of 100 parameter sets sampled from the collective posterior distributions inferred from each experimental replicate (shaded lines) and the empirical data (solid lines).

CNV reversion increases fitness

To test the effect of CNV reversion on fitness, we performed pairwise competitive fitness assays before and after evolution for the five strains that underwent CNV loss. From populations evolved for 159 or 201 generations, we isolated clones with both partial and complete loss of CNVs. For the segmental CNV in *G_4*, where the amplified region had 3 copies, 'partial' revertants lost only 1 of the extra copies, whereas 'complete' revertants lost both extra copies. For double aneuploid strains *GM_12* - *GM_15*, 'partial' revertants lost only the extra chromosome XIV, whereas 'complete' revertants lost the extra copy of both chromosomes, XIV and XI.

For all five CNVs, partial or complete reversion led to improved fitness in rich media compared to the CNV strain. Some revertants were slightly less fit than the ancestor, but in most cases, reversion restored fitness to levels comparable to or slightly higher than the ancestor. Surprisingly, complete revertants were not universally fitter than partial revertants of the same strain. For example, for strain *GM_15* , the median fitness coefficient before CNV loss was 0.89, which improved to a range of 0.93 to 1.01 for partial revertants, and from 0.94 to 0.99 for complete revertants (**Figure 6**).

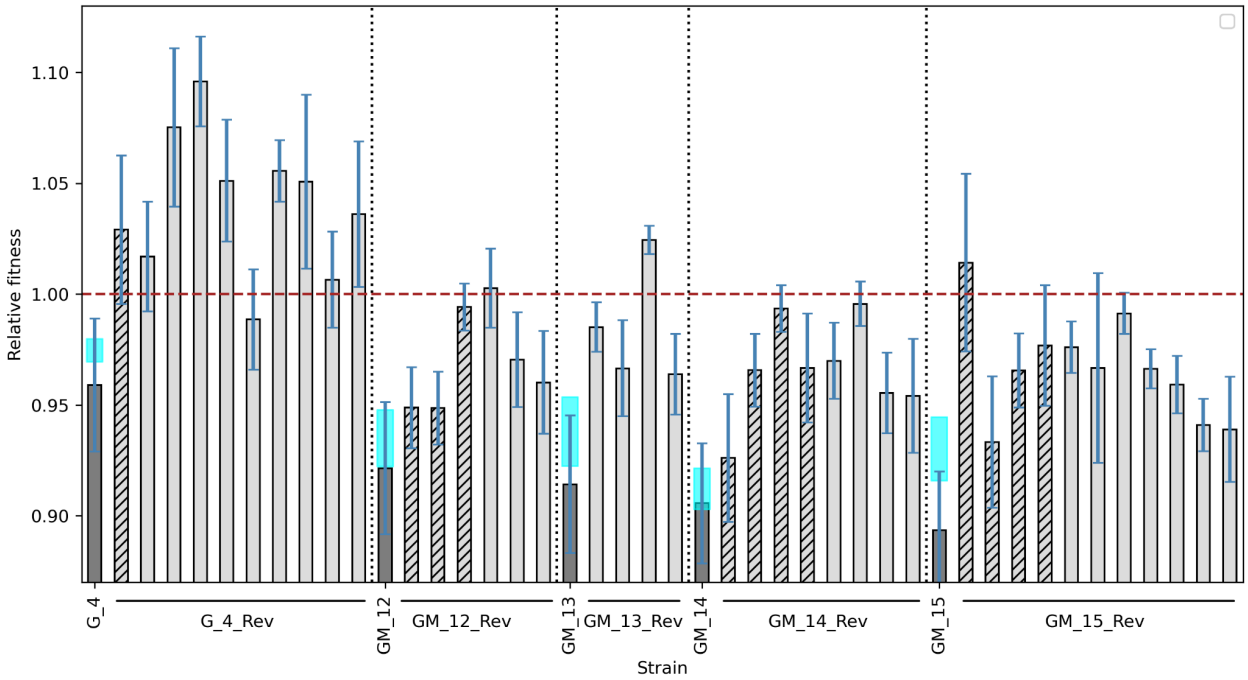

Figure 6. CNV reversion increases fitness. Pairwise competitions were performed to compare fitness before and after evolution in nutrient-rich media. Revertants were isolated after 159 or 201 generations of evolution from populations that underwent CNV loss. CNV strains are shown in dark grey, complete revertants in light grey, and partial revertants in a grey-black hatched pattern. For the segmental amplification in *G_4* which has 3 copies, the partial revertant has 2 copies, and complete revertants have 1 copy. For the double aneuploid strains *GM_12* - *GM_15*, partial revertants lost only the extra copy of chromosome XIV, whereas complete revertants lost the extra copy of both chromosome XI and chromosome XIV. Each bar shows the median relative fitness, with error bars indicating 95% confidence intervals calculated from 3 replicates for each strain. Fitness estimates for the CNV strains from our simulation-based inference, shaded in cyan, are similar to our empirical data.

CNV reversion is scarless

To study the genomes of strains after CNV reversion, we isolated a total of 56 clones from 17 revertant populations derived from five strains (*G_4*, *GM_12* - *GM_15*) at generation 159 or 201, and performed whole-genome sequencing. We calculated the copy number of all genes, relative to a reference genome resembling the single-copy ancestor (**methods**). We confirmed that for all clones that showed evidence of CNV reversion on the basis of the fluorescent reporter, the entire CNV region had reverted to a single copy.

For the only segmental amplification that was lost (strain *G_4*), we analysed sequencing read depth for 15 clones from 4 evolved populations (**Figure 7A**). One clone was a partial revertant, having reverted from 3 copies of the amplified region to 2 copies, whereas all other 14 clones were complete revertants with a single copy of all genes. We confirmed that the copy number of revertants inferred from fluorescence corresponded to that obtained from relative read depth (**Figure 7B**).

We performed breakpoint and split read analysis of all revertants to find mismatches with the reference strain. We confirmed that the parental CNV strain had 2 peaks of split reads corresponding to the two breakpoints of the amplified region of the genome (**Figure 7A**). The partial revertant also had split reads in the same positions, indicating that the breakpoints are similar to the CNV strain, although the copy number is reduced from 3 to 2. However, none of the 14 complete revertants had any trace of these peaks corresponding to the CNV breakpoints, indicating that the entire region reverted to one copy. Surprisingly, remnant split reads were not found anywhere in or adjacent to the previously triplicated region. This suggests that the reversion of gene amplifications to the one-copy genome does not leave any sequence evidence of the prior presence of gene amplifications. When we compared the nucleotide sequence in the breakpoint regions, we found that the inversion formed in the CNV strain is fully reversed in all 14 complete revertants, making the revertant sequence indistinguishable from the reference (**Figure 7C**).

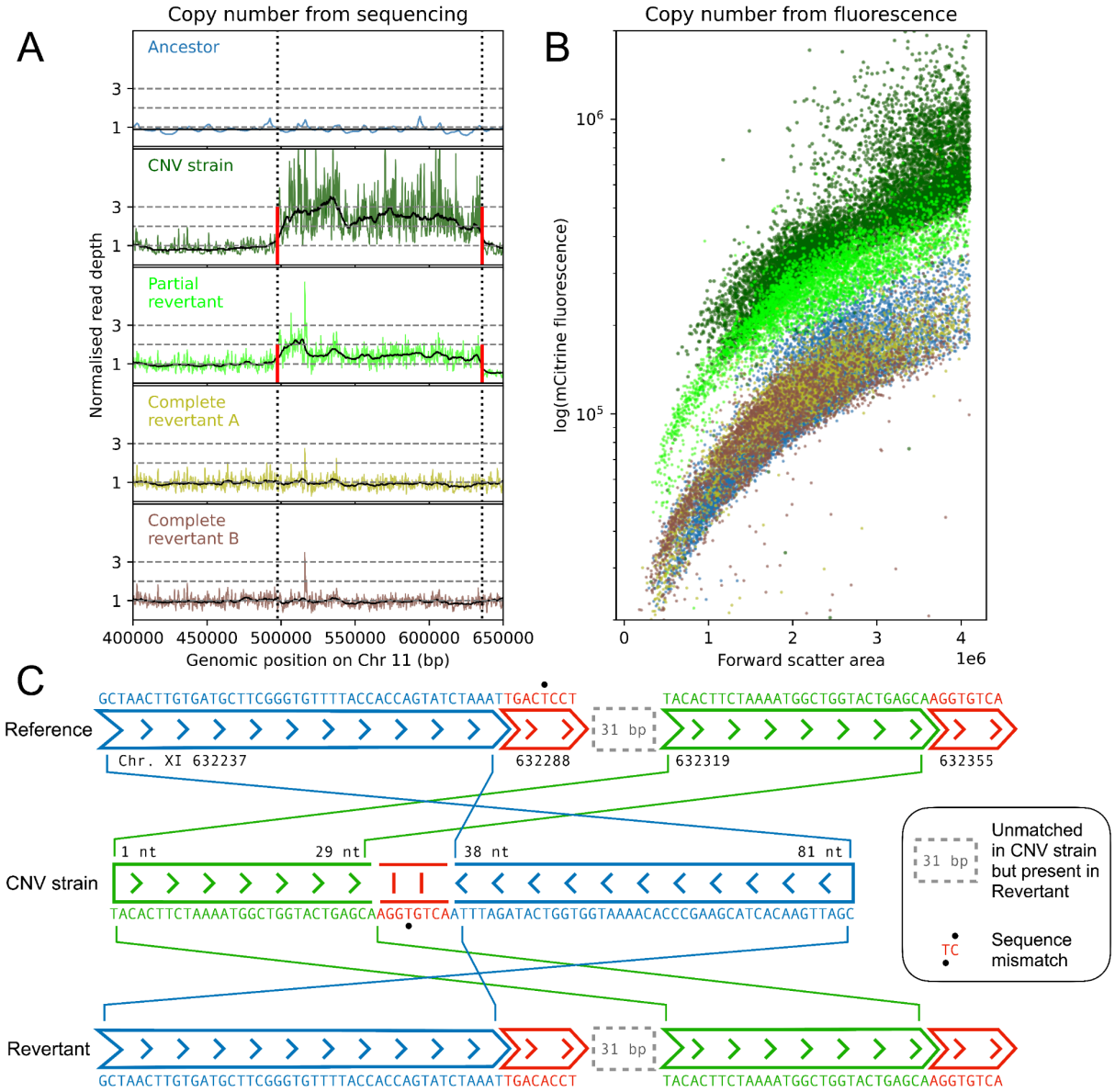

Figure 7. Scarless reversion of CNVs. A) Read depth across the chromosome indicates copy number relative to the single-copy ancestral genome. Split reads (in red) occur in regions of discrepancy of each strain's sequence compared to the ancestral reference and are indicative of CNV breakpoints or boundaries. Read depth for all positions across the chromosome have been normalized by their respective means in the chromosomal region with no CNVs; CNV boundaries are marked by dotted black lines. Solid black lines indicate median read depth over rolling 50 kb windows; dashed grey lines indicate normalized read depths of 1, 2 and 3, for visual comparison. Only the portion of chromosome XI that includes the CNV region is shown in the figure. We compared *G_4*, the 3-copy CNV strain that underwent reversion, to its 2-copy partial revertant, and to 14 complete revertants (1-copy; two are shown here). **B)** Fluorescence data also indicates 3, 2 and 1 copy number corresponding to the CNV strain, partial revertant and complete revertants, respectively. **C)** The right-hand breakpoint of the segmental amplification, showing an inversion in the CNV strain, consistent with ODIRA, which fully returns to the ancestral state in all 14 complete revertants (only a single example is shown here).

Single nucleotide variations explain some fitness differences between revertants with same copy number

Although all revertants have higher fitness than their parent CNV strain, we observed some fitness differences between revertants with similar copy numbers. This may be partly explained by certain point mutations that arose over the course of 220 generations of evolution. For example, among complete revertants of *GM_15*, which lost extra copies of both chromosomes XI and XIV, three had much lower fitness than others. Interestingly, these three revertants had a total of four confirmed SNVs, two of which are known to be missense mutations in essential proteins (*GM_15_Rev7*, *GM_15_Rev8*, *GM_15_Rev12*, see **Supplemental Table 5**). Again, among partial revertants (which lost only the extra chromosome XIV) of *GM_15*, the one with the lowest fitness (*GM_15_Rev6*) was found to have a missense mutation. Not all fitness differences between similar-copy revertants can be explained by point mutations– this may be because these slight fitness differences are simply not selected against, as long as they are more fit than the CNV parent.

Discussion

In this study, we sought to understand the long-term fate of adaptive gene amplifications in budding yeast. Specifically, we investigated the fitness and stability of copy number variants upon reversal of the selection pressure in which they arose. By evolving 15 unique CNV lineages in nutrient-rich media, we removed the original selection pressure of nitrogen limitation in chemostats, which had imposed positive selection for *de novo* CNVs containing nutrient transporter genes. We used experimental evolution, flow cytometry, fitness assays, simulation-based inference, and whole-genome sequencing to study the dynamics of these adaptive CNVs when returned to their pre-selection environment.

CNVs often incur fitness costs associated with elevated gene expression and stoichiometric imbalances of encoded products [Tang and Amon 2013, Gerstein and Berman 2015, Tsai et al. 2019, Yang et al. 2021, Rojas et al. 2024, Spealman et al. 2025]. Fitness costs are balanced against fitness benefits conferred by these CNVs, which are, typically, context dependent. We hypothesized that upon removal of the selection pressure imposed by the nitrogen-limited environment, CNVs containing nitrogen-transporter genes would no longer confer a fitness advantage, but fitness costs would remain, resulting in lower fitness relative to the single-gene copy ancestor.

Contrary to our expectation, only two of our 11 lineages containing segmental CNVs had reduced fitness compared to the ancestor in nutrient-rich media. These results differ from our prior analysis of CNV strains that arose in glutamine-limited chemostats [Avecilla et al. 2023]. We attribute this difference to the difference in conditions in the two studies (Avecilla et al. 2023 used galactose-containing media) and the increased sensitivity of our pairwise fitness assays.

Tracking CNV dynamics using fluorescent CNV reporters showed that in the absence of a fitness cost, most segmental CNVs were stably maintained across hundreds of generations in nutrient-rich conditions. By contrast, segmental CNVs with significant fitness defects in a nutrient-rich environment were lost from evolving populations as there was positive selection for spontaneously generated revertants

containing a single copy of the locus. Our results regarding general CNV stability are both similar to some previous studies that probed CNVs in yeast under selection conditions different to our study. For example, segmental duplications within a chromosome are stably inherited even in the absence of any selective advantage, as indicated by growth rates comparable to wildtype [Koszul et al. 2006]. Conversely, CNVs arising under antifungal drug treatment in *C. albicans* rapidly return to the progenitor copy number once the selection pressure is relaxed [Todd et al. 2020]. Our findings are consistent with the observation that copy number variation at the CUP1 locus is maintained among commonly used lab strains of *S. cerevisiae*, suggesting that these CNVs are maintained despite the absence of continued positive selection [Warringer et al. 2011]. Overall, we conclude that fitness costs of CNVs in absence of the original selection pressure are variable, likely depending on the specific environment, genetic background, and CNV structure. When a CNV does incur a significant fitness defect in the non-selective environment they revert and are rapidly purged from the population.

In contrast to segmental CNVs, all four strains with aneuploidies of chromosomes containing nitrogen transporter genes had lower fitness relative to the ancestor in the non-selective environment. These aneuploid lineages were positively selected in nitrogen-limited environments, but show clear fitness defects in rich media. Previous studies have found that engineered aneuploids grow more slowly than euploids, regardless of karyotype [Torres et al. 2007; Sheltzer et al. 2012; Beach et al. 2017] and adaptive aneuploids can also show antagonistic pleiotropy such that they are deleterious in other environments [Sunshine et al. 2015; Linder et al. 2017]. Aneuploidies were lost more frequently than segmental CNVs among our lineages, which agrees with previous studies that showed that large duplications on supernumerary chromosomes and disomic chromosomes are highly unstable and are spontaneously lost at high frequency [Campbell et al. 1975, Koszul et al. 2006]. Aneuploidy can serve as a fast but transient evolutionary solution during adaptation to a selection pressure [Yona et al. 2012, Kohanovski et al. 2024]. Our results are consistent with the instability and dynamic nature of aneuploidies.

As the four aneuploid lineages contained two copies of both chromosome XI and chromosome XIV, these strains provided an opportunity to directly compare the stability of two different chromosomes. We found that all evolving populations purged the extra copy of chromosome XIV much faster and more ubiquitously than the extra copy of chromosome XI. This suggests that aneuploidy of chromosome XI has a lower fitness cost than that of chromosome XIV, and hence, is better tolerated. This result is in agreement with Rojas et al. [2024], who showed that the median growth rate of chromosome XI aneuploidy was 95% of the euploid growth rate, whereas that of chromosome XIV aneuploidy was 80% of the euploid. Thus, the differing fitness costs of chromosomal aneuploidies are predictive of their long-term stability. Simulation-based inference suggests that reversion rates are similar for the two chromosomes, but the fitness cost associated with chromosome XIV aneuploidy is higher. The estimated reversion rates are higher than the CNV formation rates previously reported [Chuong et al. 2025; Avecilla et al. 2022], which had aggregated both aneuploidies and the less frequent focal CNVs. The fitness costs and rapid reversion rate indicates that spontaneous aneuploidies are expected to be rapidly lost from populations in the absence of positive selection.

Our study design allowed direct comparison of aneuploidy of chromosome XI and segmental CNVs on chromosome XI. We observed that aneuploidies of chromosome XI were negatively selected in the vast majority of cases (12 out of 13 populations), whereas segmental CNVs in chromosome XI were lost rarely

(4 out of 33 populations, or 1 of 9 unique segmental CNV lineages). We did not find a correlation between fitness costs of CNVs and the number of additional bases in the CNV region, consistent with our prior findings [Avecilla et al. 2023]. The sole segmental CNV that repeatedly reverted had no obvious distinguishing features compared with other segmental CNVs that were maintained during the evolution experiments. This suggests that fitness costs are not fully explained either by gene load or by specific dosage-sensitive genes, as concluded by Rojas et al. [2024].

Analysis of the sole segmental CNV that underwent reversion and selection revealed no evidence of the prior presence of the CNV, indicating a perfect reversion to the ancestral single-copy genome. This unexpected finding is supported by previous studies showing that antifungal drug resistance can drive CNVs that are reversible upon relaxation of this selection pressure, and can leave little trace that the CNV ever existed [Todd et al. 2020], and scarless reversion in independent instances of loss of *CUP1* amplifications [Chang et al. 2013]. This suggests that complete CNV reversion might be a common phenomenon.

Our results provide new insights into the plasticity and adaptability of eukaryote genomes. The transient and reversible nature of CNVs makes them an ephemeral adaptive class of genetic variation that may be a more widespread driver of adaptation in natural populations than inferred based on their observation in extant populations. Our results suggest CNVs can allow organisms to flexibly switch between phenotypes as is beneficial, allowing rapid adaptation to fluctuating environments. One limitation of this study is that all CNVs were nutrient transporter-driven amplifications that arose during evolution in nutrient limitation in chemostats. Although our CNV lineages include unique nutrient transporter loci on three different chromosomes, it would be interesting to study the stability and reversibility of adaptive CNVs originating in other kinds of environments.

Methods

Strains and media

We used 15 budding yeast strains with genome amplifications evolved from the haploid derivative of the *Saccharomyces cerevisiae* lab strain S288c. This strain was first modified to have an mCitrine gene under the control of the constitutively expressed *ACT1* promoter (ACT1pr::mCitrine::ADH1term) and marked by the KanMX G418-resistance cassette (TEFpr::KanMX::TEFterm). This mCitrine CNV reporter construct is on chromosome XI, adjacent to the *GAP1* gene. Strains *G_1* - *G_9* were isolated from this modified ancestor during a prior LTEE in glutamine-limited chemostats, described in Lauer et al. (2018). Cells from evolved populations of the ancestor preserved using glycerol at -80°C, were plated on glutamine-limiting media. Colonies were selected for increased mCitrine fluorescence using blue light, and grown in glutamine-limiting cultures overnight. The fluorescence of CNV-containing clones was validated using the flow cytometer Cytek Aurora and they were then preserved at -80°C using glycerol.

We then further altered the modified ancestor to include an mCherry reporter either on chromosome XIV beside the *MEP2* gene, or on chromosome 15 adjacent to the *PUT4* gene. *MEP2* and *PUT4* were amplified in ammonium-limited and proline-limited chemostats, respectively. Strains *M_10* - *GM_15* were evolved from such doubly modified ancestors with two reporters near two different genes of interest

(*GAP1* and *MEP2* or *PUT4*) during another previous LTEE (Abdul-Rahman et al., in prep) in which dual-fluorescence strains were evolved in fluctuating conditions of two different nitrogen-limiting sources: either glutamine and ammonium sulfate or glutamine and proline. Populations were revived from glycerol stocks archived at -80°C by inoculating overnight cultures of glutamine-limiting media and incubating them at 30°C. Fluorescence-Activated Cell Sorting (FACS) was used to sort subpopulations based on their FITC-A vs mCherry-A profiles in which all pairwise combinations of mCitrine:mCherry copy numbers ranging from 0 to 3 were gated separately (i.e. 0:0, 0:1, 0:2…) by using the 0-, 1-, and 2-copy controls. For each subpopulation, 100,000 - 200,000 cells were collected into 500 uL of 1X PBS, 100 uL of which was inoculated into an overnight culture of glutamine-limited media. Clones from each overnight culture were then isolated and mCitrine and mCherry copy numbers were validated by performing flow cytometry using the Cytek Aurora and they were then preserved at -80°C using glycerol.

To make fluorescence control strains, the mCitrine or mCherry reporter was incorporated at one or both of these neutral loci: *HO (YDL227C)* on chromosome IV and the dubious ORF *YLR123C* on chromosome XII. All experiments in the present study were performed in optimal growth conditions for laboratory strains of budding yeast using yeast extract peptone dextrose (YPD) media and growth at 30°C.

Long-term experimental evolution

We performed experimental evolution of all CNV strains via serial dilution in batch culture in 96-well plates. For each CNV strain, we isolated 3-4 isogenic clones to found independent populations that were placed in randomly-assorted wells. We cultured each clone in YPD, with a total volume of 200 µl per well. We back-diluted the cultures in fresh media by 1:64, such that they went through the full growth cycle of lag phase, exponential phase, and stationary phase every 6 generations. Strain *G_1 - G_3* were evolved for 110 generations, and strains *G_4 - GM_15* were evolved for 220 generations. We sealed the plates with a breathable membrane to prevent cross-contamination between wells while maintaining an aerobic environment at 30°C, shaking at 170 rpm.

Pairwise fitness competition assays

To measure the relative fitness of CNV strains and revertant clones in rich media, we performed pairwise competitive fitness assays. We co-cultured each strain of interest with the haploid reference strain lacking any fluorescent reporter, for 8-16 generations in conditions identical to the evolution experiment (96-well plate batch culture in YPD at 30°C, shaking at 170 rpm). We measured the relative abundance of each strain via the flow cytometer Cytek Aurora, and plotted the natural log of the ratio of the two strains, every 2-4 generations. This assay was done in triplicate for each strain, so that we could fit a linear regression using all three independent values. The starting ratio for each assay was set to have the same value, making it a one-parameter linear regression where we only fit for the slope. Relative fitness of each strain was calculated as (slope + 1), equal to 1 for the reference strain.

Flow cytometry

To monitor CNV dynamics in evolving strains using their fluorescent reporters, we sampled 20ul from each 200ul population and analysed them on a Cytek Aurora flow cytometer. We measured 10,000 cells per population, recording mCitrine fluorescence signal (excitation = 514 nm, emission = 530 nm, using the B2-A filter), mCherry fluorescence (excitation = 587 nm, emission = 610 nm, using the YG3-A filter),

cell size (forward scatter), and cell complexity (side scatter). We sequentially filtered out debris using side scatter area (SSC-A) and doublets using forward scatter height (FSC-H) and forward scatter area (FSC-A). We then measured fluorescence for each cell and divided this by FSC-A to account for differences in cell size. To quantify the percentage of cells in each population that have zero, one, and two or more copies of the genes of interest, we used control strains with known copy numbers of the reporter genes to manually draw gates around the range of normalized fluorescence values that correspond to each copy number. Since there is some overlap between the distribution of values corresponding to one and two copies, the process of cell selection via gating inherently involves some misclassification. We drew the one- and two-copy gates such that they were mutually exclusive, with the fraction of misclassification kept to a minimum. This ensured that each copy number gate contained 95% of cells of the corresponding control strain. The proportion of cells in the '2 or more copy' fluorescence gate at each timepoint allowed us to track the percentage of CNV-containing cells in each population over generations.

Prior to the evolution experiment, we optimized our flow cytometry protocol to ensure highest expression of fluorescent reporter genes, leading to maximal separation between the fluorescence distributions of one copy vs two copies of a reporter, and decreasing errors in gating. Fluorescence signal is reduced when cells are in stationary phase, so flow cytometry should be done in exponential phase. We measured fluorescence at different timepoints after each serial dilution, which switches cells from stationary phase back to exponential growth. We found that fluorescent signals are at their peak 4-5 hours after dilution.

Isolation of revertant clones from evolved populations

For isolating revertant clones from evolved populations preserved using glycerol at -80°C, we chose two late timepoints, generations 159 and 201. We plated cells on YPD from populations that showed CNV loss. Colonies were preliminarily screened for mCitrine fluorescence using blue light, from which randomly selected colonies were grown independently in liquid YPD overnight. We then measured the fluorescence of each culture using the flow cytometer Cytek Aurora. Clones with fluorescence corresponding to one copy of one or both of the reporter genes (mCitrine and mCherry) were designated as revertants. A total of 56 revertant clones from 17 populations across 5 strains were then preserved at -80°C using glycerol, for subsequent sequencing and fitness assays.

Whole-genome sequencing

For CNV strains and revertant clones isolated from their evolved populations, we extracted genomic DNA using the Hoffman-Winston protocol [Hoffman and Winston, 1987]. We quantified gDNA concentration using dsDNA broad range reagents on the Qubit fluorometer. DNA libraries were sequenced using a paired-end protocol (2 × 150) protocol on an Illumina Nextseq 500 or NovaSeq 6000. The reads were basecalled using Picard IlluminaBasecallsToFastq version 2.23.8 [https://broadinstitute.github.io/picard/], with APPLY_EAMSS_FILTER set to false. Following basecalling, the reads were demultiplexed using Pheniqs version 2.1.0 [Galanti et al. 2021]. The entire process was executed using a custom nextflow pipeline, GENEFLOW [https://github.com/gencorefacility/GENEFLOW].

Barcode verification of CNV strains and revertant clones

Of the 15 CNV strains, 12 strains (*G_4* - *GM_15*) had unique barcode sequences, which we used to confirm that each strain was a unique lineage that arose independently. We screened for the barcode

region using colony PCR and agarose gel electrophoresis, followed by DNA purification and quantification before Sanger sequencing. We obtained the unique barcode sequence for each strain by Clustal omega alignment on the Sanger sequences, and compared them to the known barcode template to find the varying bases.

For revertant clones, we used the barcode to confirm parental lineage and eliminate any possibility of contamination. We detected barcodes directly from whole-genome sequencing data. Across FASTQ files generated from all 56 revertants, we wrote a custom script using a global regular expression (grep) command to search for sequences matching the barcode template. The output defined the exact barcode sequence(s) present in each FASTQ file, and the number of corresponding reads. This allowed us to eliminate any barcodes present in very low frequencies, which are false positives due to index switching and not genuine detection in the genome sequence.

Gene copy number and breakpoint analysis

For each CNV and revertant strain, we estimated gene copy number using samtools to calculate the read depth of each nucleotide from whole-genome sequencing data. We calculated read depth after aligning the reads of each strain using BWA-MEM to a reference genome that includes the reporter gene constructs present in its ancestor.

To infer potential breakpoints in CNV and revertant strains, we used our custom analysis tool CVish (https://github.com/pspealman/CVish) [Spealman et al. 2025], which takes in FASTQ files of strains of interest and generates split reads and discordant reads by comparing them to the ancestral genome. We use these to locate amplified regions in the genome and to infer CNV formation and loss mechanisms. CVish also calculates relative read depth across the genome, outputting it as a bedgraph file which shows copy number differences among different genomic regions.

Single nucleotide variation analysis

For every ancestral, CNV and revertant strain, we used whole-genome sequencing data to identify genomic variations other than CNVs, including single nucleotide polymorphisms and DNA insertions and deletions. We used the bioinformatics pipeline developed by the Genomics Core at NYU CGSB (https://gencore.bio.nyu.edu/variant-calling-pipeline-gatk4/), using the Genome Analysis Toolkit 4 (GATK4) to perform variant calling and filtering. We then employed SnpEff to annotate and predict variant effects using identified SNVs, generating vcf files as the output.

GATK4 predictions typically suffer from high false-positive rates. To eliminate false-positive calls, we applied a custom pipeline (https://github.com/pspealman/heranca) [Spealman et al. 2025], that flags likely low-quality predictions arising from homopolymer or polynucleotide runs and identifies mutations that break the infinite sites assumption by occurring in at least three unrelated strains as likely false positives.

As a final step to get high-confidence SNVs, we individually evaluated SNVs by visualizing the bam files on Integrated Genome Viewer (IGV) [Robinson et al. 2011]. We compared each SNV in a revertant strain to the same position on its CNV parent, as well as its one-copy ancestor strain. Only SNVs

exclusively present in one strain, with a base conversion frequency of 90% or higher of total reads, were designated as high-confidence SNPs.

Evolutionary model and simulation-based inference

We modeled the experiments using a Wright-Fisher model with selection, mutation, and drift. First, we considered a scenario without epistasis between *GAP1* and *MEP2* CNV reversions. Without epistasis, we can consider each CNV reversion as an independent allele, with its own selection coefficient s (s_G or s_M), reversion rate δ (δ_G or δ_M), and initial frequency φ (φ_G or φ_M). Then, we infer the parameters of each experimental replicate separately. Thus, we infer three parameters per mutation, for a total of six parameters per replicate. We assume CNV reversion to be either beneficial or effectively neutral, as the fitness of CNV reversion is $1 + s > 1$. Then, estimating the fitness effect of CNVs, w_{cnv}, given s is $w_{cnv} = \frac{1}{1+s}$. The overall fitness of a strain was calculated assuming multiplicative fitness., $1 + s_{GM} = (1 + s_G)\times(1 + s_M)$. We used Neural Posterior Estimation (NPE; Greenberg et. al 2019, Papamakarios and Murray 2016) with Masked Autoregressive Flow (Papamakarios et. al 2017) for parameter inference, as in our previous studies [Avecilla et al 2022, Chuong et al 2025]. The training set consists of 10,000 evolutionary simulations and their parameter values, and the neural network is trained using a log-likelihood loss to approximate the amortized posterior distribution.

To bridge the gap between synthetic simulations and empirical observations we added a small Gaussian noise to each simulation, such that the simulation result given a parameter set θ, $x(\theta)$, becomes $x_{noisy}(\theta) = x(\theta) + \varepsilon$; $\varepsilon \sim N(0, 0.02)$. Note that for dynamics without epistasis we only require one trained neural network that can estimate the posterior distributions of both *GAP1* and *MEP2* CNV reversion parameters. We used a log-uniform prior for parameter values (**Supplemental Table 4**). Then, we computed the collective posterior distribution, a single posterior distribution conditioned on all replicates of each strain [see Chuong et al. 2025 for full details]. To validate the estimation of the individual and collective posterior distributions, we performed posterior predictive checks that measure the similarity between simulations using posterior parameter samples and the empirical observations.

Data availability

Sequencing data is available at SRA PRJNA1291234. The source code repository for simulation-based inference is on Github: https://github.com/nadavbennun1/de_et_al. Scripts for all other analysis and figures, as well as all data, are also available on Github: https://github.com/GreshamLab/CNV_Stability_without_selection_pressure.

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Supplementary Tables

Table 1. **CNV strains used in this study.** Summarised here are the different parameters by which the CNVs differed: the gene(s) under selection, copy number relative to the ancestor, size of the amplified region, molecular mechanism underlying CNV formation, number of elapsed generations before the lineage was isolated and the selection condition under which they arose.

Strain	Selected gene	Copy number	Size (kb)	Formation mechanism	Generation	Nutrient limitation
G_1	*GAP1*	4	33	ODIRA, Transposons	250	Glutamine
G_2	*GAP1*	3	191	ODIRA	250	Glutamine
G_3	*GAP1*	3	174	Segmental aneuploidy	250	Glutamine
G_4	*GAP1*	3	135	ODIRA	70	Glutamine
G_5	*GAP1*	3	62	Transposons	70	Glutamine
G_6	*GAP1*	3	45	ODIRA, NHR	70	Glutamine
G_7	*GAP1*	3	16	ODIRA	62	Glutamine
G_8	*GAP1*	2	16	ODIRA	62	Glutamine
G_9	*GAP1*	2	6	Transposons	70	Glutamine
M_10	*MEP2*	3	6	HR	265	Glutamine, Ammonium
P_11	*PUT4*	3	20	ODIRA, Transposons	88	Glutamine, Proline
GM_12	*GAP1* *MEP2*	2 2	667 784	Chr XI aneuploidy Chr XIV aneuploidy	177	Glutamine, Ammonium
GM_13	*GAP1* *MEP2*	2 2	667 784	Chr XI aneuploidy Chr XIV aneuploidy	177	Glutamine, Ammonium
GM_14	*GAP1* *MEP2*	2 2	667 784	Chr XI aneuploidy Chr XIV aneuploidy	177	Glutamine, Ammonium
GM_15	*GAP1* *MEP2*	2 2	667 784	Chr XI aneuploidy Chr XIV aneuploidy	147	Glutamine, Ammonium

Table 2. **CNV strain nomenclature and origin.** Lab collection ID, and publications which reported the experiments from which each strain originated.

Name of strain in the paper	Lab collection ID	Strain origin
G_1	DGY1741	Lauer et al. (2018)
G_2	DGY1743	Lauer et al. (2018)
G_3	DGY1744	Lauer et al. (2018)
G_4	DGY1886	Lauer et al. (2018)
G_5	DGY1917	Lauer et al. (2018)
G_6	DGY1883	Lauer et al. (2018)
G_7	DGY1988	Lauer et al. (2018)
G_8	DGY1976	Lauer et al. (2018)
G_9	DGY1920	Lauer et al. (2018)
M_10	DGY2702	Abdul-Rahman et al. (in prep)
P_11	DGY2769	Abdul-Rahman et al. (in prep)
GM_12	DGY2748	Abdul-Rahman et al. (in prep)
GM_13	DGY2750	Abdul-Rahman et al. (in prep)
GM_14	DGY2751	Abdul-Rahman et al. (in prep)
GM_15	DGY2755	Abdul-Rahman et al. (in prep)

Table 3. **Unique barcodes identifying each CNV lineage and revertants descended from them.** Each sequence was obtained from whole genome sequencing data by searching for the ancestral barcode template. Variable sequence regions in each barcode are highlighted.

Ancestral barcode template (38 bp): GGTACCNNNNNAANNNNNAANNNNNTTNNNNNATAACT		
Sequence verified barcode	**CNV strain**	**Revertant strain**
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT	*G_4*	DGY3286
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3287
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3288
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3289
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3410
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3411
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3412
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3413
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3414
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3415
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3416
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3417
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3418
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3419
GGTACCCGTAAAAAAGTAAAATTTTTTAAAATATAACT		DGY3420
GGTACCTTTACAACAATTAATAGCATTATTTTATAACT	*G_5*	No CNV reversion
GGTACCCTCTGAATGCGCAAGTCTCTTTCGGGATAACT	*G_6*	No CNV reversion
GGTACCCCAAAAACAGTTAATAACATTTGAGTATAACT	*G_7*	No CNV reversion
GGTACCCTTCAAAAAATTAAGATCCTTCATTAATAACT	*G_8*	No CNV reversion
GGTACCAATGTAAGATTTAACATCCTTTTCGCATAACT	*G_9*	No CNV reversion
GGTACCAAGGAAAGCTCCAAGTGAGTTCGAAGATAACT	*M_10*	No CNV reversion
GGTACCCACATAAATGATAAAGTTGTTCGCTCATAACT	*P_11*	No CNV reversion
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT	*GM_12*	DGY3290
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3421
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3422
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3423
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3424
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3425
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3426
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3427
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3428
GGTACCTCATGAAGAAGCAAAGGGTTTAAGGAATAACT		DGY3429

GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT	*GM_13*	DGY3291
GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT		DGY3430
GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT		DGY3431
GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT		DGY3432
GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT		DGY3433
GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT		DGY3434
GGTACCTTGAAAAATGGTAAAACAATTGTTAGATAACT		DGY3435
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT	*GM_14*	DGY3293
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3439
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3440
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3441
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3442
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3443
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3444
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3445
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3446
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3447
GGTACCTAGTCAATATATAAAGATATTTGTGCATAACT		DGY3448
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT	*GM_15*	DGY3294
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3295
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3449
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3450
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3451
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3452
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3453
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3454
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3455
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3456
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3457
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3458
GGTACCATCTTAAGTATCAACCACTTTCCGGCATAACT		DGY3459

Table 4. Marginal prior distributions of the evolutionary parameters. The overall prior distribution is the hypercube defined by these uniform marginal distributions.

Parameter	Description	Prior
s	Selection coefficients of CNV reversion	$log_{10}(s) \sim U[-3, -1]$
δ	CNV reversion rate	$log_{10}(\delta) \sim U[-5, -2]$
φ	Initial frequency of reversed CNV cells	$log_{10}(\varphi) \sim U[-5, -0.5]$

Table 5. **Confirmed SNVs across all revertants.** For each SNV, we record the genomic position, the original base and the new base to which it changed, the percentage of reads which underwent the base change, the gene or intergenic region where the SNV is located, and whether the revertant underwent partial (lost only Chr XIV) or complete (lost both Chr XI and Chr XIV) reversion.

CNV Strain	Revertant strain	Chr	Position (bp)	Original base	New base	Percentage of reads	Gene or intergenic region	Reversion type
GM_12	GM_12_Rev1	VII	245951	A	G	94% of 140	FLC3	Complete
	GM_12_Rev1	XIII	712911	C	T	95% of 169	ERG8	Complete
	GM_12_Rev2	I	54719	G	A	95% of 138	GEM1	Complete
	GM_12_Rev6	IV	675218	C	T	94% of 63	YDR109C	Complete
	GM_12_Rev10	XV	626108	C	A	96% of 77	SLP1	Partial
GM_13	GM_13_Rev1	X	536002	C	G	96% of 56	Between BFA1 and KCH1	Complete
	GM_13_Rev2	XI	669969	G	A	92% of 187	Near GEX2	Complete
	GM_13_Rev6	V	530182	A	T	96% of 117	RAD3	Complete
GM_14	GM_14_Rev1	XIII	160549	G	A	94% of 101	CMP2	Complete
	GM_14_Rev1	XIII	160563	A	G	97% of 106	CMP2	Complete
	GM_14_Rev2	III	226561	T	C	94% of 89	TVS1 (YCR061W)	Complete
	GM_14_Rev10	XVI	604956	C	A	100% of 96	SDD4 (YPR022C)	Partial
GM_15	GM_15_Rev1	X	517755	A	T	98% of 127	Between POL32 and IMT3	Complete
	GM_15_Rev3	VIII	170137	G	A	97% of 153	SLT2	Complete
	GM_15_Rev6	IV	1171363	C	T	96% of 142	PAL1	Partial
	GM_15_Rev6	V	490478	C	T	93% of 184	Between tE(UUC)E3 and YER158C	Partial
	GM_15_Rev7	XI	71825	A	T	93% of 117	PEX1	Complete
	GM_15_Rev8	VIII	240308	T	C	98% of 135	ERG7	Complete
	GM_15_Rev8	XV	410348	C	T	95% of 105	Between snR62 and WHI2	Complete
	GM_15_Rev12	IV	645268	C	G	95% of 129	Between tQ(UUG)D2 and YDR098C-B	Complete

Table 6. Strain nomenclature for all revertants. Names of all revertant as used in the paper, and their corresponding lab collection ID.

Revertant	Lab collection ID		Revertant	Lab collection ID
G_4_Rev1	DGY3286		GM_13_Rev4	DGY3432
G_4_Rev2	DGY3287		GM_13_Rev5	DGY3433
G_4_Rev3	DGY3288		GM_13_Rev6	DGY3434
G_4_Rev4	DGY3289		GM_13_Rev7	DGY3435
G_4_Rev5	DGY3410		GM_14_Rev1	DGY3293
G_4_Rev6	DGY3411		GM_14_Rev2	DGY3439
G_4_Rev7	DGY3412		GM_14_Rev3	DGY3440
G_4_Rev8	DGY3413		GM_14_Rev4	DGY3441
G_4_Rev9	DGY3414		GM_14_Rev5	DGY3442
G_4_Rev10	DGY3415		GM_14_Rev6	DGY3443
G_4_Rev11	DGY3416		GM_14_Rev7	DGY3444
G_4_Rev12	DGY3417		GM_14_Rev8	DGY3445
G_4_Rev13	DGY3418		GM_14_Rev9	DGY3446
G_4_Rev14	DGY3419		GM_14_Rev10	DGY3447
G_4_Rev15	DGY3420		GM_14_Rev11	DGY3448
GM_12_Rev1	DGY3290		GM_15_Rev1	DGY3294
GM_12_Rev2	DGY3421		GM_15_Rev2	DGY3295
GM_12_Rev3	DGY3422		GM_15_Rev3	DGY3449
GM_12_Rev4	DGY3423		GM_15_Rev4	DGY3450
GM_12_Rev5	DGY3424		GM_15_Rev5	DGY3451
GM_12_Rev6	DGY3425		GM_15_Rev6	DGY3452
GM_12_Rev7	DGY3426		GM_15_Rev7	DGY3453
GM_12_Rev8	DGY3427		GM_15_Rev8	DGY3454
GM_12_Rev9	DGY3428		GM_15_Rev9	DGY3455
GM_12_Rev10	DGY3429		GM_15_Rev10	DGY3456
GM_13_Rev1	DGY3291		GM_15_Rev11	DGY3457
GM_13_Rev2	DGY3430		GM_15_Rev12	DGY3458
GM_13_Rev3	DGY3431		GM_15_Rev13	DGY3459

Supplementary Methods

Evolutionary model and simulation-based inference

Evolutionary model (without epistasis). The following presents a model for the evolution of *GAP1* CNV reversion. We follow the frequencies of two genotypes over time: X_{G_1}, the frequency of genotype G_1, representing single-copy *GAP1*; and X_{G_2}, the frequency of genotype G_2, representing two copies of *GAP1*.

The model for *MEP2* CNV reversion is similar, with M (for *MEP2*) replacing G (for *GAP1*).

Initially, the genotype frequencies are:

$$X_{G_1}{}^{0} = \varphi_G, \; X_{G_2}{}^{0} = 1 - \varphi_G .$$

where φ_G is the initial frequency of genotype G_1 and is a model parameter.

The change in frequencies due to mutation is given by

$$X_{G_2}{}' = (1 - \delta_G)X_{G_2}{}^{t}, \; X_{G_1}{}' = X_{G_1}{}^{t} + \delta_G X_{G_2}{}^{t} .$$

where $X_g{}'$ is the frequency of genotype g after mutation.

The change in frequencies due to selection is given by

$$\bar{w} = w_{G_1}X_{G_1} + w_{G_2}X_{G_2} = (1 + s)X_{G_!} + X_{G_2} ,$$

$$X_{G_2}{}^{*} = \frac{X_{G_2}{}'}{\bar{w}}, \; X_{G_1}{}^{*} = \frac{X_{G_1}{}'}{\bar{w}}$$

where w_g is the fitness of genotype g, and $\bar{w}$ is the population mean fitness. $X_g{}^{*}$ represents the frequency of genotype g after selection.

The change in frequencies due to random genetic drift is given by

$$n_{G_1} = Binomial(N_e, X_{G_1}^{*}),\ n_{G_2} = N_e - N_{G_1}$$

where n_g is the number of cells with genotype g.

The frequencies in the next generation are then given by

$$X_{G_1}^{t+1} = \frac{n_{G_1}}{N_e},\ X_{G_2}^{t+1} = \frac{n_{G_2}}{N_e}$$

Overall, we estimate three model parameters: s_G, δ_G, φ_G. The effective population size is constant at $N_e = 1.9 \times 10^6$ (see details below).

The evolutionary model for *MEP2* CNV reversion is similar, with s_M, δ_M, φ_M substituting s_G, δ_G, φ_G, and X_{M_1}, X_{M_2} substituting X_{G_1}, X_{G_2}.

Effective population size. Populations begin each full growth cycle at $N_0 = 6.25 \times 10^5$ cells and duplicate six times before back-dilution to N_0. Using the harmonic mean [Gillespie 2004] for calculating the effective population size, we have

$$N_e = \frac{6}{\frac{1}{6.25\times10^5} + \frac{1}{2\times6.25\times10^5} + \ldots \frac{1}{2^5\times6.25\times10^5}}$$

The denominator can be calculated using a geometric sum, yielding

$$\frac{1}{6.25\times10^5} + \frac{1}{2\times6.25\times10^5} + \ldots + \frac{1}{2^5\times6.25\times10^5} = \frac{\frac{1}{6.25\times10^5}(0.5^6-1)}{0.5-1}$$

Leading to

$$N_e = \frac{6}{\frac{\frac{1}{6.25\times10^5}(0.5^6-1)}{0.5-1}} \approx 1.9 \times 10^6$$

Evolutionary model with epistasis. The following describes a model for evolution of *GAP1* and *MEP2* CNV reversions with epistasis between the two CNVs (Figure S8_3). In this model, we consider four genotypes: G_2M_2, cells with both *GAP1* CNV and *MEP2* CNV (i.e., at least one *MEP2* duplication); G_1M_2, cells with a single copy of *GAP1* and a *MEP2* CNV; G_2M_1, cells with a *GAP1* CNV and two copies of MEP2; and G_1M_1, cells with single copies of both *GAP1* and *MEP2*. The frequency of genotype g at time t is denoted by $X_g^{\ t}$.

Each genotype has a distinct fitness, $w_g = 1 + s_g$, with the fitness of the double CNV genotype G_2M_2 fixed as 1 without loss of generality. Each of the three mutant genotypes starts with a distinct initial frequency, denoted by φ_g; and the reversion rate of CNV c is denoted by δ_c, with $c = M$ for *MEP2* and $c = G$ for *GAP1*. The rate of a second reversion j is distinct and dependent on the first reversion i, denoted by $\delta_{j|i}$. This model therefore has ten parameters: $s_{G_1M_2}$, $s_{G_2M_1}$, $s_{G_1M_1}$, δ_G, δ_M, $\delta_{G|M}$, $\delta_{M|G}$, $\varphi_{G_1M_2}$, $\varphi_{G_2M_1}$, $\varphi_{G_1M_1}$.

Assuming that CNV reversion rates are independent of the genotype, and that there is no epistasis between CNV reversions, we can calculate the values of the four parameters that are unique to the model with epistasis from the six parameters of the model without epistasis: $1 + s_{G_1M_1} = (1 + s_{G_1M_2})(1 + s_{G_2M_1})$; $\delta_{M|G} = \delta_M$, $\delta_{G|M} = \delta_G$; $\varphi_{GM} = \varphi_G \times \varphi_M$.

Initially, the genotype frequencies are:

$$X_{G_1M_1}^{\ 0} = \varphi_{G_1M_1},\ X_{G_2M_1}^{\ 0} = \varphi_{G_2M_1},\ X_{G_1M_2}^{\ 0} = \varphi_{G_1M_2},$$

$$X_{G_2M_2}^{\ 0} = 1 - \varphi_{G_1M_1} - \varphi_{G_1M_2} - \varphi_{G_2M_1}.$$

where φ_G is the initial proportion of genotype G_1, and is a model parameter.

The change in frequencies due to mutation is given by

$$X_{G_2M_2}' = (1 - \delta_G - \delta_M)X_{G_2M_2}^{\ t},$$

$$X_{G_1M_2}' = (1 - \delta_M)X_{G_1M_2}^{\ t} + \delta_G X_{G_2M_2}^{\ t}$$

$$X_{G_2M_1}' = (1 - \delta_G)X_{G_2M_1}^{\ t} + \delta_M X_{G_2M_2}^{\ t}$$

$$X_{G_1M_1}' = X_{G_1M_1}^{\ t} + \delta_G X_{G_2M_1}^{\ t} + \delta_M X_{G_1M_2}^{\ t}$$

where X_g' is the frequency of genotype g after mutation.

The change in frequencies due to selection is given by

$$\bar{w} = \Sigma_g [w_g X_g] \, , X_g^* = \frac{X_g'}{\bar{w}}$$

where w_g is the fitness of genotype g, and $\bar{w}$ is the population mean fitness. X_g^* represents the frequency of genotype g after selection.

The change in frequencies due to random genetic drift is given by

$$(n_{G_1M_1}, n_{G_1M_2}, n_{G_2M_1}, n_{G_2M_2}) = Multinomial(N_e, (X_{G_1M_1}^*, X_{G_1M_2}^*, X_{G_2M_1}^*, X_{G_2M_2}^*))$$

where n_g is the number of cells with genotype g.

The frequencies in the next generation are then given by

$$X_g^{t+1} = \frac{n_g}{N_e}$$

Predictive checks for the model with epistasis. To perform a predictive check, we inferred six model parameters using the model without epistasis for each of the replicates, calculated the value of the additional four parameters assuming independence of CNV reversions, simulated using these parameter estimates in the model with epistasis, and compared the simulation results to the empirical data.
These predictions aligned well with the empirical data. Therefore, we concluded that the model without epistasis is sufficient and did not use the model with epistasis for parameter inference in the main text. In the future, the model with epistasis could be used to study results of experiments with epistasis between two-loci.

Supplementary Figures

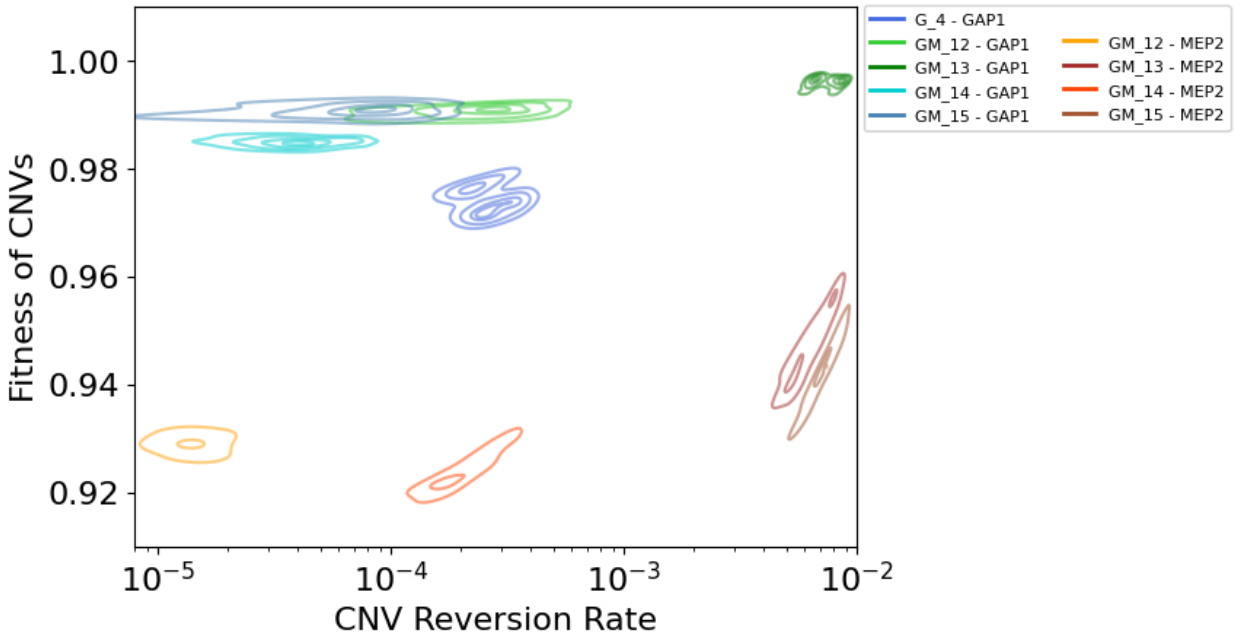

Figure S1. Parameter estimates. 95% high-density regions (HDRs) of the collective posterior distributions for the fitness cost (derived from selection coefficients, see Methods) and reversion rate of CNVs.

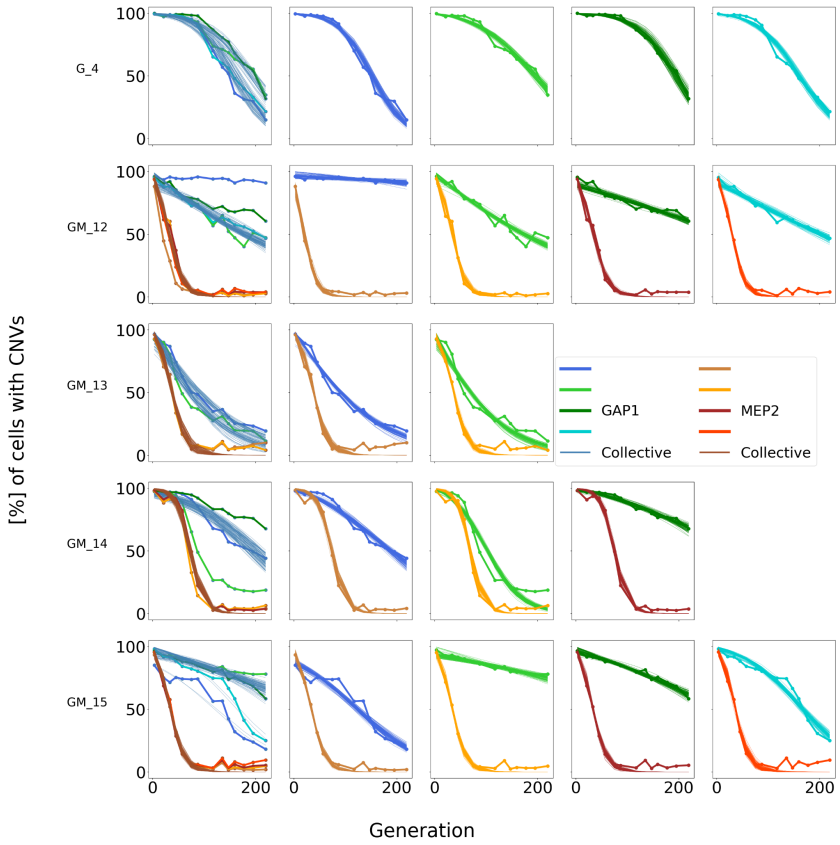

Figure S2. Posterior predictive checks. Model simulations of 100 parameter sets sampled from the posterior distributions inferred from each experimental replicate (shaded lines) against the empirical data (solid lines with markers). Leftmost (grey) panels of each row show predictive checks of the collective posterior distribution for the strain, i.e., a posterior distribution conditioned on all strain replicates.

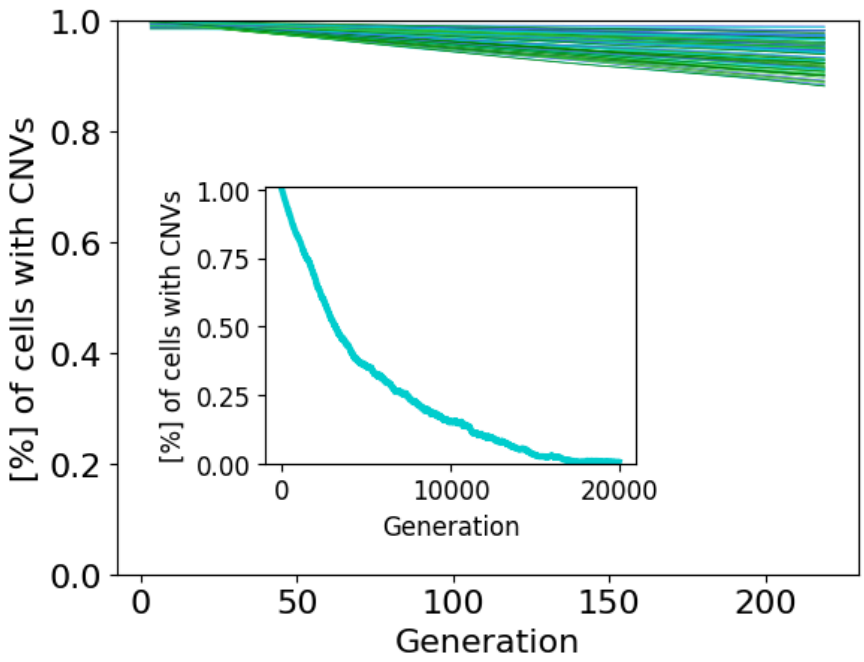

Figure S3. *GAP1* CNV reversion without selection. Evolutionary simulations with mutation rate and initial CNV proportion sampled from the collective posterior distribution of strain *G_4* and with very weak selection, $s = 10^{-10}$. Inset axis shows a simulation of the sample mean for 20,000 generations.

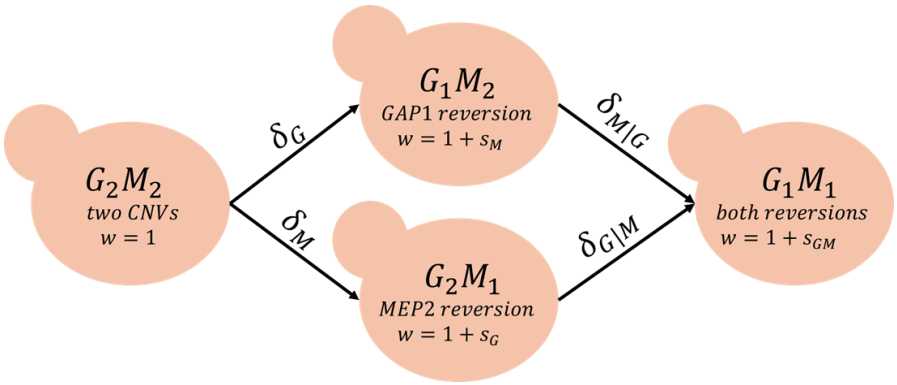

Figure S4. A genotype-tracking evolutionary model. CNV reversions are not necessarily independent, with rates $\delta_{i|j}$ for transitioning from genotype j to genotype i, and a distinct selection coefficient s_k for genotype k. We track the proportions of each genotype, from which we can calculate the total proportions of each CNV. Cell labels refer to both genes (G - *GAP1*, M - *MEP2*) and their corresponding copy numbers, i.e., indicate the entire cell's genotype.

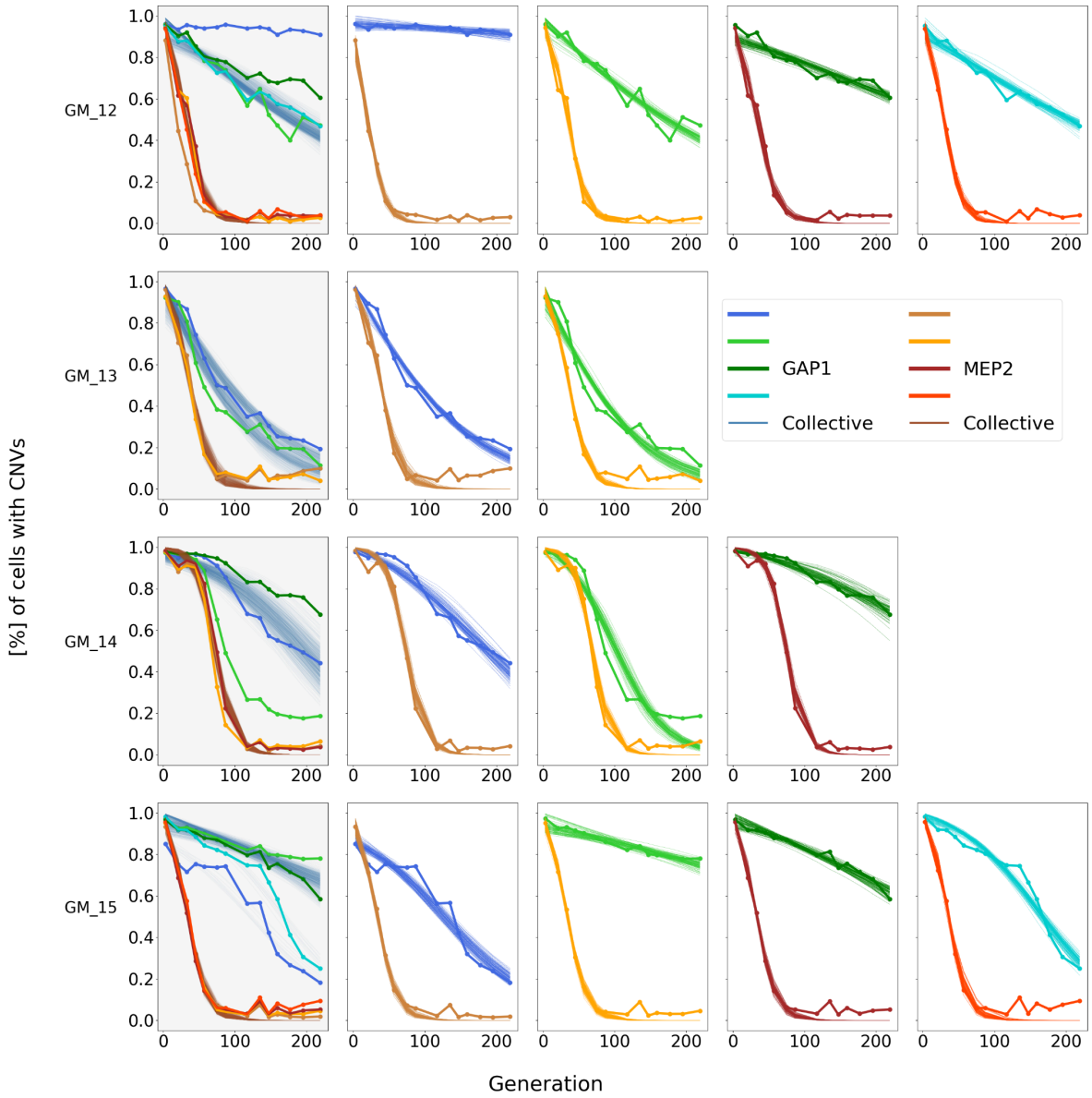

Figure S5. Posterior predictive checks with the genotype-tracking model. Genotype-tracking model simulations of 100 parameter sets sampled from the posterior distributions of each experimental replicate (shaded lines), inferred using the allele-tracking model against the empirical data (solid lines with markers). Leftmost (grey) panels of each row show predictive checks of the collective posterior distribution for the strain, i.e., a posterior distribution conditioned on all strain replicates. Strain G_4 is not shown since it does not have a *MEP2* CNV.