nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Flow cytometry and cell sorting data were collected through the commercial softwares Attune Nxt Flow Cytometer Software and BD FACSCMelody™ Software, respectively. Population based fluorescence measurements and fluorescence kinetics assay measurements were collected trough Tecan Safire 2 software.
Data analysis	The DNA sequencing data were analyzed using custom C and Python codes: pib.c, rib.c,Expression_fitness_score_notebook.ipynb and Fitness_score_notebook(activity_assays).ipynb These codes are available through the Zenodo repository https://doi.org/10.5281/zenodo.15846928 We also utilized open-source software, such as Minimap2 (version 2.19) and the BBMap alignment algorithm, for the analysis of DNA sequencing data. For structural protein analysis, we used the PyMol open-source software. More details regarding our analysis workflow and codes can be found in the method section of the manuscript.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All the data required for replicating the study, such as DNA sequencing files and the raw data associated with each figure, have been deposited in the Zenodo repository and are accessible to the public: https://doi.org/10.5281/zenodo.15846928
Information about the PDB Entry 1c0P (DAOx) are found here: https://www.wwpdb.org/pdb?id=pdb_00001c0p

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	n/a
Reporting on race, ethnicity, or other socially relevant groupings	n/a
Population characteristics	n/a
Recruitment	n/a
Ethics oversight	n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We report the results of a deep mutational scanning workflow applied to a nearly comprehensive site-saturation library of D-amino acid oxidase (DAOx) from Rhodotorula gracilis. To assess how amino acid identity influences substrate specificity, we performed the assay using five substrates spanning a range of physicochemical properties. The variant library covered 6,530 of the 6,919 possible single-mutant variants of the enzyme, and we obtained accurate specificity measurements for 6,418 unique variants—including 5,821 missense, 316 nonsense, and 281 synonymous mutations—for each of the five independent substrate assays.
Data exclusions	Variants represented by fewer than 10 cells at the single-cell sorting stage were excluded from the analysis, as population-level measurements derived from such low cell numbers are not considered reliable.
Replication	Each screening workflow was performed twice independently, and all replicates were successful. For all reported scores, we provide the mean value of the two independent screens. All activity assays described in the manuscript were repeated two or three times independently, as detailed in the Materials and Methods section.
Randomization	Randomization was not applicable to the study because of the complex nature of the mutant library analyzed, which prevented the emergence of biased observations.
Blinding	Blinding was not relevant to the study. All the samples of the replicated experiments were treated independently at the same conditions.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
[ ]	[x] Antibodies
[ ]	[x] Eukaryotic cell lines
[x]	[ ] Palaeontology and archaeology
[x]	[ ] Animals and other organisms
[x]	[ ] Clinical data
[x]	[ ] Dual use research of concern
[x]	[ ] Plants

Methods

n/a	Involved in the study
[x]	[ ] ChIP-seq
[ ]	[x] Flow cytometry
[x]	[ ] MRI-based neuroimaging

Antibodies

Antibodies used: Anti-6x-His mouse monoclonal antibodies (Thermo Fisher Scientific Cat# MA1-21315, RRID: AB_557403)
Anti-mouse antibody conjugated with Alexa Fluor™ 594 (TThermo Fisher Scientific Cat#A-11005, RRID: AB_2534073)

Validation: Anti-6x-His mouse monoclonal antibodies (Thermo Fisher, #MA1-21315): This Antibody was verified by Relative expression to ensure that the antibody binds to the antigen stated.

Anti-mouse antibody conjugated with Alexa Fluor™ 594 (Thermo Fisher #A-11005): To minimize cross-reactivity, these goat anti-mouse IgG whole antibodies have been cross-adsorbed against human IgG and human serum prior to conjugation.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s): Saccharomyces cerevisiae strain EBY100 (Background strain: BJ5465) MATa AGA1::GAL1-AGA1::URA3 ura3-52 trp1 leu2-delta200 his3-delta200 pep4::HIS3 prbd1.6R can1 GAL)

Authentication: n/a

Mycoplasma contamination: n/a

Commonly misidentified lines (See ICLAC register): n/a

Plants

Seed stocks: *Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.*

Novel plant genotypes: *Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.*

Authentication: *Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.*

Flow Cytometry

Plots

Confirm that:

[x] The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

[x] The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

[x] All plots are contour plots with outliers or pseudocolor plots.

[x] A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation: Yeast cells expressing the DAOx enzyme on the surface were assayed through antibody staining procedure or single cell

	tyramide assay before being analysed through flow cytometry.
Instrument	Attune NxT Flow Cytometer, FACSMelody cell sorter
Software	Attune Nxt Flow Cytometer Software BD FACSChorus™ Software
Cell population abundance	n/a
Gating strategy	DAOx Activity analysis towards each of the 5 selected substrates: We set the low fluorescence gate to include entirely the population of cells displaying inactive variants The remaining cells were equally divided into three populations corresponding to increasing levels of tyramide-488 signal. A figure depicting the gating strategies for each of the sorting assay is presented in Supplementary information, Figure S3. Gating strategies are essential information for presenting and reproducing the main workflow outlined in the manuscript.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.