Final Report of the *FK-128775* project entitled *"Overcoming haploinsufficiency: an experimental platform to test the evolution of dominance"*

This project was about **investigating how baker's yeast (*Saccharomyces cerevisiae*) can cope with a 50% reduction in gene dosage**. This means that the **fitness of yeast strains decreases when a single copy of a gene is not sufficient to maintain normal function (i.e. haploinsufficiency)**. Since haploinsufficiency is **often associated with various human diseases**, we aimed to **better understand the general principles underlying haploinsufficiency, with particular focus on the molecular mechanisms that might attenuate it**, thus **playing a role in the evolution of haploinsufficiency**. To achieve our goals, we have combined laboratory evolution, large-scale fitness measurements, phenomics, bioinformatics analyses and whole genome sequencing of originally slow-growing (i.e. haploinsufficient or HI in short) diploid yeast mutant strains. By integrating the above techniques, we have **achieved all our goals and prepared a manuscript to submit to a top-tier journal in the near future (Farkas et al, 2023)**, until then it has been made available in a Google Drive folder (https://rebrand.ly/NKFIH_FK_128775). Furthermore, **last year we published our results on the role of compensatory evolution on yeast morphology in a high-rank evolutionary journal** (Farkas et al., 2022).

Our results are summarised below.

According to ***Aim 1., we initiated laboratory evolutionary experiments*** with 125 diploid heterozygous gene-knockout mutant strains of *S. cerevisiae*. The slow-growth phenotype of these strains were confirmed by high-throughput in vitro fitness measurement in liquid medium. The laboratory evolutionary experiment was conducted on four independent populations of each of the selected heterozygous deletion strains. Independently evolving populations (along with control strains with no slow-growth phenotype) were **propagated for 52 transfers (approximately 320 generations**). According to ***Aim 2., we aimed to determine the extent of compensation*.** To this end, we **performed the same in vitro fitness measurements of the evolved lines as previously and compared the evolved fitness values to the corresponding initial strains**. We found that the fitness of the **500 originally haploinsufficient lines predominantly increased after evolution (75.2% of the cases**, Figure 1.).

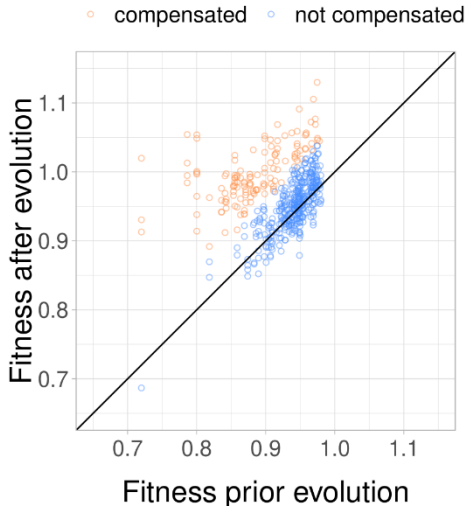

Figure 1. Fitness of haploinsufficient strains generally improves during the course of laboratory evolution. The scatterplot shows the relative fitness values of ancestor and evolved haploinsufficient strains. Relative fitness was estimated by normalizing the absolute fitness of the investigated strains with that of the wild type strain (HO/Δho). The absolute fitness of the strains was estimated by measuring the growth rate of each strain after monitoring the growth of individual strains in a plate reader. Orange and blue points correspond to compensated and not-compensated evolved lines, respectively (for definition of compensation, see the description below).

On average, **laboratory evolved, initially haploinsufficient strains showed larger fitness gains (4.1%) than the control wild-type (1.6%) and *haplosufficient*** (i.e. when a heterozygous deletion do not cause slow-growth phenotype) **strains (2.2%).** Each evolved strain was considered as a compensated strains if it showed a fitness increase disproportionately larger than that of observed in the evolving wild-type and *haplosufficient* strains. **Fifty-five out of the 125 haploinsufficient genotypes showed such a fitness gain in at least one of the four corresponding evolving populations** (Figure 2). Notably, **fitness gain differed significantly across the various genotypes**, indicating that a reduced gene dosage can be more easily compensated for certain genotypes than for others (ANOVA, P<0.001).

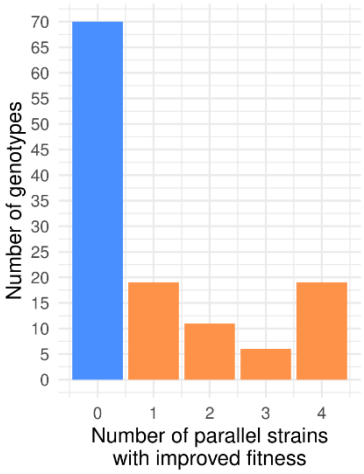

Figure 2. Genotypes with improved fitness after laboratory evolution. The histogram shows the distribution of the number of parallel evolved strains with significant fitness improvement across all genotypes (Wilcoxon rank sum test, FDR-corrected P <0.05). In total, 44.4% of the genotypes showed evidence of fitness gain in at least one of the four parallel evolved strains (orange bars).

According to ***Aim 3., we explored common genomic features that affect the propensity of compensation of HI.*** First, we investigated the differences in compensatory potential of various functional impairments. To this end, we compared the extent of fitness change and the prevalence of fitness improvement across genotypes with different disrupted functions, and found that both of them varied significantly (Figure 3, Anova, P < 0.001).

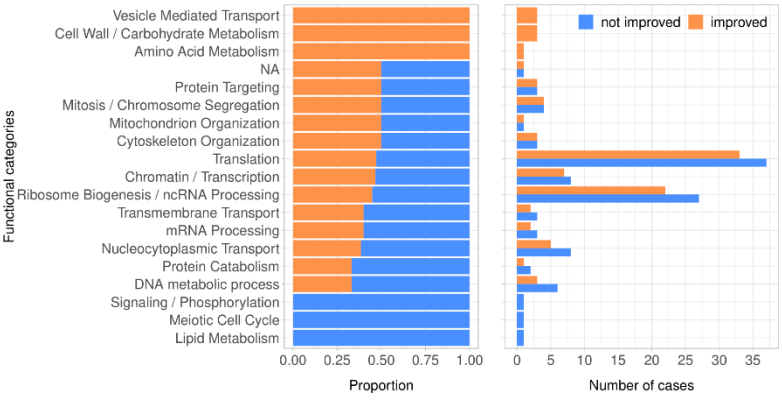

Figure 3. Distribution of fitness improvement across functional categories. The barplots show the relative fraction (left panel, 3A) and the number (right panel, 3B) of evolved strains with and without fitness improvement as a function of the major cellular role of the disrupted genes (Ryan et al., 2012). Strains with a significantly higher fitness change compared to the corresponding evolving wild-type strains (one-sided Wilcoxon rank sum test, FDR-corrected P < 0.05) are indicated as *improved* (orange), whereas those without a significant change are indicated as *not improved* (blue). Please note, that a given genotype could fall into both categories (improved / not improved) based on the fitness change of the independently evolved strains. Relative fitness improvement (RFI) was calculated as detailed in Figure 1B.

We **collected various** genomic and functional genomic **gene properties** covering many aspects of baker's budding yeast genetics, such as expression level, protein abundance or

pleiotropy. We subsequently **tested whether the extent of compensation is influenced by any of these genomic features**. Our observations were as follows:

a.) Initial fitness: This was the **strongest gene property that influenced fitness compensation**. Consistent with prior studies on null mutations(Moore et al., 2000; Poon and Chao, 2005; Szamecz et al., 2014), gene disruptions with remarkably severe fitness defects were especially likely to be mitigated during the course of laboratory evolution (Figure 4).

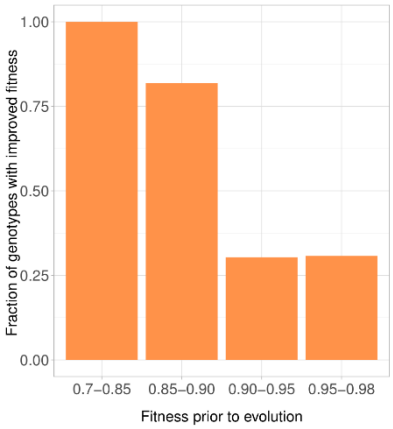

Figure 4. Impact of the initial fitness on fitness improvement. The barplot shows the fraction of genotypes with improved fitness in the evolved lines as a function of initial fitness of the haploinsufficient genotype. Genotypes with a more severe initial fitness defect were more likely to improve fitness during laboratory evolution (chi-square test for trend in proportions, $P < 0.0001$). Fitness was estimated by normalizing the growth rate of the strains in liquid medium to that of the wild type.

b.) Context dependency of haploinsufficiency. Compensability of HI do not depend on either the environmental or the genetic context. To investigate environment dependency, we used a previous systematic study that measured the fitness of heterozygous deletion strains in various conditions and established different set of haploinsufficient mutants across different environments (Delneri et al., 2008). Surprisingly, we found no association between the compensability and environment-dependency of haploinsufficiency (Odd's ratio = 0.76, P = NS, Fisher's test). In a similar vein, we tested the association between the compensability of haploinsufficiency and its conservation across species using orthologous genes of *S. cerevisiae* and *S. pombe* (Kim et al., 2010). Unexpectedly, we found similar compensability of conserved haploinsufficient genes (6/13 = 46.2%) with that of the species-specific haploinsufficient genes (43/95 = 45.2%). These results suggest that the plasticity of haploinsufficiency across genetic and environmental factors does not have a large contribution in the compensability of haploinsufficiency.

c.) Potential mechanisms of dosage sensitivity. Compensability of haploinsufficiency was independent on dosage sensitivity. Haploinsufficiency might be induced by at least three molecular mechanisms: i.) dosage imbalance in case the corresponding gene encodes a protein complex member (Papp et al., 2003); ii.) insufficient expression after copy number reduction (Wilkie, 1994) and iii.) increased susceptibility to stochastic variations in gene expression due to the absence of one active allele (Cook et al., 1998; Kemkemer et al., 2002). We investigated the possibilities that any of these mechanisms affect the compensability of HI by using several previously published datasets. First, we found no evidence that membership in protein complexes (Pu et al., 2009) has any effect on the compensability of a haploinsufficient gene (Fisher's test, P = NS). Second we compared the expression level of the haploinsufficient genes in

compensated genotypes to that of not compensated ones using a genome-scale database (Greenbaum et al., 2003) and found no significant difference in neither gene expression, nor protein abundance (Wilcoxon rank sum test, P = NS). Third, we found no significant difference in either the stochastic noise or responsiveness (Choi and Kim, 2009) of the corresponding haploinsufficient gene in the compensated strains compared to that of not compensated ones.

According to ***Aim 4., we explored the molecular mechanisms of compensatory adaptation by functional genomics.*** There are at least two potential routes towards enhanced fitness in haploinsufficient strains. First, the expression level of the wild-type, intact allele could be increased through duplication of the corresponding genomic segment. Second, evolution may promote the emergence of point mutations in other genomic regions, leading to enhanced dominance of the wild-type allele over the loss-of-function allele, possibly without changing its expression level.

First we **investigated the changes in DNA copy number of the genomic region carrying the haploinsufficient gene of interest**. For this purpose, a real-time qPCR analysis was performed both on the ancestor genotype and the evolved strains of 24 initially haploinsufficient genotypes, all of which displayed improved fitness. An **extra copy number of the DNA segment carrying the wild-type allele was observed in 55.4% of the 83 investigated laboratory evolved strains** (Figure 5). As expected, there was a strong positive association (Spearman's correlation, R = 0.77, p = 0.00068) between the change in DNA copy number and the transcript level of the haploinsufficient gene (based on gene expression analysis of the corresponding HI genes on all evolved lines of 3 selected genotypes, data not shown).

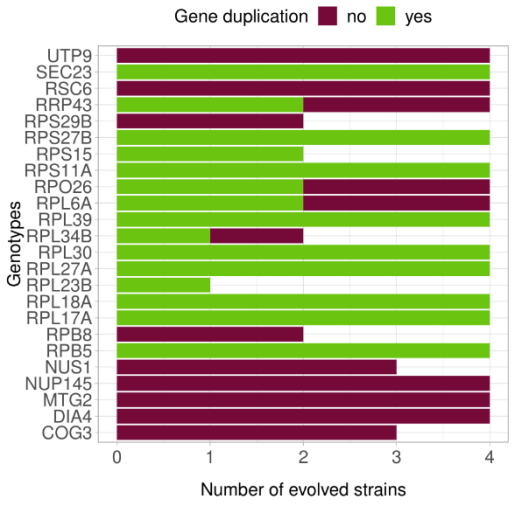

Figure 5. Evolution by gene duplication in haploinsufficient strains. The barplot shows the total number of evolved strains per genotype with (green) or without (purple) gene duplication of the haploinsufficient gene. Only strains with improved fitness (N = 83) of the 24 initially haploinsufficient genotypes are indicated. In total, 55.4% of the improved strains evolved by duplication of the haploinsufficient gene.

Notably**, independently evolved strains carrying the same disrupted haploinsufficient gene occasionally followed different evolutionary paths**: some but not all strains improved fitness via duplication of the haploinsufficient gene (Figures 3A and 3B). Moreover, **not all genotypes were equally likely to restore fitness through gene duplication**. Laboratory evolved strains with a disrupted gene involved in protein production-related functions (mRNA and ncRNA processing, ribosome biogenesis, transcription or translation) were especially likely to display an increase in copy number (Fisher's test, odds ratio = 6.1, P < 0.0001, Figure 6).

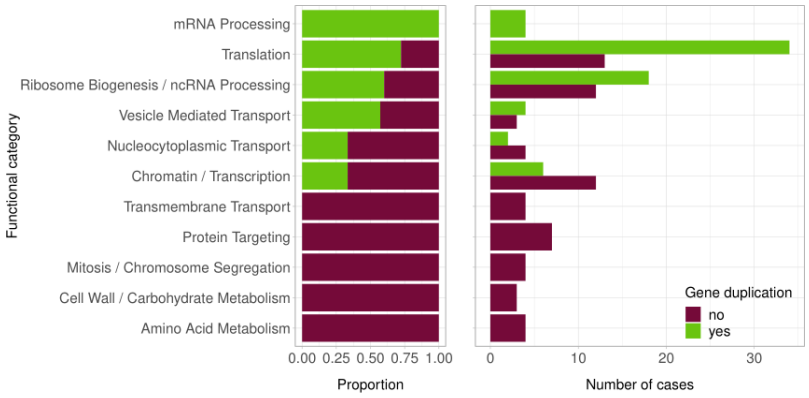

Figure 6. Evolution by gene duplication as a function of the haploinsufficient genes. The barplots show the relative fraction (left panel) and the number (right panel) of the evolved strains with (green) and without (purple) duplication of the focal gene as a function of the major cellular role of the disrupted genes (Ryan et al., 2012). Mutants with haploinsufficient genes involved in protein production-related functions (mRNA processing, transcription, ribosome biogenesis, ncRNA processing, translation) were especially likely to be improved by gene duplication during evolution (Fisher's test, odds ratio = 6.1, P < 0.0001).

However, **fitness improvement of the evolved strains with or without gene duplication of the haploinsufficient gene were found to be indifferent** (Figure 7). On a related note, we found that the severity of haploinsufficiency (i.e. initial fitness) do not predispose either of the two potential compensatory trajectories (Figure 7).

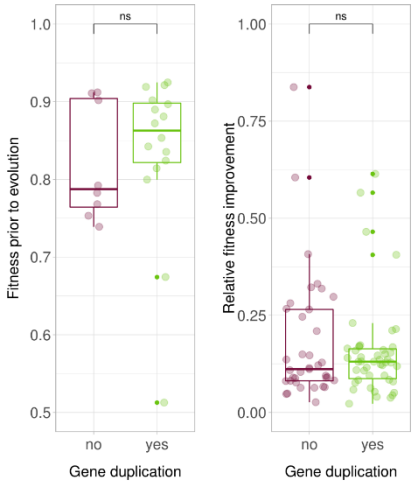

Figure 7. No relationship between evolution by gene duplication and fitness improvement. The boxplots show the initial fitness of the ancestor genotype (left panel) and the relative fitness improvement of the evolved strains (right panel) with or without duplication of the haploinsufficient gene. Each point represents the median initial fitness and relative fitness improvement of the ancestor genotype and evolved strains, respectively, based on at least 6 biological replicates. Boxplots show the median, first and third quartiles, with whiskers showing the 5th and 95th percentiles of the fitness values of the tested strains. Fitness and fitness improvement were calculated as detailed above (see Figure 1). Wilcoxon rank sum test was used to assess significant differences between the two groups (ns indicates P = not significant).

To explore the genomic changes that have accumulated during laboratory evolution ***(part of Aim 4.), we re-sequenced the complete genomes of all four independently evolved strains and the corresponding ancestors*** of **five initially haploinsufficient genotypes**, by using Illumina platform. **In total, we observed 125 mutational events among the 20 genomes analysed,** including **point mutations (77.6%), small insertions or deletions (10.4%), as well as duplication and loss of chromosomal segments (12%). Sixty-seven percent** of the point mutations **affected protein coding regions**, while the rest were intragenic mutations (Figure 8).

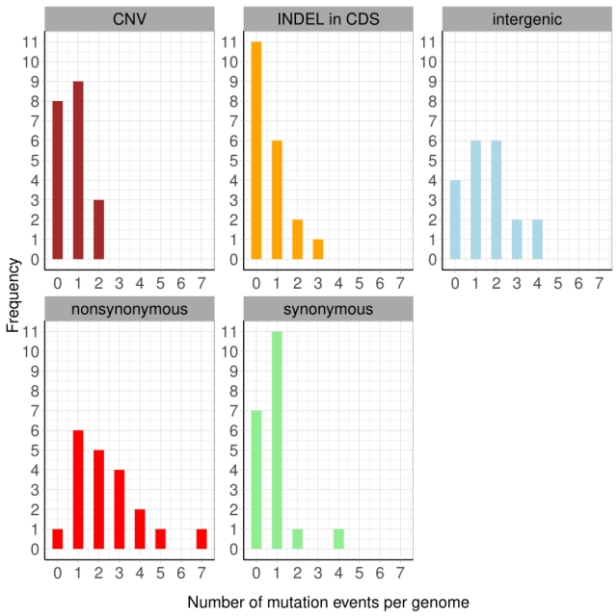

Figure 8. Distribution of the detected mutations in the evolved strains. CNV indicates large chromosomal duplication or deletion events, while INDELS in CDS indicate insertions and deletions in protein coding regions. Point mutations are divided into three main categories (intergenic, nonsynonymous and synonymous). On average, 4.8 point mutations, 0.6 small insertions or deletions and 0.76 copy number variations per evolved strain were detected.

On average, we detected 6.25 genomic mutations per strain. Notably, two evolved lines of *RPO26/Δrpo26* displayed an increased *RPO26* copy number (Figure 5). Genome sequence analysis of these evolved strains revealed a duplication of chromosome XVI that harbours the intact copy of the *RPO26* gene. Notably, **we did not find less genomic mutations in the parallel evolved strains of *RPO26* with chromosome duplication compared to that of without duplication**.

We **found no mutations in either the coding regions of transcriptional regulators, or the promoter region of the haploinsufficient gene**. However, **several notable functional links were apparent** between the mutated genes and the haploinsufficient gene. As expected, we also found **several links between the mutated and haploinsufficient genes using unbiased measures of functional relationships** (e.g. coexpression, genetic- or protein-interaction). To conclude, we found **several types of mutations, preferentially in functionally related genes that could provide an evolutionary escape from haploinsufficiency**.

According to ***Aim 5., we investigated fitness costs and epistatic effects of compensatory mutations.*** To this end, we conducted an **in-depth genetic analysis of the *RPO26/Δrpo26* genotype** with the aim of exploring the potential pleiotropic side effects of mutations shaping haploinsufficiency. Independently evolved lineages starting from the same *RPO26/Δrpo26* genotype **compensate** for the reduced *RPO26* dosage **either by chromosome duplication and/or by accumulation of point mutations elsewhere**.

We examined **how mutations that have accumulated during the course of laboratory evolution in the heterozygote genetic background affect fitness in the wild-type**. This was achieved by **increasing the transcript level of the individual genes causing haploinsufficiency using the MoBY-ORF 1.1 single-copy plasmid library** (Ho et al., 2009). This system is well-suited to increase the expression of individual genes by two to threefold only (Morrill and Amon, 2019). Another advantage of this experiment is that by restoring the wild-type expression level, it **simultaneously investigate the total impacts of all compensatory mutations in a background analogous to wild-type yeast** The MoBY-ORF plasmid carrying the *RPO26* gene was

introduced into the wild-type, the *RPO26* haploinsufficient ancestor and the corresponding four evolved strains.

Next, we measured the **fitness of these strains with and without the corresponding MoBY-ORF plasmid** in the conditions employed during the course of laboratory evolution. One might expect that mild gene overexpression of the specific focal gene is beneficial in the haploinsufficient, but not in the wild-type genetic background. This was indeed so: **MoBY-ORF increased fitness by 150% in the haploinsufficient strain prior to laboratory evolution, while it was neutral in the wild-type**. Introduction of MoBY-ORF into the four *RPO26/Δrpo26* laboratory evolved lines had distinct effects on fitness. Notably, the relative **fitness change was significantly higher in those lines that carried no duplication of chromosome XVI harboring the *RPO26* gene itself** (58% and 48% increase in Ev1 and Ev3, respectively), whereas it had only **very minor or no fitness effect in the lines harboring an extra copy of chromosome XVI** (8% and 1% increase in Ev2 and Ev4, respectively, Figure 9).

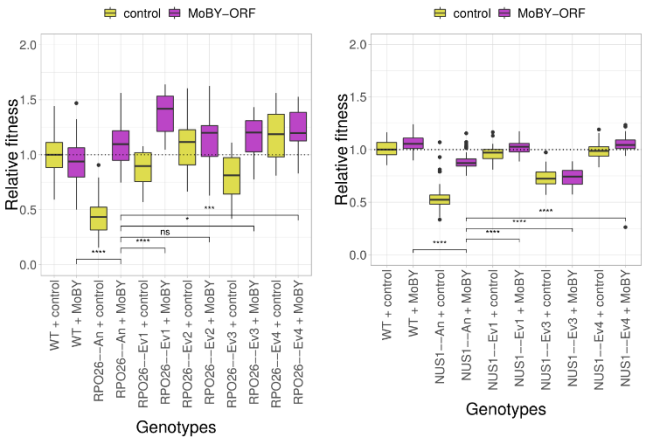

Figure 9. Fitness costs and benefits of mutations in the evolved lines. The boxplots show the relative fitness of the wild type, initially haploinsufficient heterozygote strains (left panel *RPO26/Δrpo26, NUS1/Δnus1,* right panel), and the corresponding evolved lineages (Ev1 to Ev4). All strains were investigated in the presence of a control (yellow), or the corresponding MoBY-ORF single copy plasmid (*RPO26* or *NUS1)* under the control of the native promoter (purple), respectively. Two evolved lines of *RPO26/Δrpo26 (*EV2 and EV4) carried trisomies of chrXVI harboring the focal gene; whereas the other two evolved lines (EV1 and EV3) of the same genotype and all evolved line of the *NUS1/Δnus1* genotype displayed no duplication of the corresponding focal gene. Boxplots show the median, first and third quartiles, with whiskers showing the 5th and 95th percentiles of the relative fitness of each investigated strain based on 30 biological replicates. Mann Whitney U-test was used to assess significant differences of relative fitness of the control and MoBY-ORF carrying mutants for each genotype tested. */**/***/**** indicates p < 0.05/0.01/0.001/0.0001, ns indicates p = not significant. Relative fitness was calculated by normalizing the absolute fitness values of the lines with that of the wild-type harboring a control plasmid.

This indicates that the **accumulated mutations also have an independent (potentially additive) beneficial effect in a genotype with restored copy number of the HI gene**. Introduction of MoBY-ORF into a nuclear undecaprenyl pyrophosphate synthase mutant strain, the *NUS1/Δnus1* genotype resulted in similar patterns, although in the case of NUS1-ev3 the accumulated mutation had a significant fitness cost in the genotype with restored dosage of the HI gene. Together, these results indicate that **ectopic mutations and duplication of the haploinsufficient genes modulate haploinsufficiency in an independent manner**.

According to ***Aim 6., we performed experiments to study the trade-off mechanisms of compensatory adaptation.*** Our results so far indicate that several haploinsufficient genotypes rapidly mitigate a fitness defect. However, the above analyses were restricted to studying the evolution of haploinsufficiency in a single laboratory environment only. Hence, the results raise two related questions. **First, is the initial haploinsufficient phenotype environment-specific? Second, do the beneficial effects of compensatory mutations depend on the environmental settings?**

We addressed the first question by monitoring fitness of 24 heterozygous mutant yeast strains in 16 alternative environmental settings, including previously tested nutrients and stress factors (Dudley et al., 2005; Szamecz et al., 2014). We detected haploinsufficiency in 81% and *haploproficiency* in 6.8% of all tested genotype-environment combinations. For most genotypes, the haploinsufficient phenotype was detected in only a fraction of the environments considered. Together, these results indicate that the **haploinsufficient phenotype depends on the environmental context**. In specific instances, halving the copy number of a gene reduces fitness in most environments, but increases fitness in others.

The second question is equally important. **Do the observed compensatory mutations provide full functional restoration or are the associated beneficial effects restricted to specific genetic and environmental settings only**. To **investigate the generality of compensation we monitored the growth phenotypes of 83 laboratory evolved, initially haploinsufficient heterozygous yeast strains** (along with the corresponding ancestor genotypes) **in the same environmental settings**. We found a **large number of positive and negative fitness changes** of the compensated lines across different environments (Figure 10).

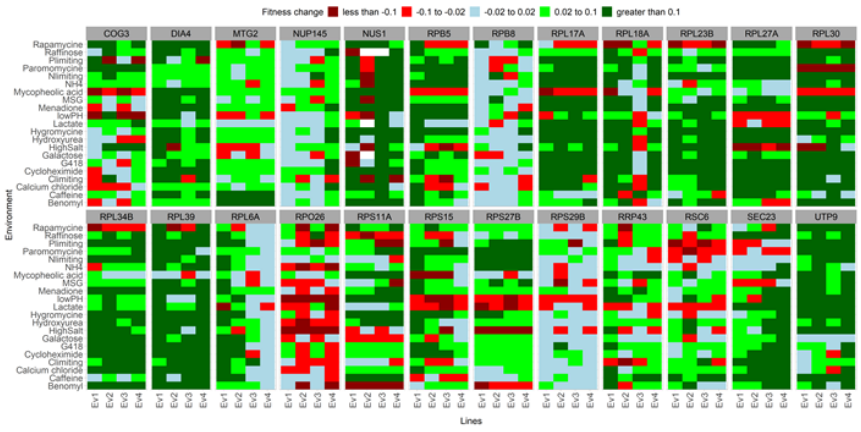

Figure 10. Fitness changes in evolved lines across different environments. The heatmap shows the fitness change of the evolved lines of 24 haploinsufficient genotypes across 16 environments. All of the investigated genotypes were confirmed to be haploinsufficient prior to evolution in a nutrient-rich (YPD) liquid medium. Fitness change was calculated as previously. Colors indicate fitness decline (red) or fitness improvement (green) compared to the corresponding heterozygous ancestor genotypes in the given environment. The rows correspond to the evolved lines (EV1 to EV4) of a given genotype; the columns correspond to the environmental conditions tested.

Next, we focused on only those genotype-environment combinations where haploinsufficiency was confirmed and found **that fitness improved in 81.8%, whereas no beneficial effect was observed in the remaining cases: fitness declined in 5.4% of the cases, and were neutral elsewhere (12.8%)**. The copy number analysis of the haploinsufficient genes enabled us to make a direct comparison of the two alternative molecular strategies of fitness compensation. **Compensation through point mutations elsewhere in the genome were more likely to be neutral in other environments (Fisher's test, Odds ratio = 3.1, $p < 0.001$). Intriguingly, fitness decline was especially frequent in evolved lines with duplication of the haploinsufficient gene (Fisher's test, Odds ratio = 2.2, $p < 0.01$).** Notably, a similar pattern holds when the analysis was restricted to the evolved lines started from the same genotype *RPO26/Δrpo26*: lineages with duplication of the disrupted gene, achieved via whole chromosome trisomy of chromosome XVI, displayed fitness decline in especially high number of environments (Figure 10). Together, the data demonstrate that **gene duplications mitigate the fitness loss in haploinsufficient genetic background via**

functional restoration, but these genetic alterations tend to have pleiotropic side-effects in specific environmental settings. A plausible explanation for such side-effects would be partial/whole chromosome duplications, such as can be seen in the two evolved lines of *RPO26/Δrpo26,* potentially via the **combined fitness effect of multiple linked genes located on the same chromosome as the corresponding gene**.

Next, we examined **how compensatory evolution affects fitness in response to changes in not just environmental, but also in genetic settings**. We focused on five haploinsufficient genotypes and the corresponding evolved strains, all of which have only a single DNA copy of the disrupted gene. We compared the fitness of the evolved and the corresponding ancestral haploinsufficient strains in multiple environmental settings. Considering only those cases where the ancestor genotype was haploinsufficient, we found **numerous positive and negative fitness trade-offs across the various environmental settings tested**. Specifically, fitness of the evolved strains **improved in 37.9% and declined in 13.2% of the cases, and was neutral elsewhere (48.9%**, Figure 11, left panel). This indicates that in most cases (62.1%), the **mutations that have accumulated during laboratory evolution do not provide benefit or are even deleterious in specific environmental settings**.

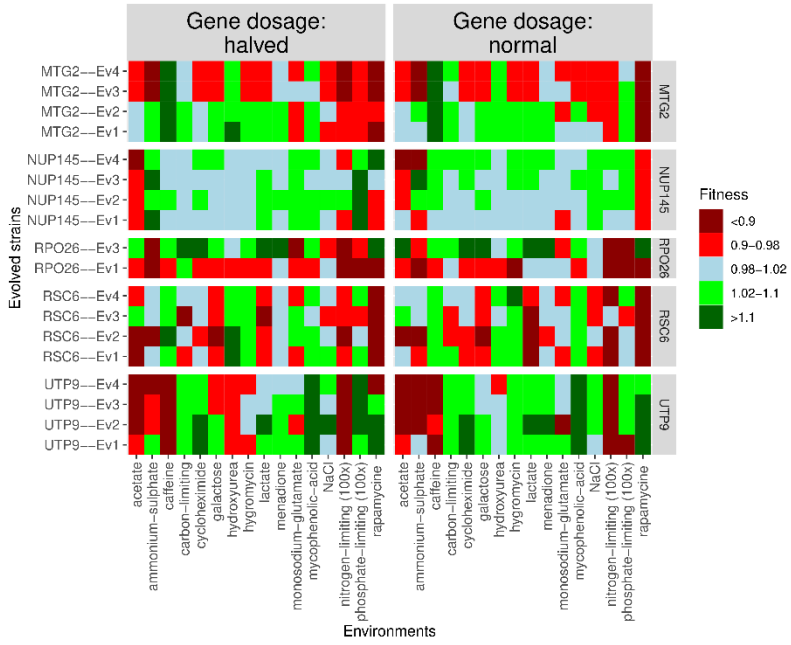

Figure 11. Fitness trade-offs in the evolved strains across the environmental settings tested. The heatmap shows the fitness change of the evolved strains prior to (left panel) and after (right panel) restoring the DNA copy number of the haploinsufficient gene. Colony size was used as a proxy for fitness. Fitness of the evolved lines was calculated as previously and was normalized to that of the corresponding ancestor genotype. All studied strains carried either the control (left panel) or the appropriate MoBY plasmid (right panel). Red and green indicate a lower and higher fitness of an evolved line, respectively, compared to the fitness of the corresponding ancestor genotype carrying the same plasmid.

Next, **we investigated how these mutations affect fitness in genotypes with a restored gene dosage of the haploinsufficient gene**. This issue is all the more relevant as compensatory mutations are less likely to be favored by selection in sexual populations, if they impose a fitness cost on the wild-type. After elevating the expression of the haploinsufficient gene through the MoBY-ORF system in all strains studied, we compared the fitness of the evolved strains with the fitness of the corresponding ancestor genotypes in multiple laboratory environments, as previously. We found that the **evolved strains have higher fitness in 27% and reduced fitness in 18.9% of the cases** (Figure 11, right panel). Although these figures are qualitatively similar to that of those observed prior to DNA copy restoration (Figure 11, left panel), **we noted a slight increase in the fraction of cases with reduced fitness upon dosage restoration (18.9% versus 13.2%)**. This

indicates that **compensatory mutations are more likely to have harmful side effects on the wild type background**. In addition, a closer inspection of the data indicated major differences in fitness in response to DNA copy restoration. We **focused on 69 strain-environment combinations** that fulfilled the following two criteria: the ancestral strain is haploinsufficient, and the corresponding evolved strains display improved fitness in the particular environment. Intriguingly, **an elevated expression level of the corresponding haploinsufficient gene increased fitness in 25 cases, whereas it decreased fitness in 10 cases, and was neutral elsewhere (34 cases**, Figure 12). Together, these results indicate that the **accumulated mutations in the evolved strains can have deleterious and beneficial side effects alike in genotypes with a restored copy number of the haploinsufficient gene**. This suggests that **ectopic mutations and duplication of the haploinsufficient genes modulate haploinsufficiency in an independent manner**. Consistently with this theory, we found that **such mutations are occasionally harmful under certain circumstances** (Figure 12).

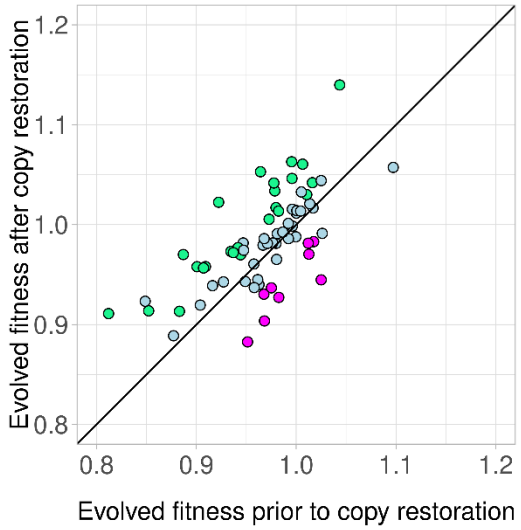

Figure 12. Fitness effects of compensatory mutations. The scatterplot shows the median fitness of 69 evolved strains prior to and after copy restoration of the corresponding haploinsufficient gene. Only those environmental conditions were considered where the evolved strains showed increased fitness compared to the corresponding ancestor genotypes prior to copy restoration. Fitness values significantly higher/lower after DNA copy restoration are indicated with green/magenta dots, respectively (Wilcoxon rank sum test, FDR-corrected $P < 0.05$), whereas blue dots represent no significant difference (Wilcoxon rank sum test, FDR-corrected P = not significant). Fitness was estimated by normalizing the colony sizes of the evolved strains under a given condition to that of the wild type.

According to ***Aim 7., we investigated how does compensatory evolution modulate alternative phenotypic traits.*** What are the physiological consequences of these genomic changes? We opted to investigate this on a large-scale, thereby we decided not to perform transcriptome profiling and in vitro assays for two reasons: i.) these would have been possible in a limited set of strains as both are low-throughput methods, ii.) these would not give a direct answer to the following questions. What are the exact phenotypic consequences of compensatory evolution? In other words, how do these evolved strains perform in conditions other than the medium which was used during the laboratory evolution (i.e. home medium)? This issue was already tested in Aim 6 by measuring fitness in alternative environments. Apart from this, we previously reported that **compensatory evolution does not restore wild-type genomic expression state, but rather drives the cell towards novel genomic expression states** (Szamecz et al., 2014). In addition, changes in the transcriptome does not necessarily reflect similar changes in the proteome due to a large contribution of post-transcriptional/translational/post-translational regulation in the process of protein production/maturation. Hence, **instead of measuring molecular phenotypic traits, we focused on the role of compensatory evolution in creating new organismal phenotypes**.

This is of great importance as it is generally believed that phenotypic innovations driven by selection arise as a result of step-by-step accumulation of multiple beneficial mutations. Accordingly, the **contribution of deleterious mutations to the evolution of phenotypic innovations is generally disregarded** (Covert et al., 2013). However, **slightly deleterious mutations are far more common than adaptive mutations and can reach high frequencie**s in natural populations, potentially because of compensatory evolution.

In our previous work - **published last year in Nature Ecology and Evolution with myself as being a co-first author** (Farkas et al., 2022) - we demonstrated that **compensatory evolution following fixation of deleterious loss-of-function mutations initiate major changes in cellular and macroscopic morphological traits without direct selection on these traits** in *Saccharomyces cerevisiae*.

The main results were as follows:

1.) We used **high-dimensional phenotyping** (including 149 single cell morpholoigcal traits) of laboratory-evolved budding yeast lineages to demonstrate that **new cellular morphologies emerge exceptionally rapidly** as a by-product of gene loss and subsequent compensatory evolution.

a) The cellular morphology of the compensated and the wild-type strains significantly differed from each other, hence **restoration of the wild-type morphology was relatively rare** (15% of the 142 cases).

b) In **25% of cases, the ancestral and the corresponding compensated strains showed similar cellular morphologies, but they substantially differed from that of the wild-type**. Hence, in these cases, compensatory evolution improved fitness, but left the knock-out's cellular morphology unaltered.

c) In **46% of the cases, the compensated strains displayed markedly different morphology compared to that of the wild-type and the corresponding ancestral strain** as well.

d) Additionally, when only ancestral knock-out strains with wild-type morphology were considered, **compensatory evolution generated novel morphological states in 16 out of the 36 cases (44%).**

e) Hierarchical clustering of the morphological profiles coupled with a bootstrap analysis (Suzuki and Shimodaira, 2006) identified **11 morphologically distinct groups of compensated strains** with statistical support, indicating the existence of **multiple distinct morphotypes**.

2.) **We focused on three morphological traits**; cell size, cell elongation and bud neck position, and **compared the morphological diversity of laboratory evolved lines to that of 29 natural strains with diverse ecological origins**. Despite vast differences in the extent of genomic divergence, the **overall extent of morphological variation in the compensated strains was comparable to that of natural strains.**

3) **We found rapid evolution of multicellular phenotypes**. Different species of yeasts typically undergo a **developmental transition from a single-cell form into multicellular forms upon environmental change** (Roberts and Fink, 1994; Madhani and Fink, 1998). We studied three different forms of multicellular phenotypes: i) **invasive growth** phenotype that permits penetration into solid agar, ii) **biofilm formation** that allows adherence to semi-solid agar and iii) **cellular aggregation** in liquid medium via flocculation or clump formation. These three traits **enable survival under stressful conditions**, aid nutrient acquisition (Cullen and Sprague, 2000) and contribute to

virulence in pathogenic yeasts (Madhani and Fink, 1998; Desai et al., 2014). However, several natural *S. cerevisiae* strains have lost their capacity to display these multicellular traits (Hope and Dunham, 2014).

a) Strikingly, **several compensated strains gained the capacity of invasive growth (13%), formed enlarged biofilms (2.8%) or displayed multicellular aggregates in liquid (12.4%)**.

b) Multicellular aggregation in the compensated strains was achieved by **incomplete daughter cell separation (i.e. clumping** (Kuzdzal-Fick et al., 2019)**), rather than by flocculation** of previously separated cells.

c) Overall, **23.4% of the compensated strains showed an enhanced capacity to display at least one multicellular trait**, that is unlikely to confer any benefit in the well-shaken liquid medium employed during the course of laboratory evolution.

d) The **extent of gain in invasive growth phenotype in the compensated strains reaches as high as ~50% of the range of invasiveness displayed by natural isolates with different ecological origins**.

e) These multicellular phenotypes were achieved by **diverse mutational routes and without re-activating the canonical regulatory pathways (i.e. not in a Flo11p-dependent manner)**.

4) We showed that **both the initial gene loss and subsequent compensatory mutations contribute to new morphologies, with their synergistic effects underlying specific morphological changes**. We concluded that **compensatory evolution is a previously unrecognized source of morphological diversity and phenotypic novelties**. This work was published in **Nature Ecology & Evolution**:

> **Farkas, Zoltán**, Károly Kovács, Zsuzsa Sarkadi, Dorottya Kalapis, Gergely Fekete, Fanni Birtyik, Ferhan Ayaydin, et al. 'Gene Loss and Compensatory Evolution Promotes the Emergence of Morphological Novelties in Budding Yeast'. Nature Ecology & Evolution, 28 April 2022, 1–11. https://doi.org/10.1038/s41559-022-01730-1.

Our work was also spotlighted by Linda Koch in **Nature Review Genetics** in an In Brief article:

> 'Turning Gene Loss into Phenotypic Gain | Nature Reviews Genetics'. Accessed 24 May 2022. https://www.nature.com/articles/s41576-022-00504-6.

Moreover, **I published a Behind the Paper blogpost** based on our results in **Nature Portfolio Ecology & Evolution Community site**:
https://ecoevocommunity.nature.com/posts/do-harmful-mutations-have-a-constructive-role-in-evolution

In addition to the above results, we further **expanded our knowledge on how compensatory evolution modulate morphology (*Aim 7.*) by using the set of haploinsufficient strains**. This allowed us to:

1. investigate whether reducing the dosage of genes that cause haploinsufficiency would also change the organism's morphology;
2. explore how compensating for haploinsufficiency would affect its morphology;

3. compare the morphology of compensated strains that had their gene dosage increased by duplication against those that were compensated through ectopic mutations

To investigate these issues, we **focused on invasive growth** for several reasons. First, previous works already demontrated **that aneuploidy, which involves changes in the gene dosage, could have major effect on multicellular phenotypes** (Tan et al., 2013), and ii) the **throughput of invasive growth assay can be easily scaled up**, hence repeating these experiments **in multiple conditions is feasible** using a colony-replicating robot. The set of strains for these experiments was the same that we used throughout the environmental pleiotropy screen. **Three media** were used for these experiments: the same **optimal medium** used during the course of laboratory evolution (**YPD**), **a synthetic medium with low nitrogen source (SLAD**, synthetic low ammonium defined medium, **traditionally used to investigate mutlicellular phenotypes in dipoid yeasts**) and the same SLAD medium, but supplemented with **tryptophol (i.e. 3-(2-Hydroxyethyl)-indole, a.k.a. HEI), an aromatic compound that can induce a morphogenetic switch** (through quorum sensing) from unicellular growth to multicellular filaments composed of elongated, attached cells spread over and into surfaces (Lenhart et al., 2019).

Regarding the **first question related to the effect of gene dosage on invasive growth**, we found that **haploinsufficient mutants,** i.e. heterozygous deletion strains known to be slow-growers in optimal condition, **showed environment-dependent invasive growth** (Figure 13.). In the same condition (**YPD**) where they show haploinsufficiency, **only a limited number of genotypes (*SEC23,RPS11A, UTP9*) were able to invade** the solid medium, that is they i) showed significantly higher invasiveness score than the wild-type HO heterozygous deletion strain, and ii) their invasiveness score reached 10% of the level of invasiveness of the positive control strain (sigma 1278b, a.k.a. SIGMA). The **situation was different in SLAD medium**: the genotype with the strongest invasive growth in YPD (*SEC23*) lost its capacitiy to invade the agar, whereas other became capable to invade (*RSC6, RRP43*). As expected, the strains had a **general increase in the invasiveness score in the SLAD medium supplemented with the aromatic alcohol** (even in the negative control strain), but still, we **observed both significant increase and decrease in the level of invasiveness of HI mutant strains compared to the wild-type strain**.

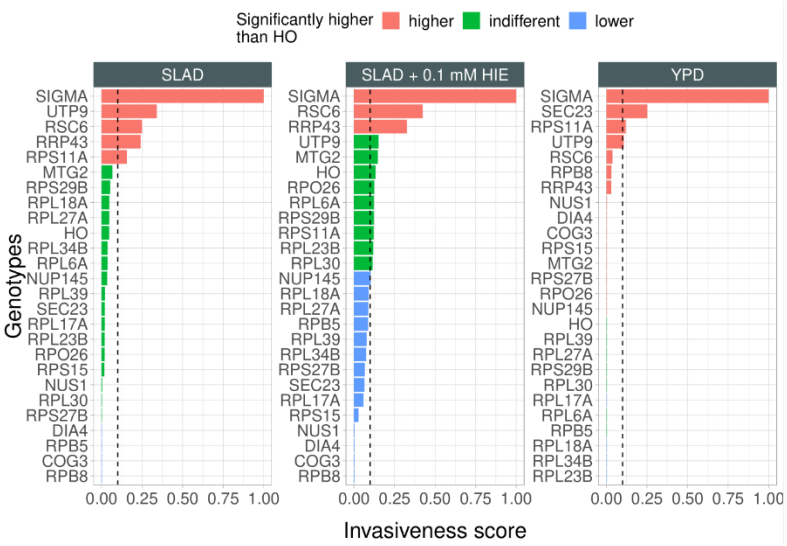

Figure 13. Invasive growth phenotype of heterozygous deletion strains is environment-dependent. The barplot show the invasiveness score of multiple heterzygous deletion strains across 3 different environments, including an optimal (YPD), a nitrogen starvation (SLAD) and a nitrogen starvation medium supplemented with an aromatic alcohol (known to induce multicellular phenotypes, SLAD + 0.1 mM HEI). Invasiveness score was calculated by normalizing the invasiveness of the investigated strains to that of the positive control strain (sigma 1278b, SIGMA). As a negative control strain, we used the wild-type strain (HO). The level of invasive growth, that is, absolute invasiveness, was determined as the ratio of the intensity of yeast spots after washing to the intensity that of before washing (based on 12 biological replicates of each). Colors indicate the significant differences of mutant invasiveness values compared to that of the wild-type. Strains that met the following criteria were considered to display an invasive growth phenotype: (1) the relative invasiveness value was significantly higher than that of the wild type (one-sided Wilcoxon rank-sum test, 10% FDR) and (2) the relative invasiveness value was higher than 10% of the corresponding value of the positive control strain (sigma 1278b, a.k.a. SIGMA). To calculate intensity of spots, we used our previously published image analysis pipeline (Farkas et al., 2022).

Regarding the **second question, related to the effect of compensatory evolution on the invasive growth phenotype** of originally HI mutant strains, we made **direct comparison of the ancestor and evolved strains of HI mutants** (Figure 14.). We observed that the **limited number of originally invasive genotypes in optimal conditions all lost their capacity to invade the agar**. In the case of **SLAD medium** we found **similar patterns**, that is originally invasive strains became non-invasive after laboratory evolution, nevetheless we **detected several instances when originally non-invasive strains became capable for that** (e.g. evolved lines of *RPL17A, RPL27A, NUS1, SEC23*, etc.)

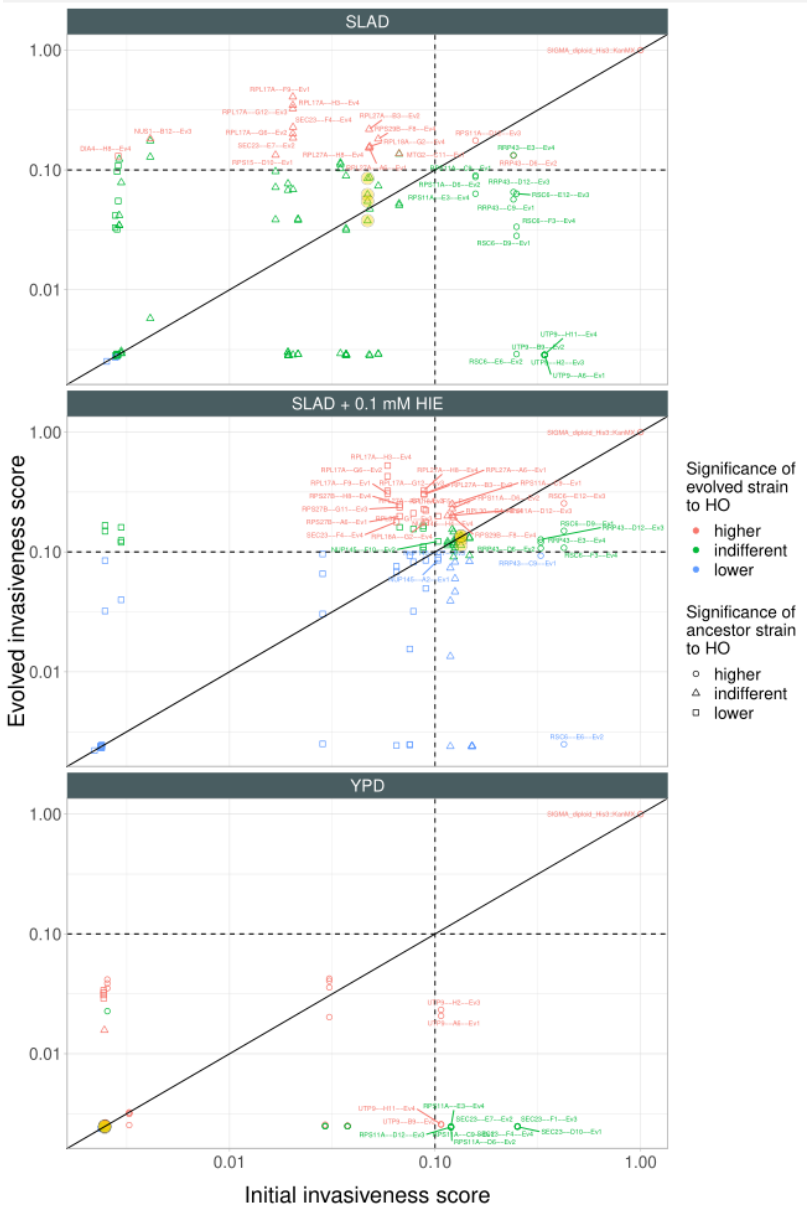

Figure 14. Compensatory evolution modulate invasive growth phenotype in an enviroment-dependent manner. The scatterplot show the invasiveness score (log_{10}-scale) prior to and after compensatory evolution. Three media were tested, as previously (Figure 13.). Each point correspond to a single, independently evolved line. Invasiveness score is the median of 3 and 12 biological replicates for the evolved and ancestor strains, respectively. The color and shape of points indicate significant differences in the invasiveness score of evolved and ancestor strains compared to the wild-type, respectively. Significance was assessed using a Wilcoxon-rank sum test (FDR-corrected P-value < 0.1). Dashed lines represent the cutoff used to invasive growth, that is the 10% of the invasiveness values relative to the positive control strain (SIGMA). Gold points with black stoke represent the wild-type strain (HO), but please note that these point show the same value in the initial invasivensss score, whereas there is a variation in these value after laboratory evolution (ie. these are considered to be control evolved lines).

The third question relates to the issue whether the **two types of mitigation of haploisufficiency, i.e. compensation for by ectopic mutations versus gene duplication of the focal gene, modulates invasive growth** in a similar way. Direct comparison of evolved strains belonging to these groups revealed a similar observation about environment dependency: there was **no signififcant differences in the SLAD medium, whereas even though a significant difference was appearant in YPD** medium, **the overall invasiveness was negligible in optimal condition**. On the other hand, we found a **significant difference between the two types of mitigation in the presence of the aromatic compound**, moreover, the median invasiveness value of the **strains that underwent copy number variation during the course of evolution exceeded the threshold value for invasive growth**. This implies that **gene duplication, possibly via chromosome aneuploidy, as a means of compensating for haploinsufficiency could trigger a clinically relevant virulence trait**, at least in the presence of certain environmental stresses. However, it is important to note that these

results seems to be specific to not just the environmental conditions, but also to the particular gene studied, and may not be generalizable to all cases of haploinsufficiency or gene duplication. **Further research is needed to explore the mechanisms underlying these observations and their potential implications for understanding the evolution of invasive growth in fungal pathogens**. Such knowledge might open a new avenue in the **treatment of fungal infections,** by providing useful information on **targeting genes involved in invasive growth pathways that are prone to copy number variations**.

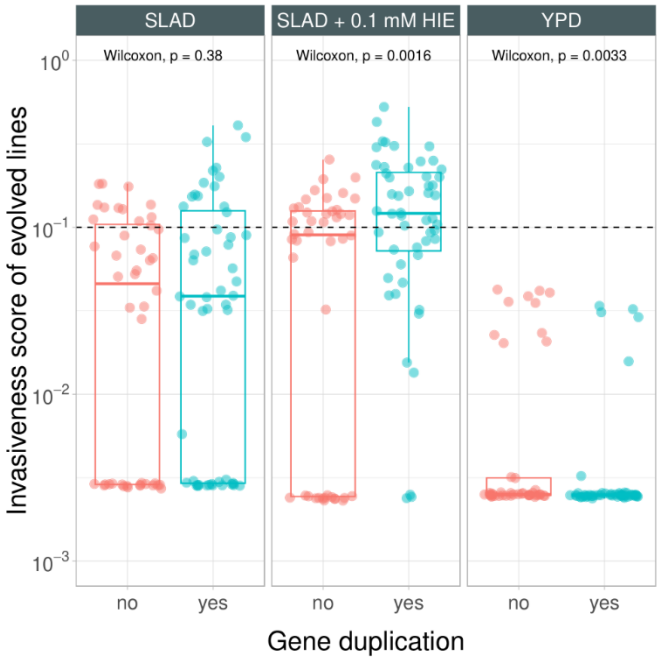

Figure 15. Gene duplication triggers invasive growth in a context-dependent manner. The boxplots show the invasiveness scores (log_{10}-scale) of evolved lines categorized into two groups, strains with gene duplication (yes, colored by red) and strains without gene duplication that got compensated for by accumulation ectopic mutations (no, colored by green). Invasiveness was investigated in three different media as previously (Figures 13. and 14.). Dashed line represent the invasiveness score of the positive control strain (SIGMA). To assess significant differences in the invasiveness score of the two groups, Wilcoxon rank-sum test was used.

Overall, our work investigating compensatory evolution in diploid and haploid baker's yeasts has **provided useful insights into the principles of evolution** by different means.

First, **we examined the genetic basis of the evolution of dominance**. We **identified two equally common genetic ways of fitness compensation** in slow-growing diploid mutant strains with heterozygous loss-of-function mutations: i**) functional restoration by restoring the dosage of the intact allel**e and ii) **accumulation of compensatory mutations in ectopic genomic region**s that do not affect the expression of the focal gene but **alter the dominance of the remaining allele**. Thus, we demonstrated a **high prevalence of dominance modifier alleles that can attenuate the harmful effects of heterozygous loss-of-function alleles** and suppress the loss of fitness. As such, we provided **experimental evidence for Ronald Fisher's hypothesis on dominance modifier alleles.**

Second, our results on the **constructive role of deleterious mutations on morphology shed fresh light on how we think phenotypes evolve.** Since the early 1920s, Ronald Fisher, one of the founders of population genetics, pioneered the view that

phenotypic evolution is by and large a "hill-climbing" process, i.e. that it proceeds through a progressive accumulation of beneficial mutations. More recently, **computer simulations have shown that deleterious mutations followed by compensatory evolution can sometimes play a constructive role in evolution**. **Our work was the first that systematically tested this hypothesis and provided empirical evidence that deleterious mutations can often contribute to new phenotypes**. Biotechnological applications often use the principles of natural selection to "breed" proteins with new desirable properties. However, based on our results, **it is conceivable that mutations that are deleterious to protein function could be combined with other beneficial mutations to produce qualitatively new enzymes.**

Conferences/publications

Workshops/conferences:

These works were presented at 13 workshops/conferences:

Presenter	Type of presentation	Title of presentation	Conference name	Conference location	Conference type	Year	Date
Daraba Andreea	Conference Talks	Haploinsufficiency mechanisms and evolution	Hungarian Molecular Life Sciences	Eger, Hungary	personal	2019	29-31 March 2019
Daraba Andreea	Conference Talks	Haploinsufficiency mechanisms and evolution	EvolBiolDay Szeged	Szeged, Hungary	personal	2019	23 April 2019
Daraba Andreea	Posters	Haploinsufficiency mechanisms and evolution	Straub Days	Szeged, Hungary	personal	2019	30 May 2019
Daraba Andreea	Posters	Haploinsufficiency mechanisms and evolution	International Conference on Yeast Genetics and Molecular Biology (ICYGMB_2019)	Göteborg, Sweden	personal	2019	August 18-22, 2019
Farkas Zoltán	Conference Talks	Compensated deleterious mutations as drivers of morphological evolution	International Conference on Yeast Genetics and Molecular Biology (ICYGMB_2019)	Göteborg, Sweden	personal	2019	August 18-22, 2019
Károly Kovács	Conference Talks	Compensated Deleterious Mutations as Drivers of Morphological Evolution	Molecular Mechanisms in Evolution (GRS), Gordon Research Seminar	Easton, USA	personal	2019	08-09.06.2019
Károly Kovács	Conference Talks	Compensated deleterious mutations as drivers of morphological evolution	23rd Evolutionary Biology Meeting at Marseilles	Marseille, France	personal	2019	24-27.09.2019
Zsuzsa Sarkadi	Conference Talks	Compensatory mutations drive morphological evolution	SMBE19	Manchseter	personal	2019	21 to 25 July 2019
Farkas Zoltán	Posters	Fitness trade-offs shape the evolution of dosage sensitivity	EMBL Conference: Molecular Mechanisms in Evolution and Ecology	Heidelberg, Germany	personal	2020	September - 3 October 2020
Zsuzsa Sarkadi	Posters	Deleterious mutations as drivers of morphological evolution	Molecular Mechanisms in Evolution and Ecology - Virtual	Heidelberg, Germany	virtual	2020	30.09-02.10.2020.
Farkas Zoltán	Posters	Compensatory evolution promotes the emergence of morphological novelties	Evolutionary Systems Biology 2022 - VIRTUAL event		Virtual	2022	09-11 February 2022
Balázs Papp	Conference Talks	Gene loss and compensatory evolution promotes the emergence of morphological novelties	EMBO Workshop, Molecular mechanisms in evolution and ecology 2022	Heidelberg, Germany	personal	2022	5-8.OCT, 2022
Károly Kovács	Posters	Gene loss and compensatory evolution promotes the emergence of morphological novelties in budding yeast	Understanding and Predicting Microbial Evolutionary Dynamics	Manchester, UK	personal	2022	22-23. NOV, 2022
Károly Kovács	Conference Talks	Gene loss and compensatory evolution promotes the emergence of morphological novelties in budding yeast	Straub days	Szeged	personal	2022	25-27 May, 2022

Publications:

The results of the proposed reasearch plan were used in one publication and one manuscript, both including me as a first author (marked with *):

> **Farkas, Zoltán***, Károly Kovács, Zsuzsa Sarkadi, Dorottya Kalapis, Gergely Fekete, Fanni Birtyik, Ferhan Ayaydin, et al. 'Gene Loss and Compensatory Evolution Promotes the Emergence of Morphological Novelties in Budding Yeast'. **Nature Ecology & Evolution**, 28 April 2022, 1–11. https://doi.org/10.1038/s41559-022-01730-1.

> **Zoltán Farkas***, Andreea Daraba, Gábor Boross, Zoltán Bódi, Dorottya Kalapis, Karola Almási, Éva Boros, István Nagy, Balázs Papp & Csaba Pál. 'Fitness trade-offs shape the evolution of dominance'. **In preparation**, https://rebrand.ly/NKFIH_FK_128775

Other publications:

Related to compensatory evolution:

Besides the current results, which come from studies of compensatory evolution in yeast, I was also involved in another study that **investigated the efficacy and phenotypic impact of compensatory evolution in *Escherichia coli* strains carrying multiple resistance mutations** (Dunai et al., 2019). Although resistance mutations frequently have associated fitness costs, such costs may decline subsequently through the accumulation of compensatory mutations. It has been argued that such compensatory mutations mitigate the fitness costs of resistance mutations *without* affecting the level of resistance, suggesting that limiting antibiotic usage may not have much practical utility in clinical settings. In order to test this, we **started a lab evolution with 23 drug-resistant *E. coli* strains and found that 60 days of evolution under antibiotic-free conditions** has led to i) **rapid fitness increase in antibiotic free-medium**, ii) **associated loss of antibiotic resistance**. The extent of **resistance loss was found to be generally antibiotic-specific**, driven by **mutations that reduce both resistance level and fitness costs of antibiotic-resistance mutations**. Our study indicates that **restricting antimicrobial usage could be a useful policy, but for certain antibiotics only**. This work was published in *eLife* in which I share the first authorship with Anett Dunai and Réka Spohn (Dunai et al., 2019).

> Dunai, Anett, Réka Spohn, **Zoltán Farkas***, Viktória Lázár, Ádám Györkei, Gábor Apjok, Gábor Boross, et al. 'Rapid Decline of Bacterial Drug-Resistance in an Antibiotic-Free Environment through Phenotypic Reversion'. ELife 8 (16 August 2019). https://doi.org/10.7554/eLife.47088.

Unrelated to compensatory evolution:

Several publications were published during the grant period (2 having me as a first author, marked with *), all of them having the current grant number (NKFIH FK-128775) mentioned in the acknowledgment section:

Kovács, Károly, **Zoltán Farkas***, Djordje Bajić, Dorottya Kalapis, Andreea Daraba, Karola Almási, Bálint Kintses, et al. 'Suboptimal Global Transcriptional Response Increases the Harmful Effects of Loss-of-Function Mutations'. **Molecular Biology and Evolution**, no. msaa280 (3 November 2020). https://doi.org/10.1093/molbev/msaa280.

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