EMBO reports

Rapid evolutionary repair by secondary perturbation of a primary disrupted transcriptional network

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DOI: 10.15252/embr.202256019

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Review Timeline:

Submission Date:	24th Aug 22
Editorial Decision:	21st Sep 22
Revision Received:	20th Dec 22
Editorial Decision:	3rd Feb 23
Revision Received:	22nd Feb 23
Editorial Decision:	15th Mar 23
Revision Received:	16th Mar 23
Accepted:	17th Mar 23

Editor: Ioannis Papaioannou

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 21st Sep 2022

Dear Dr. Hsu,

Thank you for the submission of your research manuscript to EMBO reports and for your patience while it was peer-reviewed. We have now received the full set of referee reports that is copied below.

All referees acknowledge that the study is interesting and largely well-performed, and -as you will see- they all provide broadly favorable reports. However, referees 1 and 3 also point out some concerns that should all be addressed for the improvement of the study, and they provide a number of suggestions that should be considered. In line with their recommendations, interpretation of results should be strongly supported by the presented data and carefully explained, and the rationale for key decisions in the experimental design provided. Furthermore, all referees provide a number of suggestions for minor changes that would further improve the study and the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (December 20th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

*****IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.*****

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
(<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their

respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <
https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Specifically, we would kindly ask you to provide public access to the following datasets:
- Genome sequencing data
- RNA sequencing data

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the new policy (<https://www.embopress.org/competing-interests>) and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and place it after the Acknowledgements section.

9) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

10) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession

number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

12) Please also note our reference format:
<http://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

13) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. See also guide to authors: <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

14) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

You can use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

In their manuscript titled "Rapid compensatory evolution by secondary perturbation of a primary disrupted transcriptional network", Hsu et al. have used the Lachancea yeast system to investigate how perturbation of organismal fitness due to gene dysregulation can be compensated by the evolution of second site suppressors. Briefly, Hsu et al. exploit the rapid development of genetic suppressors in a Δsef1 strain to understand how perturbation in gene regulatory networks can be rapidly, but conditionally, 'fixed' during evolution. They have previously shown that Sef1 is a transcription factor that has been repurposed in Lachancea to regulate the expression of metabolic genes. The authors observe that suppressors of the Δsef1 mutant fall into two main categories, generalists (mediated by ira1 inactivation) that resolve the growth defects of Δsef1 under fermentative and respiratory conditions and multiple temperatures, and specialists (mediated by azf1 inactivation) that resolve growth defects of Δsef1 only under respiratory conditions. They further show, using genetic experiments and RNA-seq transcriptomics that the effects of these suppressors can be recapitulated at the level of transcription of TCA cycle genes and heat shock proteins. They finally also show that the frequency of the suppressor in a population can be maintained due to cell non-autonomous effects.

Overall, I find this to be an interesting study, particularly since it tries to address an important question in molecular and systems evolution. This study attempts to bridge the gap between molecular mechanism and organismal phenotype/fitness, which can be challenging even in model organisms like Saccharomyces and E. coli. The major strength of this study is exploiting a spontaneously arising suppressor using careful genetic and transcriptomics analyses to understand how cells can mutationally rewire their metabolic and gene expression networks. This rewiring is often on a 'need-basis', as seen for the azf1 mutant and can be conditional. This is an important point which is often missed by researchers working in the area. The weakness of this study, in its present form, lies in firstly not having direct evidence linking loss of a transcription factor (azf1 for instance) with its specific effects on transcription, which are likely only a subset of those observed at the whole-transcriptomics level. Secondly, the interesting observation that cells can access either a generalist or a specialist suppressor has not been satisfactorily explored. The co-existence, relative abundances and evolutionary pressures driving each of these pathways for gene rewiring remain relatively un-investigated. Thirdly, a few interpretations and data representations are difficult to understand.

Therefore, I would request that the authors address the following queries/concerns before the manuscript can be accepted for

publication:

Major comments:

1. The relative frequencies of occurrence of generalist and specialist suppressors is not apparent from the figures or the text. This information is crucial to understand which of the two suppressor strategies is more common and how that correlates with the trade-off associated with specialist suppression.
2. Have the authors tried directly competing the generalist and specialist suppressors? Are there environments that select one over the other?
3. The growth phenotypes as well as transcriptional profiles of Δazf1 and Δsef1Δazf1 are extremely similar. This means that in a Δazf1 strain the presence of absence of sef1 is immaterial. Given this fact, I am unclear on why the authors chose to represent sef1 and azf1 as two alternative pathways/transcriptional programs (Figure 3J). Would it not make more sense to position azf1 downstream of sef1?
4. Though the authors have used extensive RNA-seq experiments to demonstrate that there is transcriptional rewiring in the suppressors of Δsef1, it is unclear to me where the rewiring exactly is. For example, the authors have previously identified several binding sites for sef1 in the genome of Lachancea. Do these targets also respond to azf1, or are the effects of azf1 through an independent set of gene promoters? Further, since both sef1 and azf1 mutations used in this study are gene deletions and loss-of-function mutations, do the authors believe that loss of repression by these factors in driving the observed transcriptomic changes? Finally, how many of the effects that the authors report at the gene expression level are primary rewiring effects, and how many are secondary/tertiary effects? Some clarity on this issue is crucial if the authors want to make the claim that the compensatory effects are due to gene regulatory rewiring. One possible approach to address this could be by performing one-hybrid assays with some of the TCA (and other) gene targets from the RNA-seq.
5. Azf1 is known to be a prion-like protein in Saccharomyces. Given the contribution of heat shock proteins to the phenotypes of Azf1, have the authors considered the possibility that some of the phenotypes may be due to loss of the prion form of azf1 at high temperature in the Δazf1 strain rather than its transcriptional roles?
6. The last section of the study that deals with frequency dependent phenotypes and cell non-autonomous effects is interesting, but not sufficiently fleshed out in terms of mechanism. As a result, its relevance in the current manuscript is difficult to understand. Can the authors demonstrate, for example, that the frequency at which the azf1 mutant occurs in the population of Δsef1 strain is higher than expectation? Further, without the molecular mechanisms of these effects that they see I would be wary of just the phenomenological findings. My suggestions would be to remove these observations from the present study and report them once more mechanistic and population-level details are available.

Minor comments:
1. In Figure 1B, the growth rate of the Δsef1 seems lower on YPD-PDS than on YPGly. However, plate assays in Figure 1A suggest that growth yield in higher on YPGly than on YPD. Are there trade-offs possibly between yield and growth rate for this mutant?
2. Quantification for Western blots is missing and will significantly improve the reach of the data in this manuscript.
3. Line 80-84. This is an important statement that sets up the question addressed in the paper. However, as it is frames currently, it is very difficult to understand. Perhaps the authors could make this sentence crisper?
4. The authors have presented many as part of the figures associated with this manuscript. However, their legibility and readability are very poor. It may be better to keep them as separate tables rather than as part of figure. This would significantly simplify reading the manuscript.

Referee #2:

The study with title 'Rapid compensatory evolution by secondary perturbation of a primary disrupted transcriptional network' focuses on the characterization of two targets that emerged from a suppressor screen of a transcriptionally perturbed (sef1Δ) L. kluyveri strain and in depth characterization of one of them. The characterization involved differential expression data of the perturbed and suppressed strains, growth assays in various media, genetic, biochemical and pathway analyses. This is a thorough investigation and a well-written paper.

I only have very minor comments and suggestions.

Fig 3F and 3G: It is unclear to me why the WT in these two panels is different. Are they different constructs or backgrounds? Please clarify or explain the discrepancy.

Throughout the manuscript (for ex. Lines 258-260), ira1 mutants are treated as generalists. Additionally, the authors used that as an argument to justify focusing the study on the azf1 mutants. In fact, RAS PKA perturbations are not a generalist strategy, but typically emerge as a response to conditions that involve changes in nutrient abundance. (Had the majority of our evolution experiments in yeast been done in chemostats, RAS PKA would not have been as popular of a target). That is mutations in azf1 and mutations in ira1 probably emerged in response to different selective pressures within the same environment. Please re-

visit the document to account for that and consider including a different argument (less explored target?) on why azf1 mutants were chosen for further analysis. In line 482, it is mentioned that ira1 generalist impact is specific to the particular set of experiments. That can be introduced earlier on, to avoid confusion.

Is the YPD-YPD in fig S11B some sort of control? It was unclear at first what the media transitions are, because the YPD-YPD is never mentioned in the text or legends or materials and methods. Please add a note explaining in the legend or methods.

The left-most columns in figures S12A and S13 showing DE data are unreadable, unfortunately. The authors can put these data in excel with col-coded wells and turn these panels into summary data, or put the gene columns only in excel with identifiers and use the same identifiers for the figure every a few or several rows.

Please provide a guide for the RNAseq data tables. For example, a brief description of the sheet contexts and the columns at the supplementary tables legends (for example a longer legend in the first table should suffice).

Fig 1B: the two conditions could be combined in a single plot

Given that two adaptive strategies were found, corresponding to perturbations in two genes, it would be interesting to know whether the rest of the isolated clones have mutations in these two genes and whether their phenotypes match the authors' predictions. If in the meantime sequencing information was recovered on these loci for other clones, it would be nice to see them included.

Can data like those in fig 1D be used to approximate suppression rates? And then speculate whether there are other loci that contribute to suppression?

Genetic analysis in fig S5 and S6: Was the phenotyping performed on the parental diploids? It would be nice to have dominance/ recessiveness information.

Language and typos
The language in the abstract in general could use a little 'tightening' (Line 31: 'minimalize the deleterious effects' to minimize deleterious effects', Lines 33-37: clanky and inefficient writing, Lines 39-42: difficult sentence)
Line 158: 'selecting' - it seems that 'plating' is a more appropriate term
Lines 192 and 195: mention 'three clones' per temperature (l. 192) or mating type (l. 195), but in fact it is three clones per temperature AND mating type, and there are data for 12 clones total. Please re-word.
Lines 204-206: Confusing wording, please re-word for accuracy. Were three clones checked via backcrossing, tetrad dissection and phenotyping of one full tetrad each? This can be gathered by figures S5 and S6, but the writing needs improvement.
Line 755: Fig 16C to Fig S16C

Referee #3:

The manuscript is an in-depth examination of the causes and pleiotropic consequences of compensatory evolution of a single transcription factor, using various genetic and molecular biology methods. Specifically, the authors deleted SEF1, a transcription factor gene involved in respiration from the yeast L. kluyveri and then selected for better growing colonies in respiratory medium (glycerol as carbon source). They also initiated selection experiments with added heat stress, as a second selection pressure. The evolved lines were able to compensate their fitness in both sets of experiments, but the compensation was caused by two different mutations: loss-of-function of two transcription factors (IRA1 and AZF1, respectively). As a consequence, the evolved lines behaved differently when exposed to different conditions: only the high-temperature selected lines showed genetic trade-off/antagonistic pleiotropy by growing worse than the wild-type under fermentative conditions (rich glucose medium). Using transcriptomics, the authors showed that the initially deleted and compensatory genes are functionally related through the Ras-cAMP-PKA pathway. Further experiments suggest that AZF1 deletion can increase fitness via multiple simultaneous mechanisms: by adapting to heat-shock and glycerol, and by restoring the level of TCA cycle genes, downregulated by the SEF1 deletion, indicating deletion-specific compensation. The authors additionally show that the fitness impact of loss of AZF1 function is density-dependent, which have relevance to the population genetic mechanisms driving compensatory evolution.

Overall, while the dissection of compensatory mutations is not conceptually novel, to our knowledge, this is the first such detailed work focusing on a transcription factor mutation. Also, demonstrating that a key compensatory mutation shows density-dependent fitness effect represent a conceptual advance. Thus the work is an important step towards understanding how transcription networks may evolve through compensatory evolution. We found most of the presented analyses and methodologies convincing. However, some limitations of the experimental design raised questions about the interpretation of the results, which should be addressed (see below).

Main comments:

1. There are two methodical shortcomings, which might make some of the conclusions less convincing. First, the fitness measurement is mostly based on visual inspection of colony growth, making it difficult to compare the sizes of the changes or to detect epistasis. Importantly, the two compensatory mutations increase/decrease fitness not only in the SEF1 deletion background but also in the wild-type. This raises the question if these fitness effects are larger in the deletion background than in the wild-type background (i.e. genetic interaction/epistasis). More precisely epistasis can only be claimed if the effect of the two mutations together are different to what we would expect based on single mutation effects (e.g. van Leeuwen et al. 2017). Therefore, the conclusion in the following sentence (Line 214) is not followed by its premises: 'Interestingly, the "double-compensation" effect of ira1∆ and the "Dex-trade-off and Gly-compensation" effect of azf1∆ were retained in the wild-type SEF1 background, and the high-activity Sef1-VP16 (Hsu et al., 2021) was unable to mask its effects, indicating that these two genes can function independently of and epistatic to SEF1.' Showing that there is synergistic epistasis would be important because that would answer the question whether the 'compensatory mutations' are really compensating for the harmful mutation, or simply increase fitness because of adaptation to the medium/heat stress.
Even if the authors cannot provide direct evidence, they should discuss this question and present their existing indirect pieces of evidence, suggesting that the mutations might be compensatory: i) sef1∆ lines evolved under heat stress are also able to partially compensate fitness when growing in normal temperature (FigS3 D), ii) transcript changes suggest that azf1 deletion can increase fitness by all three mechanisms: adaptation to the medium, adaptation to heat stress and compensation for the sef1 deletion.

2. A related shortcoming is the lack of wild-type control in the evolution experiment. Fitness increase can be also expected for the wild-type when grown under the same conditions as the sef1 deletion mutant (especially under heat stress). Using wild-type controls would show whether the same 'compensatory' mutants appear during their evolution (relating to the first question of their specificity), and how well the compensated sef1∆ lines would compete with them. That the wild-type are also expected to evolve and increase its fitness should be at least discussed, since it would affect the potential evolutionary fate of the compensated sef1∆ lines.

3. There are some key decisions made by the authors without much explanation given. It would be nice to provide some rationale for the following decisions:
- Why choose L. kluyveri and SEF1 deletion?
- What is the rationale behind applying heat stress together with the deletion? What are the authors' expectations?
- What was the motivation to perform the evolutionary experiment using both MATa and MATalpha founder strains? Did the authors expect mating type to influence any outcomes? Was there any difference found?

4. The density-dependent fitness effect of AZF1 deletion is important from a conceptual point of view as it might influence how genetic polymorphism in AZF1 could be maintained in the population. This finding would definitely deserve mentioning in the abstract. Also, the authors may elaborate more on the possibility of compensatory mutations arising first and forming a subpopulation in which subsequent loss-of-function mutations may occur that would otherwise be highly deleterious (i.e. acting as permissive mutations).

Minor comments:
- Prior works reported that compensation of loss-of-function mutations can result in massive transcriptomic rewiring despite fitness restoration, see Szamecz et al. 2014 PloS Biol and McCloskey et al. 2018 Nat Comm. These works appear to be relevant for the central concept of the manuscript.
- We found the opening sentences of the Abstract confusing: 'The discrete steps of transcriptional rewiring have been proposed to occur neutrally to ensure steady gene expression under stabilizing selection, especially when a regulon is being transferred from one transcription factor (TF) to another. An evolutionarily conflict-free switch of a regulon may require an immediate compensatory evolution to minimalize the deleterious effects'. The first sentence seems to suggest an evolutionary scenario of neutral mutations, while the second sentence suggests a different scenario, consisting of a deleterious and a later adaptive (compensatory) step. We guess the authors meant that transcriptional rewiring can result in multiple equally fit outcomes.

- How was 'inconsistent phenotype' defined? In table S3 strains evolved under the same conditions were labelled either inconsistent or consistent even if they had the exact same fitness scores.
- Please indicate sample sizes, when applicable, e.g. in the figure legend of plots with error bars.
- Missing explanation in figure legend of Figure 1 c) about the name "SEF1R". Did the authors mean sef1∆::SEF1 ?
- The Figure 3 panel B) C) shows the transcriptional activity of Sef1 in the different media that are labelled by distinct colors. At panel B) the Azf1 transcriptional activities are also colored by the media. However, this systematic labelling breaks at the panel F) and G) where the colors already distinguish between negative control (lexA) and the lexA-LkAzf1. It would be good to have one system for all of the graphs.
At panel E) "Azf-lexA" should be corrected to Azf1-lexA.
At panel E) it is not clear whether the two bars on the right represent a strain that contains both the plasmid based lexA-Azf1 and the chromosome inserted one or it indicates a strain that carry only the chromosome inserted Azf1-lexA.
Most of the panels (except E) and H)) do not indicate whether the experiments were done by the native (chromosome based) promoter or the constative (plasmid based) promoter. However, the authors make the point that they behave differently (panel

E), line 245).
At panel F) and G) the lexA-fused TF has a name that includes the species name as well (lexA-LkAzf1). What is the purpose of this labelling? One can think that the Sef1 at panel B) and C) does not derive from L. kluyveri, because its name does not include the "Lk".
- Line 340 - The authors say "Indeed, deletion of AZF1 not only partially restored expression of TCA cycle genes under the YPGly condition (Fig. 5C)" . At Figure 5C the expression data are normalized to the sef1Δ strain. One could see the restoration better when the data would be normalized to the WT strain.
- At Figure 6 B) and C) labelling of the Y axis should be corrected to HGBR/ HGBS.
- Some of the tables cannot be read because of the too low resolution e.g. Figure S12, S13
- There is a high background of the Figure 1 E lower image (YPGly), therefore it is hard to compare it with the image above (YPD).

References:
van Leeuwen, Jolanda, Charles Boone, and Brenda J. Andrews. "Mapping a diversity of genetic interactions in yeast." Current opinion in systems biology 6 (2017): 14-21.

Response to Reviewer 1 (Reviewer's comments in **bold** and responses in red):

- **Referee #1:**

Comments to the Authors

In their manuscript titled "Rapid compensatory evolution by secondary perturbation of a primary disrupted transcriptional network", Hsu et al. have used the Lachancea yeast system to investigate how perturbation of organismal fitness due to gene dysregulation can be compensated by the evolution of second site suppressors. Briefly, Hsu et al. exploit the rapid development of genetic suppressors in a Δsef1 strain to understand how perturbation in gene regulatory networks can be rapidly, but conditionally, 'fixed' during evolution. They have previously shown that Sef1 is a transcription factor that has been repurposed in Lachancea to regulate the expression of metabolic genes. The authors observe that suppressors of the Δsef1 mutant fall into two main categories, generalists (mediated by ira1 inactivation) that resolve the growth defects of Δsef1 under fermentative and respiratory conditions and multiple temperatures, and specialists (mediated by azf1 inactivation) that resolve growth defects of Δsef1 only under respiratory conditions. They further show, using genetic experiments and RNA-seq transcriptomics that the effects of these suppressors can be recapitulated at the level of transcription of TCA cycle genes and heat shock proteins. They finally also show that the frequency of the suppressor in a population can be maintained due to cell non-autonomous effects.

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27 an important question in molecular and systems evolution. This study attempts
28 to bridge the gap between molecular mechanism and organismal
29 phenotype/fitness, which can be challenging even in model organisms like
30 Saccharomyces and E. coli. The major strength of this study is exploiting a
31 spontaneously arising suppressor using careful genetic and transcriptomics
32 analyses to understand how cells can mutationally rewire their metabolic and
33 gene expression networks. This rewiring is often on a 'need-basis', as seen for
34 the azf1 mutant and can be conditional. This is an important point which is often
35 missed by researchers working in the area. The weakness of this study, in its
36 present form, lies in firstly not having direct evidence linking loss of a
37 transcription factor (azf1 for instance) with its specific effects on transcription,
38 which are likely only a subset of those observed at the whole-transcriptomics
39 level. Secondly, the interesting observation that cells can access either a
40 generalist or a specialist suppressor has not been satisfactorily explored. The
41 co-existence, relative abundances and evolutionary pressures driving each of
42 these pathways for gene rewiring remain relatively un-investigated. Thirdly, a
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44

45 Therefore, I would request that the authors address the following
46 queries/concerns before the manuscript can be accepted for publication:
47

48 Major comments:

49 (1) The relative frequencies of occurrence of generalist and specialist
50 suppressors is not apparent from the figures or the text. This information is
51 crucial to understand which of the two suppressor strategies is more common
52 and how that correlates with the trade-off associated with specialist

53 **suppression.**

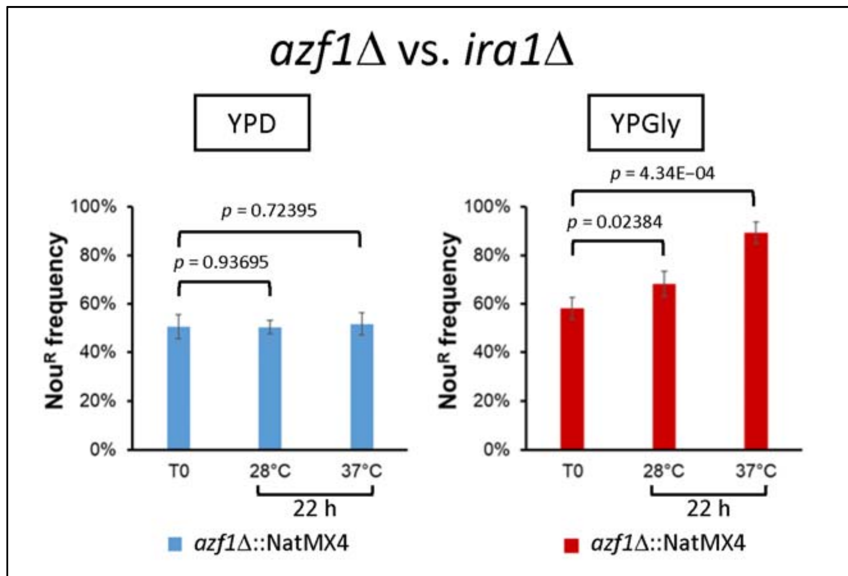
54 We add the information about the relative frequencies of occurrence of generalist (*ira1*
55 loss-of-function or related mutations) and specialist (*azf1* loss-of-function and related
56 mutations) suppressors in the results (lines 216-244) and displayed in Fig EV1 and
57 EV2. The frequencies were estimated based on direct sequencing and
58 mutation-specific phenotypic assays (desiccation hypersensitivity for *ira1*
59 loss-of-function or related mutations; "Dex-trade-off" growth for *azf1* loss-of-function
60 and related mutations).

61 **(2) Have the authors tried directly competing the generalist and specialist**
62 **suppressors? Are there environments that select one over the other?**

63 According to the growth (colony size and viability) on the agar plates (Fig 4F), the
64 generalist (e.g., *ira1*Δ) suppressors were more competitive than the specialist (e.g.,
65 *azf1*Δ) suppressors in all tested conditions. Interestingly, if under the *sef1*Δ
66 background (Appendix Fig S9C), the generalist (e.g., *sef1*Δ*ira1*Δ) suppressors were
67 still more competitive than the specialist (e.g., *sef1*Δ*azf1*Δ) suppressors in YPD. In
68 contrast, the generalist suppressors were more competitive than the specialist
69 suppressors in YPGly at 28°C but the specialist suppressors became more
70 competitive under heat stress.

71 Surprisingly, when we competed the *ira1*Δ with the *azf1*Δ strains directly in the
72 liquid broth for 22 hours (see the Review-only figure below), we found that they
73 showed similar competitiveness in YPD while the *azf1*Δ strain became more and more
74 competitive in YPGly from 28 to 37°C. We think one possibility of making this
75 discrepancy is the different availability of nutrients between agar plates and liquid
76 broth. However, figuring this out requires more experiments but this is not the key
77 question that we want to address in this paper. Therefore, we decide not to put this
78 discussion into the article.

79 <Review-only figure>:



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81

82 **(3) The growth phenotypes as well as transcriptional profiles of Δazf1 and**
83 **Δsef1Δazf1 are extremely similar. This means that in a Δazf1 strain the presence**
84 **of absence of sef1 is immaterial. Given this fact, I am unclear on why the**
85 **authors chose to represent sef1 and azf1 as two alternative**
86 **pathways/transcriptional programs (Figure 3J). Would it not make more sense**
87 **to position azf1 downstream of sef1?**

88 The *sef1Δ* effect is masked by *azf1Δ* (as shown in the transcriptional profiles). This
89 result can be explained by two possible mechanisms, one is that Azf1 is epistatic to
90 Sef1 and the other is that Azf1 and Sef1 regulate similar target genes but work
91 independently at the molecular level. We did not choose to put Azf1 downstream of
92 Sef1 is due to the lack of solid regulatory information from Sef1 to Azf1 (i.e., the Sef1
93 does not bind to the promoter of Azf1 (Hsu et al., 2021)). Moreover, the reason why
94 we represented Sef1 and Azf1 as two alternative pathways in Fig 3J is that these two
95 TFs are both regulated by the upstream Ras1-Ira1-PKA signaling pathway and that is
96 the clear conclusion we got from the experiments in Fig 3.

97 **(4) Though the authors have used extensive RNA-seq experiments to**
98 **demonstrate that there is transcriptional rewiring in the suppressors of Δsef1, it**
99 **is unclear to me where the rewiring exactly is. For example, the authors have**
100 **previously identified several binding sites for sef1 in the genome of Lachancea.**
101 **Do these targets also respond to azf1, or are the effects of azf1 through an**
102 **independent set of gene promoters? Further, since both sef1 and azf1**
103 **mutations used in this study are gene deletions and loss-of-function mutations,**
104 **do the authors believe that loss of repression by these factors in driving the**
105 **observed transcriptomic changes? Finally, how many of the effects that the**
106 **authors report at the gene expression level are primary rewiring effects, and**
107 **how many are secondary/tertiary effects? Some clarity on this issue is crucial if**
108 **the authors want to make the claim that the compensatory effects are due to**
109 **gene regulatory rewiring. One possible approach to address this could be by**
110 **performing one-hybrid assays with some of the TCA (and other) gene targets**
111 **from the RNA-seq.**
112 First, we would like to clarify the "rewiring" concept discussed in this study. The
113 consequence of *azf1* loss-of-function is to create a new genetic background that can
114 stabilize a new transcriptional network without Sef1 (*sef1*Δ). This evolutionary change
115 will potentially stabilize the initial stage of transcriptional rewiring to allow this new
116 transcriptional network without Sef1 to keep evolving rather than being purged due to
117 misexpression of important genes (e.g., TCA cycle genes) under selective conditions.
118 And that is how compensation works to reduce the misexpression of TCA cycle genes.
119 We did not have any evidence saying that *azf1*Δ directly triggers the rewiring of Sef1
120 target genes to another new regulator at the molecular level.
121 Then we conducted a simple experiment to test whether Azf1 affects Sef1 target
122 genes expression directly or indirectly (Appendix Fig S17) and put it in the Discussion

123 (lines 505-516). Briefly, we deleted the putative Azf1 binding motif on the *IDH2*
124 promoter (a Sef1 direct target TCA cycle gene) in the wild type and found out this motif
125 loss did not lead to the upregulation of *IDH2* the same as azf1Δ did. This finding
126 suggests that *azf1*Δ upregulates TCA cycle genes indirectly, possibly through other
127 down-regulated transcriptional regulators in response to *azf1*Δ (Table EV15),
128 especially through those potentially transcriptional repressors (Appendix Fig S17D),
129 which were downregulated in response to *azf1*Δ and then caused derepression of
130 many genes such as the TCA cycle genes. Notably, although these transcriptional
131 regulators are conserved between *L. kluyveri* and *S. cerevisiae*, it is guaranteed that
132 their target genes are all conserved. Elucidating these mechanisms, especially
133 figuring out the primary and secondary/tertiary effects resulting from *azf1*Δ requires
134 more new experiments and we think it has been beyond the scope of this study.
135 **(5) Azf1 is known to be a prion-like protein in Saccharomyces. Given the**
136 **contribution of heat shock proteins to the phenotypes of Azf1, have the authors**
137 **considered the possibility that some of the phenotypes may be due to loss of**
138 **the prion form of azf1 at high temperature in the Δazf1 strain rather than its**
139 **transcriptional roles?**
140 After carefully reading the papers discussing *S. cerevisiae* Azf1 as a prion-like protein
141 (Chakrabortee et al., 2016, Cell 167, 369–381; Stewart et al., 2021, PLoS ONE 16(5):
142 e0247285), we think *L. kluyveri* Azf1 is less likely to regulate the gene expression
143 indirectly similar to ScAzf1 through its prion toxicity for the following reasons: (1)
144 unlike ScAzf1 which carries both N-terminal poly-N and poly-Q disordered domains
145 characterized as the features of a prion-like protein, LkAzf1 does not contain a clear
146 poly-N domain. Although it still has a poly-Q domain, poly-Q domains are commonly
147 known to act as a transcriptional activation domain in many TFs; (2) By mating the
148 *AZF1* cells with the *azf1* cells, the meiotic progenies did not have prion-like

149 inheritance patterns (non-Mendelian fashion)(as shown in our tetrad dissection
150 assays); (3) the transcriptional responses of *azf1*Δ did not require a higher
151 temperature. Therefore, the hypothesis that the loss of the prion form of Azf1 at a high
152 temperature in the *azf1*Δ strain is less likely; (4) usually the prion toxicity induces a
153 protein homeostasis response (e.g., upregulation of heat-shock proteins). In contrast,
154 in our study, the deletion of *AZF1* triggered the upregulation of heat-shock proteins
155 and many other stress-related genes (Appendix Fig S12). Hence, we do not think that
156 the transcriptional effects of *azf1*Δ come from the loss of Azf1 prion proteins.
157 Also, we think this discussion is beyond the scope of this study. Therefore, we
158 decided not to put this discussion into the article.
159 **(6) The last section of the study that deals with frequency dependent**
160 **phenotypes and cell non-autonomous effects is interesting, but not sufficiently**
161 **fleshed out in terms of mechanism. As a result, its relevance in the current**
162 **manuscript is difficult to understand. Can the authors demonstrate, for example,**
163 **that the frequency at which the azf1 mutant occurs in the population of Δsef1**
164 **strain is higher than expectation? Further, without the molecular mechanisms**
165 **of these effects that they see I would be wary of just the phenomenological**
166 **findings. My suggestions would be to remove these observations from the**
167 **present study and report them once more mechanistic and population-level**
168 **details are available.**
169 We fully understand the concerns of Reviewer 1 about the cell-density-dependent
170 phenotypes of *azf1* mutants due to the lack of a clear molecular mechanism in this
171 paper. However, the density-dependent fitness effect of *AZF1* deletion is important
172 from a conceptual point of view as it might influence how genetic polymorphism in
173 *AZF1* could be maintained in the population. Moreover, Reviewer 3 is very
174 appreciative and positive about this part of the experiments and strongly encourages

175 us to put more descriptions about it in the Abstract. Therefore, we contacted the editor,
176 Dr. Ioannis Papaioannou, to discuss with him this conflict between Reviewer 1 and
177 Reviewer 3, and got the editorial advice that we should keep the experiment in the
178 revised manuscript as long as its reproducibility is convincing. We are very confident
179 about the reproducible results of this experiment and then decide to keep it. Still, we
180 want to express our sincere appreciation to Reviewer 1 for your prudent attitude
181 toward this data.

182

183 **Minor comments:**

184 **(1) In Figure 1B, the growth rate of the Δsef1 seems lower on YPD-PDS than on**
185 **YPGly. However, plate assays in Figure 1A suggest that growth yield is higher**
186 **on YPGly than on YPD. Are there trade-offs possibly between yield and growth**
187 **rate for this mutant?**

188 We do not think that it is the consequence of a trade-off between yield and growth rate.
189 We think it is simply that *sef1*Δ did not favor growing by using ethanol, which is the
190 major carbon source during the post-diauxic phase growth (YPD-PDS). This is the
191 reason why we used glycerol as the respiratory carbon source in our study and this
192 can explain why *sef1*Δ showed a severer growth defect in the YPD-PDS phase than in
193 YPGly.

194 **(2) Quantification for Western blots is missing and will significantly improve**
195 **the reach of the data in this manuscript.**

196 The normalized band intensities are displayed below the blots for Fig 3D, 3I, and
197 Appendix Fig S7B as ratios of TAP to α-tubulin signals.

198 **(3) Line 80-84. This is an important statement that sets up the question**
199 **addressed in the paper. However, as it is frames currently, it is very difficult to**
200 **understand. Perhaps the authors could make this sentence crisper?**

201 In short, the key point of this section is: “mutations causing larger phenotypic effect
202 may trigger the evolutionary changes of the current transcriptional network, but
203 generate trade-offs simultaneously. Therefore, compensatory evolution is
204 subsequently required to diminish the trade-offs in order to stabilize the newly evolved
205 transcriptional network”. Moreover, we focused on a situation that “there is no
206 intermediate stage of transition in which a redundant regulatory machinery evolves
207 first before the old regulatory connection has broken” by deleting *SEF1* (i.e., the
208 broken old regulatory connection) directly.

209 We modify this section (lines 80-85) to be “However, such large-effect mutations
210 may be a double-edged sword due to their deleterious pleiotropic effects (Dittmar *et al*,
211 2016). Therefore, we aimed to investigate whether and how compensatory evolution
212 works efficiently to deal with this conflict (trade-offs from new large-effect mutations)
213 when the “redundancy” mechanism (redundant and/or cooperative machinery of
214 regulation) is unavailable as new transcriptional networks evolve”.

215 **(4) The authors have presented many as part of the figures associated with**
216 **this manuscript. However, their legibility and readability are very poor. It may be**
217 **better to keep them as separate tables rather than as part of figure. This would**
218 **significantly simplify reading the manuscript.**

219 Although not specified, we believed that the reviewer is talking about the
220 low-resolution heatmaps. Therefore, we provide high-resolution source tables for all
221 heatmaps (Fig 4D, 5C, 5D, Appendix Fig S8, S11A, S12A, and S13A) in Table EV17.
222 Each gene ID, expression level, fold change, and a color gradient of heatmaps are
223 very clearly provided. However, we still keep the original small heatmap figures
224 because we aim to visualize the global gene expression patterns across samples (e.g.,
225 co-upregulation and co-downregulation) to facilitate result interpretations.

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229 Response to Reviewer 2 (Reviewer's comments in **bold** and responses in red):

230

231 - **Referee #2:**

232

233 **Comments to the Authors**

234

235 **The study with title 'Rapid compensatory evolution by secondary perturbation**
236 **of a primary disrupted transcriptional network' focuses on the characterization**
237 **of two targets that emerged from a suppressor screen of a transcriptionally**
238 **perturbed (sef1Δ) L. kluyveri strain and in depth characterization of one of them.**
239 **The characterization involved differential expression data of the perturbed and**
240 **suppressed strains, growth assays in various media, genetic, biochemical and**
241 **pathway analyses. This is a thorough investigation and a well-written paper.**

242

243 **I only have very minor comments and suggestions.**

244

245 **Minor comments:**

246 **(1) Fig 3F and 3G: It is unclear to me why the WT in these two panels is**
247 **different. Are they different constructs or backgrounds? Please clarify or**
248 **explain the discrepancy.**

249 The WT strains used in Fig 3F and 3G are the same strain. We think the reading
250 difference was just a signal shifting of biochemical reactions between these two
251 experiments. The LacZ assays in Fig 3F and 3G were performed independently.
252 Because the LacZ assay is a biochemical assay in which the enzymatic activity can be

253 affected by a lot of technical factors, it is not easy to make sure that when handling
254 different batches of samples, we can always get the same absolute reading values of
255 biochemical reactions. That is the reason why every time we need to have a WT as a
256 positive control and all the tested samples are compared with the control. In Fig 3F
257 and 3G, the conclusions stay unchanged (*ira1*Δ, *RAS1*^{G20V}, *pde2*Δ, and *bcy1*Δ
258 decrease the Azf1 activity under the YPD condition while increasing it under the
259 YPGly condition) even though the wild-type controls had different values.

260 **(2) Throughout the manuscript (for ex. Lines 258-260), ira1 mutants are treated**
261 **as generalists. Additionally, the authors used that as an argument to justify**
262 **focusing the study on the azf1 mutants. In fact, RAS PKA perturbations are not**
263 **a generalist strategy, but typically emerge as a response to conditions that**
264 **involve changes in nutrient abundance. (Had the majority of our evolution**
265 **experiments in yeast been done in chemostats, RAS PKA would not have been**
266 **as popular of a target). That is mutations in azf1 and mutations in ira1 probably**
267 **emerged in response to different selective pressures within the same**
268 **environment. Please re-visit the document to account for that and consider**
269 **including a different argument (less explored target?) on why azf1 mutants were**
270 **chosen for further analysis. In line 482, it is mentioned that ira1 generalist**
271 **impact is specific to the particular set of experiments. That can be introduced**
272 **earlier on, to avoid confusion.**

273 We completely agree with this comment. We add "However, some arguments suggest
274 that they typically emerge as a response to conditions that involve changes in nutrient
275 abundance, such as leading to uncontrolled cell growth in the absence of glucose
276 (Cazzanelli *et al*, 2018)" in this section (lines 287-290). Moreover, we indeed chose
277 the *azf1* mutant for further analysis because it is not previously characterized. This
278 statement has been mentioned in the same paragraph (lines 290-291).

279 **(3) Is the YPD-YPD in fig S11B some sort of control? It was unclear at first what**
280 **the media transitions are, because the YPD-YPD is never mentioned in the text**
281 **or legends or materials and methods. Please add a note explaining in the**
282 **legend or methods.**

283 The YPD→YPD samples were the control without the amino acid pre-starvation. The
284 method has been described in the Materials and Methods/Phenotypic assays section.
285 The descriptions for "YPD→YPD" and "SM→YPD" are added to the Appendix Figure
286 legend.

287 **(4) The left-most columns in figures S12A and S13 showing DE data are**
288 **unreadable, unfortunately. The authors can put these data in excel with**
289 **col-coded wells and turn these panels into summary data, or put the gene**
290 **columns only in excel with identifiers and use the same identifiers for the figure**
291 **every a few or several rows.**

292 We provide high-resolution source tables for all heatmaps (Fig 4D, 5C, 5D, Appendix
293 Fig S8, S11A, S12A, and S13A) in Table EV17. Each gene ID, expression level, fold
294 change, and a color gradient of heatmaps are very clearly provided. However, we still
295 keep the original small heatmap figures because we aim to visualize the global gene
296 expression patterns across samples (e.g., co-upregulation and co-downregulation) to
297 facilitate result interpretations.

298 **(5) Please provide a guide for the RNAseq data tables. For example, a brief**
299 **description of the sheet contexts and the columns at the supplementary tables**
300 **legends (for example a longer legend in the first table should suffice).**

301 We add a "README" sheet to each excel file of RNA-seq data tables (Table EV1, EV2,
302 EV5-EV12) to explain the sheet contexts and the columns.

303 **(6) Fig 1B: the two conditions could be combined in a single plot**

304 Fig 1B is modified as suggested.

305 **(7) Given that two adaptive strategies were found, corresponding to**
306 **perturbations in two genes, it would be interesting to know whether the rest of**
307 **the isolated clones have mutations in these two genes and whether their**
308 **phenotypes match the authors' predictions. If in the meantime sequencing**
309 **information was recovered on these loci for other clones, it would be nice to see**
310 **them included.**

311 We add the information about the relative frequencies of occurrence of generalist (*ira1*
312 loss-of-function or related mutations) and specialist (*azf1* loss-of-function and related
313 mutations) suppressors in the results (lines 216-244) and displayed in Fig EV1 and
314 EV2. The frequencies were estimated based on direct sequencing and
315 mutation-specific phenotypic assays (desiccation hypersensitivity for *ira1*
316 loss-of-function or related mutations; "Dex-trade-off" growth for *azf1* loss-of-function
317 and related mutations). Notably, we identified some clones without *azf1* or *ira1*
318 mutations but they still showed evolved phenotypes (Fig EV1B and 2B), suggesting
319 that there are other causal mutations possibly playing similar roles in the same
320 pathways as Azf1 and Ira1 do, respectively.

321 **(8) Can data like those in fig 1D be used to approximate suppression rates?**
322 **And then speculate whether there are other loci that contribute to suppression?**

323 We estimate the suppression rates by using fluctuation assays (Appendix Fig S18)
324 and briefly discussed them in the Discussion (lines 521-523). As mentioned above, we
325 indeed identified some clones without *azf1* or *ira1* mutations but they still showed
326 evolved phenotypes (Fig EV1B and 2B). Therefore, there must be some other loci that
327 contribute to suppression but use the same or similar mechanisms.

328 **(9) Genetic analysis in fig S5 and S6: Was the phenotyping performed on the**
329 **parental diploids? It would be nice to have dominance/ recessiveness**

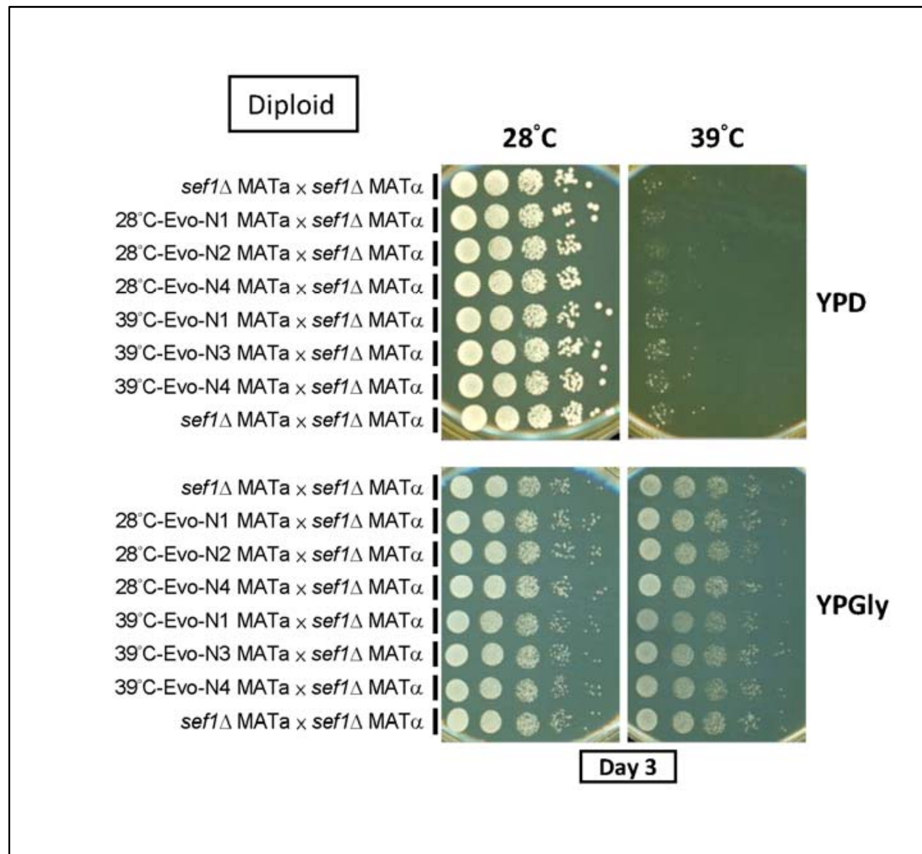
330 **information.**

331 Due to all the mutations being loss-of-function or hypomorphic, they are recessive in

332 the diploids as shown in the figure below. We just add a simple description of the

333 recessiveness of these mutations in the legends of Appendix Fig S5 and S6.

334 <Review-only figure>:



335

336 **(10) Language and typos**

337 **The language in the abstract in general could use a little 'tightening'**

338 **Line 31: 'minimalize the deleterious effects' to minimize deleterious effects'**

339 The sentence is modified as suggested.

340 **Lines 33-37: clanky and inefficient writing, Lines 39-42: difficult sentence**

341 The sentence is rephrased to: “Our results not only indicate that secondary

342 transcriptional perturbation provides rapid and adaptive mechanisms potentially

343 stabilizing the initial stage of transcriptional rewiring, but also suggest how genetic
344 polymorphisms of pleiotropic mutations could be maintained in the population" (lines
345 39-42).

346 **Line 158: 'selecting' - it seems that 'plating' is a more appropriate term**

347 The sentence is modified as suggested.

348 **Lines 192 and 195: mention 'three clones' per temperature (l. 192) or mating**
349 **type (l. 195), but in fact it is three clones per temperature AND mating type, and**
350 **there are data for 12 clones total. Please re-word.**

351 The sentence is changed to: "Therefore, only three clones each of 28°C-Evo MATa,
352 28°C-Evo MATα, 39°C-Evo MATa, and 39°C-Evo MATα lines were re-stocked" (lines
353 193-194).

354 **Lines 204-206: Confusing wording, please re-word for accuracy.**

355 The whole section (lines 201-206) is reworded to: "Subsequent examination of
356 mutation types revealed that 28°C-Evo lines carry deletion, missense, or
357 loss-of-function (premature stop codon-gained) mutations in the *IRA1* loci (Fig 2C)
358 and 39°C-Evo lines carry missense and another type of loss-of-function mutations
359 (frameshift) in the *AZF1* loci (Fig 2D), supporting that *ira1* and *azf1* loss-of-function
360 alleles are the causal mutations in the 28°C-Evo and 39°C-Evo suppressors,
361 respectively.

362 **Were three clones checked via backcrossing, tetrad dissection and**
363 **phenotyping of one full tetrad each? This can be gathered by figures S5 and S6,**
364 **but the writing needs improvement.**

365 Yes, only one representative tetrad of each mating pair was dissected and shown. The
366 whole section (lines 207-213) is reworded to: "To prove that the suppressive
367 phenotypes are monogenic, we performed tetrad dissection analyses by backcrossing
368 the MATa suppressor clones with their MATα founders. We checked three each of the

369 MATa 28°C-Evo and MATa 39°C-Evo clones. After sporulation, one tetrad of each
370 mating pair was dissected. All four spores of each tetrad were phenotyped and the
371 candidate causal mutation loci were sequenced. All tetrads showed a perfect 2-to-2
372 ratio between suppressive vs wild-type phenotypes, consistent with the 2-to-2
373 genotypes (Appendix Fig S5 and S6), indicating a clear monogenic effect of the
374 suppressive mutation".

375 **Line 755: Fig 16C to Fig S16C**

376 This typo is changed to "Appendix Fig S16C" (line 900).

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399 Response to Reviewer 3 (Reviewer's comments in **bold** and responses in red):

400

401 - **Referee #3:**

402

403 **Comments to the Authors**

404

405 **The manuscript is an in-depth examination of the causes and pleiotropic**
406 **consequences of compensatory evolution of a single transcription factor, using**
407 **various genetic and molecular biology methods. Specifically, the authors**
408 **deleted SEF1, a transcription factor gene involved in respiration from the yeast**
409 **L. kluyveri and then selected for better growing colonies in respiratory medium**
410 **(glycerol as carbon source). They also initiated selection experiments with**
411 **added heat stress, as a second selection pressure. The evolved lines were able**
412 **to compensate their fitness in both sets of experiments, but the compensation**
413 **was caused by two different mutations: loss-of-function of two transcription**
414 **factors (IRA1 and AZF1, respectively). As a consequence, the evolved lines**
415 **behaved differently when exposed to different conditions: only the**
416 **high-temperature selected lines showed genetic trade-off/antagonistic**
417 **pleiotropy by growing worse than the wild-type under fermentative conditions**
418 **(rich glucose medium). Using transcriptomics, the authors showed that the**
419 **initially deleted and compensatory genes are functionally related through the**
420 **Ras-cAMP-PKA pathway. Further experiments suggest that AZF1 deletion can**

421 **increase fitness via multiple simultaneous mechanisms: by adapting to heat-shock and glycerol, and by restoring the level of TCA cycle genes downregulated by the SEF1 deletion, indicating deletion-specific compensation. The authors additionally show that the fitness impact of loss of AZF1 function is density-dependent, which have relevance to the population genetic mechanisms driving compensatory evolution.**

427

428 **Overall, while the dissection of compensatory mutations is not conceptually novel, to our knowledge, this is the first such detailed work focusing on a transcription factor mutation. Also, demonstrating that a key compensatory mutation shows density-dependent fitness effect represent a conceptual advance. Thus the work is an important step towards understanding how transcription networks may evolve through compensatory evolution. We found most of the presented analyses and methodologies convincing. However, some limitations of the experimental design raised questions about the interpretation of the results, which should be addressed (see below).**

437

438 **Major comments:**

439

440 **(1) There are two methodical shortcomings, which might make some of the conclusions less convincing. First, the fitness measurement is mostly based on visual inspection of colony growth, making it difficult to compare the sizes of the changes or to detect epistasis. Importantly, the two compensatory mutations increase/decrease fitness not only in the SEF1 deletion background but also in the wild-type. This raises the question if these fitness effects are larger in the deletion background than in the wild-type background (i.e. genetic**

447 **interaction/epistasis). More precisely epistasis can only be claimed if the effect**
448 **of the two mutations together are different to what we would expect based on**
449 **single mutation effects (e.g. References: van Leeuwen, Jolanda, Charles Boone,**
450 **and Brenda J. Andrews. "Mapping a diversity of genetic interactions in yeast."**
451 **Current opinion in systems biology 6 (2017): 14-21). Therefore, the conclusion**
452 **in the following sentence (Line 214) is not followed by its premises:**
453 **'Interestingly, the "double-compensation" effect of ira1∆ and the "Dex-trade-off**
454 **and Gly-compensation" effect of azf1∆ were retained in the wild-type SEF1**
455 **background, and the high-activity Sef1-VP16 (Hsu et al., 2021) was unable to**
456 **mask its effects, indicating that these two genes can function independently of**
457 **and epistatic to SEF1.' Showing that there is synergistic epistasis would be**
458 **important because that would answer the question whether the 'compensatory**
459 **mutations' are really compensating for the harmful mutation, or simply increase**
460 **fitness because of adaptation to the medium/heat stress. Even if the authors**
461 **cannot provide direct evidence, they should discuss this question and present**
462 **their existing indirect pieces of evidence, suggesting that the mutations might**
463 **be compensatory: i) sef1∆ lines evolved under heat stress are also able to**
464 **partially compensate fitness when growing in normal temperature (FigS3 D), ii)**
465 **transcript changes suggest that azf1 deletion can increase fitness by all three**
466 **mechanisms: adaptation to the medium, adaptation to heat stress and**
467 **compensation for the sef1 deletion.**

468 To avoid confusion, we first completely removed the statement "Interestingly, the
469 "double-compensation" effect of ira1∆ and the "Dex-trade-off and Gly-compensation"
470 effect of *azf1*∆ were retained in the wild-type *SEF1* background, and the high-activity
471 Sef1-VP16 (Hsu et al., 2021) was unable to mask its effects, indicating that these two
472 genes can function independently of and epistatic to *SEF1*."

473 Then, we performed an epistasis analysis using the maximal growth rates and the
474 multiplicative model according to the reference (Leeuwen et al., Curr Opin Syst Biol.
475 2017, 6:14-21) (Table EV13). We add a new section (lines 518-544) in the Discussion
476 to elaborate on the results of this analysis. Briefly, the *ira1*Δ and *azf1*Δ surprisingly did
477 not provide better fitness improvement (positive epistasis) in the *sef1*Δ than in the
478 wild-type backgrounds, indicating that the effects of suppressive mutations do not
479 dependent on the genetic background (at least in our study).

480 **(2) A related shortcoming is the lack of wild-type control in the evolution**
481 **experiment. Fitness increase can be also expected for the wild-type when**
482 **grown under the same conditions as the sef1 deletion mutant (especially under**
483 **heat stress). Using wild-type controls would show whether the same**
484 **'compensatory' mutants appear during their evolution (relating to the first**
485 **question of their specificity), and how well the compensated sef1Δ lines would**
486 **compete with them. That the wild-type are also expected to evolve and increase**
487 **its fitness should be at least discussed, since it would affect the potential**
488 **evolutionary fate of the compensated sef1Δ lines.**

489 We performed a new batch of suppressor development experiments including the
490 wild-type strain and then analyzed the evolved clones from the wild-type population.
491 We add a new section (lines 518-544) in the Discussion to elaborate on the results.
492 Interestingly, although at different frequencies, the wild-type population could also
493 develop evolved clones with similar *azf1* or *ira1* loss-of-function-like phenotypes. Our
494 findings raise an alternative hypothesis that the *ira1* and *azf1* mutants can form
495 subpopulations in the wild-type population first and then alleviate the deleterious
496 effects of the following spontaneous *sef1* mutations (Fig EV 4). This strategy may
497 allow the *sef1* mutations to be fixed in the population as long as the *sef1* mutants are
498 not less competitive in future environments.

499 **There are some key decisions made by the authors without much explanation**
500 **given. It would be nice to provide some rationale for the following decisions:**
501 **- Why choose L. kluyveri and SEF1 deletion?**
502 We add the statement “The *L. kluyveri* Sef1 was chosen due to its known
503 condition-dependent phenotypes, completely characterized direct target genes,
504 simple condition-responsive regulation, and proper evolutionary divergence from the
505 model baker’s yeast (Hsu *et al*., 2021). All these advantages will help to simplify
506 subsequent investigation after the evolutionary repair experiments” into the
507 Introduction (lines 98-102).
508 **- What is the rationale behind applying heat stress together with the deletion?**
509 **What are the authors' expectations?**
510 We did not have a specific rationale or hypothesis behind the strategy of applying heat
511 stress. That is why we did not mention it in the article.
512 According to our previous work (Hsu et al., 2021), the *L. kluyveri sef1*Δ mutant is
513 more sensitive to heat stress (37−39°C) than the wild type. We just intuitively tried to
514 trigger the compensatory evolution of *sef1*Δ under two different conditions (with and
515 without heat stress) and expected to get different suppressive mutations in response
516 to different conditions. However, we indeed expected that beneficial mutations gained
517 in one condition might cause trade-offs in the other conditions (it is a well-accepted
518 knowledge), but this was not the original purpose for applying heat stress before we
519 got trade-off phenotypes.
520 **- What was the motivation to perform the evolutionary experiment using both**
521 **MATa and MATalpha founder strains? Did the authors expect mating type to**
522 **influence any outcomes? Was there any difference found?**
523 We do not expect mating types to affect any outcomes. On the contrary, we expected
524 two mating types will generate similar evolutionary outcomes (and this is truly what we

525 observed). We took MATa and MATalpha lines as two biological repeats of the
526 evolution repair experiments. Moreover, we got suppressor clones from two
527 mating-type lines so that the subsequent genetic analysis for the incompatibility
528 between suppressive mutations (e.g., Appendix Fig S10) is feasible and easy to be
529 done.

530 **(3) The density-dependent fitness effect of AZF1 deletion is important from a**
531 **conceptual point of view as it might influence how genetic polymorphism in**
532 **AZF1 could be maintained in the population. This finding would definitely**
533 **deserve mentioning in the abstract.**

534 A brief description (lines 38-39) of the cell density-dependent fitness effect of *azf1*
535 mutations is added to the Abstract due to the length limitation (175 words).

536 **Also, the authors may elaborate more on the possibility of compensatory**
537 **mutations arising first and forming a subpopulation in which subsequent**
538 **loss-of-function mutations may occur that would otherwise be highly**
539 **deleterious (i.e. acting as permissive mutations).**

540 We add a new section (lines 518-544) in the Discussion to elaborate on this possibility
541 according to the new control suppressor development experiment including the
542 wild-type strain. We also put a new figure to explain this hypothetical model (Fig EV4).

543

544 **Minor comments:**

545

546 **(1) Prior works reported that compensation of loss-of-function mutations can**
547 **result in massive transcriptomic rewiring despite fitness restoration, see**
548 **Szamecz et al. 2014 PloS Biol and McCloskey et al. 2018 Nat Comm. These**
549 **works appear to be relevant for the central concept of the manuscript.**

550 Thanks for the suggestion of citing these two good references. We incorporate them

551 into the Discussion (lines 502-505).

552 **(2) We found the opening sentences of the Abstract confusing: 'The discrete**
553 **steps of transcriptional rewiring have been proposed to occur neutrally to**
554 **ensure steady gene expression under stabilizing selection, especially when a**
555 **regulon is being transferred from one transcription factor (TF) to another. An**
556 **evolutionarily conflict-free switch of a regulon may require an immediate**
557 **compensatory evolution to minimalize the deleterious effects'. The first**
558 **sentence seems to suggest an evolutionary scenario of neutral mutations, while**
559 **the second sentence suggests a different scenario, consisting of a deleterious**
560 **and a later adaptive (compensatory) step. We guess the authors meant that**
561 **transcriptional rewiring can result in multiple equally fit outcomes.**

562 The first sentence means the mutational changes in the transcriptional network are
563 not always necessary to be beneficial at the time when they appear and the
564 destabilizing mutations on gene expression are usually negatively selected in order to
565 maintain the optimal gene expression level. The second sentence indicates that the
566 scenario to keep the destabilizing mutations that alter the topology of the current
567 transcriptional network is to let compensatory mutation fix the deleterious effect of the
568 destabilizing mutations. Within a long-enough evolutionary period, the whole changing
569 process looks neutral and the consequent transcriptional networks with different
570 topologies may produce equal fitness under some conditions but different phenotypic
571 plasticity under changing environments.

572 **(3) How was 'inconsistent phenotype' defined? In table S3 strains evolved**
573 **under the same conditions were labelled either inconsistent or consistent even**
574 **if they had the exact same fitness scores.**

575 Thanks for helping us to find out the mistakes. We carefully examined the whole table
576 again and reassigned the “inconsistency” information, based on the criterion that “any

577 clone with a simple fitness score higher than the mean score of the same group +1 or
578 lower than the mean score of the same group −1 is defined as an inconsistent clone.
579 Table EV3, Appendix Fig S3F, and the S3F legend are updated.

580 **(4) Please indicate sample sizes, when applicable, e.g. in the figure legend of**
581 **plots with error bars.**

582 The sample sizes of plots with error bars in all figures are indicated in their figure
583 legends, including Fig 1B, 3B, 3C, 3E-3H, 6A, 6B, 6F, Appendix Fig S7A, S9A, S9B,
584 S12B, S16D, S16E, S17C.

585 **(5) Missing explanation in figure legend of Figure 1 c) about the name "SEF1R".**
586 **Did the authors mean sef1Δ::SEF1 ?**

587 "*SEF1*R" indicates the reconstituted strain *sef1*Δ::*SEF1*. The description is added to
588 the figure legend.

589 **(6) The Figure 3 panel B) C) shows the transcriptional activity of Sef1 in the**
590 **different media that are labelled by distinct colors. At panel B) the Azf1**
591 **transcriptional activities are also colored by the media. However, this**
592 **systematic labelling breaks at the panel F) and G) where the colors already**
593 **distinguish between negative control (lexA) and the lexA-LkAzf1. It would be**
594 **good to have one system for all of the graphs.**

595 Fig 3E, 3F, and 3G are modified to be displayed by a consistent labeling system (YPD
596 in blue and YPGly in red) the same as what is used in Fig 3B and 3C.

597 **At panel E) "Azf-lexA" should be corrected to Azf1-lexA.**

598 The mislabeling is corrected.

599 **At panel E) it is not clear whether the two bars on the right represent a strain**
600 **that contains both the plasmid based lexA-Azf1 and the chromosome inserted**
601 **one or it indicates a strain that carry only the chromosome inserted Azf1-lexA.**

602 The right-most strain carries two copies of *AZF1* (one on the plasmid and the other on

603 the chromosome). Because Azf1 is a weak activator, we used a strain with two copies
604 of *AZF1* to confirm the detection of its transcriptional activation activity. The
605 description is added in the figure legend and clearer figure labeling is made.

606 **Most of the panels (except E) and H)) do not indicate whether the experiments**
607 **were done by the native (chromosome based) promoter or the constative**
608 **(plasmid based) promoter. However, the authors make the point that they**
609 **behave differently (panel E), line 245).**

610 For Fig 3B, 3C, and 3E-3H, either the chromosome-based or the plasmid-based
611 system is labeled directly in the figures.

612 **At panel F) and G) the lexA-fused TF has a name that includes the species name**
613 **as well (lexA-LkAzf1). What is the purpose of this labelling? One can think that**
614 **the Sef1 at panel B) and C) does not derive from L. kluyveri, because its name**
615 **does not include the "Lk".**

616 All Azf1 genes come from *L. kluyveri*. To avoid confusion, "Lk" labeling is all removed.

617 **(7) Line 340 - The authors say "Indeed, deletion of AZF1 not only partially**
618 **restored expression of TCA cycle genes under the YPGly condition (Fig. 5C)".**
619 **At Figure 5C the expression data are normalized to the sef1Δ strain. One could**
620 **see the restoration better when the data would be normalized to the WT strain.**

621 For Fig 5C, we still prefer to display it by normalization to the *sef1*Δ strain. Because
622 the expression of TCA cycle genes is only partially restored (not all TCA cycle genes
623 are restored or restored to the wild-type level), it is not easy to distinguish the
624 difference of some TCA cycle genes between *sef1*Δ and *sef1*Δ*azf1*Δ strains when
625 normalized to the WT. However, we provide the alternative heatmap by normalization
626 to the WT in Table EV17.

627 <Review-only figure>: normalized to *sef1*Δ

Systemic name	Gene	WD	AzD	SD	SAzD	WG	AzG	SG	SAzG
SAKL0H24046g	*ACO1*								
SAKL0C04180g	*ACO2*								
SAKL0E07876g	*IDH1*								
SAKL0G03520g	*IDH2*								
SAKL0H02860g	*IDP1*								
SAKL0D08426g	*IDP3*								
SAKL0E08866g	*KGD1*								
SAKL0H15422g	*KGD2*								
SAKL0B01958g	*LPD1*								
SAKL0G03630g	*LSC1*								
SAKL0H02464g	*LSC2*								
SAKL0G11440g	*SDH1*								
SAKL0H25850g	*SDH2*								
SAKL0E04136g	*SDH3*								
SAKL0H14146g	*SDH4*								
SAKL0E00946g	*FUM1*								
SAKL0G19140g	*MDH1*								
SAKL0C12760g	*MDH2*								
SAKL0E04928g	*MDH3*								
SAKL0D09152g	*CIT1*								
SAKL0B02926g	*CIT3*								

628

629 <Review-only figure>: normalized to WT

Systemic name	Gene	WD	AzD	SD	SAzD	WG	AzG	SG	SAzG
SAKL0H24046g	*ACO1*								
SAKL0C04180g	*ACO2*								
SAKL0E07876g	*IDH1*								
SAKL0G03520g	*IDH2*								
SAKL0H02860g	*IDP1*								
SAKL0D08426g	*IDP3*								
SAKL0E08866g	*KGD1*								
SAKL0H15422g	*KGD2*								
SAKL0B01958g	*LPD1*								
SAKL0G03630g	*LSC1*								
SAKL0H02464g	*LSC2*								
SAKL0G11440g	*SDH1*								
SAKL0H25850g	*SDH2*								
SAKL0E04136g	*SDH3*								
SAKL0H14146g	*SDH4*								
SAKL0E00946g	*FUM1*								
SAKL0G19140g	*MDH1*								
SAKL0C12760g	*MDH2*								
SAKL0E04928g	*MDH3*								
SAKL0D09152g	*CIT1*								
SAKL0B02926g	*CIT3*								

630

631 **(8) At Figure 6 B) and C) labelling of the Y axis should be corrected to HGBR/**

632 **HGBS.**

633 The original labeling of the Y-axis is correct. We used HGB^R and HGB^S (R and S are

634 given in superscript) to represent “HGB-resistant” and “HGB-sensitive”, respectively.

635 The description is added to the figure legend.

636 **(9) Some of the tables cannot be read because of the too low resolution e.g.**

637 **Figure S12, S13**

638 We provide high-resolution source tables for all heatmaps (Fig 4D, 5C, 5D, Appendix

639 Fig S8, S11A, S12A, and S13A) in Table EV17. Each gene ID, expression level, fold

640 change, and a color gradient of heatmaps are very clearly provided. However, we still

641 keep the original small heatmap figures because we aim to visualize the global gene

642 expression patterns across samples (e.g., co-upregulation and co-downregulation) to

643 facilitate result interpretations.

644 **(10) There is a high background of the Figure 1 E lower image (YPGly), therefore**
645 **it is hard to compare it with the image above (YPD).**
646 The new Fig 1E lower image (YPGly) is modified by a 20% decrease in brightness to
647 decrease the background.

1st Revision - Editorial Decision 3rd Feb 2023

Dear Dr. Hsu,

Thank you for the submission of your revised manuscript to EMBO reports and for your patience during peer review. We have now received the full set of reports from the three referees that agreed to re-evaluate your study. Please find their comments appended below.

As you will see, all referees find that the manuscript has been substantially improved, new data have been added, and most of the previous concerns have been satisfactorily addressed. However, referee #2 identified an erroneous statement that should be removed, because it is not supported by the presented data. Furthermore, referee #3 points out that the recovered mutations are general beneficial mutations that should not be described as suppressor or compensatory mutations. Therefore, several statements and claims throughout the paper (including the title, the abstract, the Discussion, and the conceptual model presented in Fig. EV4) should be revised to accurately reflect the nature of the mutations. Please make sure that all changes are highlighted (or "tracked") to be clearly visible in the revised manuscript file.

From the editorial side, there are also a few things that we need from you:

- The revised title should be short (up to 100 characters including spaces), informative, and accurate, and it should not contain any abbreviations.

- The abstract should be a single paragraph describing all key novel findings of the study, written in present tense, and it should not exceed 175 words. Please revise it accordingly.

- Please provide up to 5 keywords in your revised manuscript (you currently have 7).

- The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also guide to authors:
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

- According to our journal's policy, "data not shown" (stated on page 18 of your manuscript) is not permitted. All data referred to in the paper should be displayed in the main or Expanded View figures, or in the Appendix. Please add these data or change the text accordingly if these data are not central to the study and its conclusions.

- Figure callouts for Fig. EV4 and Appendix Figs. S5, S6, S10, and S18 are missing. Please make sure that all panels are called out in your revised manuscript.

- All EV Tables should be renamed as Dataset EV# and uploaded individually using the file type Data Set. Please note that the legends should be added to the corresponding files, and callouts should be revised accordingly.

- Please revise the title and remove line numbering from your Appendix file.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes).

- Please note that EMBO press papers are accompanied online by
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-4 bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size.
Please send us this information along with your revised manuscript.

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a

cover.

We look forward to seeing a revised version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

In the revised version of the manuscript, the authors have addressed all my queries satisfactorily. I think that this is an interesting study, and the revised manuscript can be accepted for publication.

Referee #2:

The manuscript was adequately revised and my concerns for the most part addressed. There was only one point that I insist to be modified in the manuscript prior to publication.

My point on what triggers RAS-PKA mutations was either missed or my comment was not clear enough. In particular, the statement 'All such mutations, which arose repeatedly, proved beneficial, globally enhancing fitness seemly irrespective of the diverse laboratory conditions' in lines 286-287 is erroneous, can be misleading, does not add to the manuscript and thus should be omitted. A careful dissection of the conditions that favor these mutations (all citations that proceed the statement) will show that they arise as a response to nutrient limitation (either in a chemostat or at some point of the cycle in batch culture). In fact, the cited Wenger et al 2011 explicitly showed that the related evolved mutants display tradeoffs when glucose limitation is removed. The manuscript's own desiccation hypersensitivity assay also shows that ira1 perturbations increase desiccation sensitivity. Additionally, if they indeed globally enhance fitness, then we would have to assume that the activity of the RAS-PKA is suboptimal in the wild parental strain, which does not really make sense. Please omit statement.

Referee #3:

The authors made a significant effort to improve the manuscript, and I greatly appreciate the new experiments performed, i.e. the epistasis analysis and the evolution experiments using the WT as a control. I also appreciate the honesty with which the new data are presented. However, the new data raises a serious concern about the interpretation of the original suppressor screen.

Specifically, the epistasis analysis (Table EV13) clearly show that the slow-growing sef1 knockout is not compensated by either azf1 or ira1 deletion under the conditions where the suppressor screen was performed (YPGly). It is true that the fitness of sef1 is enchanced by azf1 or ira1 deletion, however, wild-type fitness is also enhanced to a similar extent by these mutations. Thus, there is no evidence for compensation / suppression, which would manifest itself as positive epistasis between sef1 and azf1 or ira1 (i.e. that's the widely used definition of suppression / compensation, see van Leeuwen et al. Science 2016, Moore, Rozen, Lenski Proc Roy Soc B 2000). In a similar vein, the authors now report the possible emergence of azf1 and ira1 mutations when the wild-type background was subjected to 'suppressor' screening. Together these observations indicate that the recovered mutations are general beneficial mutations in these environments and not suppressor or compensatory mutations. Simply, I don't see any evidence for compensatory evolution. This is more than a terminological nuance. It implies that several of the claims of the paper (including title and abstract) has to be rewritten to reflect the fact that the suppressor screen identified mutations that are general beneficial mutations and can be selected in the WT background as well. It further implies that the conceptual model presented on Fig EV4 should also be heavily revised: ira1 or azf1 deletions do not actually act as permissive mutations because they don't change the relative fitness impact of SEF1 loss.

Overall, I still think that the study is highly worthy of publication in EMBO Reports as it uncovers an important molecular rewiring

by which fitness can be increased. However, the conceptual framework and story must be substantially changed to accommodate the totality of evidence presented by the authors.

Response to Editor (Editorial comments in **bold** and responses in red):

- **Editorial side:**

Comments to the Authors

The revised title should be short (up to 100 characters including spaces), informative, and accurate, and it should not contain any abbreviations.

We revise our title to “Rapid evolutionary repair by secondary perturbation of a primary disrupted transcriptional network” (98 characters including spaces). Specifically, we use “evolutionary repair” to replace “compensatory evolution” in the old title.

The abstract should be a single paragraph describing all key novel findings of the study, written in present tense, and it should not exceed 175 words. Please revise it accordingly.

We revise the abstract according to the guidelines (written in present tense; 170 words).

Please provide up to 5 keywords in your revised manuscript (you currently have 7).

We keep only 5 keywords, including “compensatory evolution, trade-off, Sef1, Azf1, and *Lachancea kluyveri*”.

The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the contributions of each author in the journal submission system. Please use the free text box to provide more detailed descriptions. See also guide to authors: <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.

We remove the author contributions statement entirely.

According to our journal's policy, "data not shown" (stated on page 18 of your manuscript) is not permitted. All data referred to in the paper should be displayed in the main or Expanded View figures, or in the Appendix. Please add these data or change the text accordingly if these data are not central to the study and its conclusions.

We remove the “data not shown” related statements completely since it is not required. Please check the revised text at L494-497 in the manuscript with hidden tracked changes.

Figure callouts for Fig. EV4 and Appendix Figs. S5, S6, S10, and S18 are missing. Please make sure that all panels are called out in your revised manuscript.

The figure callouts to describe the panels of each figure are added into or further specified in each figure legend. Please note that the previous Fig EV4 is changed to Fig EV5.

- All EV Tables should be renamed as Dataset EV# and uploaded individually using the file type Data Set. Please note that the legends should be added to the corresponding files, and callouts should be revised accordingly.

We rename each previous Table EV# to Dataset EV# and add the legend in a new sheet into each corresponding file. The callouts can be found in either the legends or the README sheet in each excel file.

- Please revise the title and remove line numbering from your Appendix file.

We revise the title to “Rapid evolutionary repair by secondary perturbation of a primary disrupted transcriptional network” and also remove the line numbering from the Appendix file.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes).

We modify the figure legends according to the editorial suggestions in the revised manuscript.

- Please note that EMBO press papers are accompanied online by

A) a short (1-2 sentences) summary of the findings and their significance,

B) 2-4 bullet points highlighting the key results, and

C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size.

Please send us this information along with your revised manuscript.

We add a summary and four highlights for the synopsis in the revised manuscript before the Abstract.

Response to Reviewer 1 (Reviewer's comments in **bold** and responses in red):

- **Referee #1:**

Comments to the Authors

In the revised version of the manuscript, the authors have addressed all my queries satisfactorily. I think that this is an interesting study, and the revised manuscript can be accepted for publication.

We appreciate you taking the time to review our manuscript and bring us good suggestions and comments.

Response to Reviewer 2 (Reviewer's comments in **bold** and responses in red):

- **Referee #2:**

Comments to the Authors

The manuscript was adequately revised and my concerns for the most part addressed. There was only one point that I insist to be modified in the manuscript prior to publication.

My point on what triggers RAS-PKA mutations was either missed or my comment was not clear enough. In particular, the statement 'All such mutations, which arose repeatedly, proved beneficial, globally enhancing fitness seemly irrespective of the diverse laboratory conditions' in lines 286-287 is erroneous, can be misleading, does not add to the manuscript and thus should be omitted. A careful dissection of the conditions that favor these mutations (all citations that proceed the statement) will show that they arise as a response to nutrient limitation (either in a chemostat or at some point of the cycle in batch culture). In fact, the cited Wenger et al 2011 explicitly showed that the related evolved mutants display tradeoffs when glucose limitation is removed. The manuscript's own desiccation hypersensitivity assay also shows that ira1 perturbations increase desiccation sensitivity. Additionally, if they indeed globally enhance fitness, then we would have to assume that the activity of the

RAS-PKA is suboptimal in the wild parental strain, which does not really make sense. Please omit statement.

Thanks for the clarification of the previous comments. We remove the inappropriate statement “All such mutations, which arose repeatedly, proved beneficial, globally enhancing fitness seemly irrespective of the diverse laboratory conditions”. Please check the modified paragraph at L305-315.

Response to Reviewer 3 (Reviewer's comments in **bold** and responses in red):

- **Referee #3:**

Comments to the Authors

The authors made a significant effort to improve the manuscript, and I greatly appreciate the new experiments performed, i.e. the epistasis analysis and the evolution experiments using the WT as a control. I also appreciate the honesty with which the new data are presented. However, the new data raises a serious concern about the interpretation of the original suppressor screen. Specifically, the epistasis analysis (Table EV13) clearly show that the slow-growing sef1 knockout is not compensated by either azf1 or ira1 deletion under the conditions where the suppressor screen was performed (YPGly). It is true that the fitness of Δsef1 is enhanced by azf1 or ira1 deletion, however, wild-type fitness is also enhanced to a similar extent by these mutations. Thus, there is no evidence for compensation / suppression, which would manifest itself as positive epistasis between Δsef1 and Δazf1 or Δira1 (i.e. that's the widely used definition of suppression / compensation, see van Leeuwen et al. Science 2016, Moore, Rozen, Lenski Proc Roy Soc B 2000). In a similar vein, the authors now report the possible emergence of Δazf1 and Δira1 mutations when the wild-type background was subjected to 'suppressor' screening. Together these observations indicate that the recovered mutations are general beneficial mutations in these environments and not suppressor or compensatory

mutations. Simply, I don't see any evidence for compensatory evolution. This is more than a terminological nuance. It implies that several of the claims of the paper (including title and abstract) has to be rewritten to reflect the fact that the suppressor screen identified mutations that are general beneficial mutations and can be selected in the WT background as well. It further implies that the conceptual model presented on Fig EV4 should also be heavily revised: ira1 or azf1 deletions do not actually act as permissive mutations because they don't change the relative fitness impact of SEF1 loss.

Overall, I still think that the study is highly worthy of publication in EMBO Reports as it uncovers an important molecular rewiring by which fitness can be increased. However, the conceptual framework and story must be substantially changed to accommodate the totality of evidence presented by the authors.

We are sorry for not highlighting the evidence of compensatory evolution from this study. We agree with Referee #3 about the comment that “fitness” displayed as maximal growth rates, it is not typical “compensation” according to the “positive epistasis” definition. However, evidence supports that loss-of-function *azf1* mutations are compensatory in the “gene expression” under the *sef1*Δ background compared with the wild type. We show this evidence in the newly added Fig EV3 and add a clear interpretation at L421-434. Generally, the *azf1*Δ induced a higher restoration in the expression of TCA cycle genes and a more dramatic differential expression in glycerol and stress responsive pathways (shown as fold-changes of gene expression) in the presence of *sef1*Δ than in the absence (i.e., the wild type).

Then, we carefully go through the whole manuscript and modify it more conservatively.

First, we use “evolution repair” to replace “compensatory evolution”. Second, we use “adaptive” to replace “compensatory/suppressive”. Third, we clarify the effects of adaptation using more specific terms, i.e., (1) “beneficial” in fitness and (2) “compensatory” in gene expression. Finally, we use the “historical contingency” model to replace the “permissive” model in Fig EV 5 (which is Fig EV4 in the previous version of the manuscript). To be noted, we still use “suppressor” to describe our evolved clones because we think it has been a historically well-accepted genetic term used in suppressor-selecting experiments.

Generally, we believe that our conceptual framework and significance of the story are still properly maintained, that is, “the *sef1*Δ cells rapidly acquire adaptive mutations whose beneficial effects in fitness allows cells to survive under selective environment and compensatory effects in gene expression allows the *sef1*Δ transcriptional network having the chance to further evolve”.

2nd Revision - Editorial Decision 15th Mar 2023

Dear Dr. Hsu,

Thank you for submitting your revised manuscript to EMBO reports. Referee #3 has now re-evaluated your improved manuscript and recommends publication.

Before we can proceed to accept your manuscript, we need you to make sure that all Figure panels are called out in your revised manuscript. We noticed that Figure callouts for Appendix Figs. S5A-C, S6A-C, S10A&B, and S18A&B are still missing.

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You can opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #3:

I appreciate the author's further efforts to clarify the manuscript. They now much more clearly distinguish between mutational effects that are compensatory versus beneficial. I agree with their interpretation and I recommend the paper for publication in its present form.

The authors have addressed all minor editorial requests.

3rd Revision - Editorial Decision 17th Mar 2023

Dr. Po-Chen Hsu
Institute of Molecular Biology, Academia Sinica
128 Sec.2, Academia Road
Nankang, Taipei 115
Taiwan

Dear Dr. Hsu,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table EV

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Table EV

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Legends of Figures and Figure EV
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Legends of Figures and Figure EV, Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Not Applicable	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Legends of Figures and Figure EV

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods