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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	All neuronal functional data was collected using custom and proprietary instrumentation developed in-house at Quiver Bioscience. High content imaging of immunofluorescence for quantifying FMRP expression was collected using a GE IN Cell Analyzer 6000.
Data analysis	All data analysis (for functional data and high content imaging data) was performed using custom code that may be made available upon reasonable request (see "code availability" statement).

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

The data that support the findings of this study are available as Supplementary Data in the form of excel worksheets.

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	We have specified the sex of the donors for each of the cell lines used in the manuscript. In all cases, cell lines were obtained from male donors. Males were chosen due to (a) the increased severity of FXS in males vs. females to enhance chances of finding phenotype differences, (b) the increased prevalence of FXS in males, and (c) to control for random X inactivation that is observed in female iPS lines.
Reporting on race, ethnicity, or other socially relevant groupings	We have not reported on race, ethnicity, or other socially relevant groupings in the manuscript.
Population characteristics	For FXS patient samples, all patients were males ages 18-32 (at time of collection) with an FXS diagnosis and where known, CGG repeat lengths >200. Patient-matched controls were male subjects (brother or father) ages 35-62 with no neurological diagnosis.
Recruitment	Participants with FXS were recruited from the RUMC Fragile X Clinic and a family member (immediate relative) willing to participate was recruited at the time the FXS participant was identified. All participants signed informed consent to participate through a protocol approved by the RUMC IRB.
Ethics oversight	Sample collection protocols and consent forms were approved by RUSH University Medical Center's IRB to obtain peripheral blood.

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	The 720 patient-control wells plated for feature selection were designed to yield n samples > p features with fully counterbalanced plate locations. Isogenic reagents were plated concurrently, with a parallel plate design. Lentivirus rescue experiments were powered to minimally detect at least 50% functional rescue. Given the relatively modest screening window, the pilot screen was designed after performing bootstrap aggregations of 1-6 wells of already-collected phenotype data, finding that 3-well averages were required to yield sufficient separation for reliable hit detection (plated in duplicated at a rotated plate location, leaving 6 wells per compound). Dose-response hit confirmation retained these 3-well replicates.
Data exclusions	Source quality criteria were pre-specified prior to data collection and are detailed in the Methods. Individual movies from within a well were rejected if affected by clear technical artifact (e.g. a stuck shutter, overheated laser, out-of-focus images, or other such rare occurrences).
Replication	As described in the paper, experiments were all generally replicated across several rounds (typically 3) and/or many cell lines. This included the full pilot screen and hit confirmation, which was conducted in both patient/control and isogenic cell models.
Randomization	FXS status is intrinsic to the cell lines, there is no relevant randomization of disease label. Individual differences were partially mitigated by collecting samples from familial controls of each of the FXS donors.
Blinding	Personnel conducting cell culture, compound treatment, imaging, and analysis of screening experiments were blind to the identities of the compounds.

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
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	FMRP antibody (Cell Signaling, 7104S, 1:100)
Validation	This antibody was validated by showing detecting of FMRP in several control lines (WT) and absence of FMRP in all patient lines and the FMR1-/y CRISPR knockout line.

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	For the generation of FXS patient-derived iPSC cell models, we used sample collection protocol and consent forms approved by RUSH University Medical Center's IRB to obtain peripheral blood samples (2–4 mL) from 5 male Fragile X Syndrome patients and from 5 healthy control male relatives (brother or father). For the generation of FMR1+/y and FMR1-/y isogenic iPSC lines, we used CRISPR/Cas9 to target the disruption of the FMR1 gene in the control iPSC line (FMR1+/y) M28, which was initially derived from a neurologically healthy male donor.
Authentication	All cell lines were authenticated by a series of quality control metrics as outlined in the Methods section of the manuscript
Mycoplasma contamination	Mycoplasma testing was part of the QC stated above (and described in the manuscript; see "Methods"). All cell lines tested negative for mycoplasma.
Commonly misidentified lines (See ICLAC register)	Not available

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	Male Fmr1 knockout mice (Fmr1-KO) on the C57BL/6J background were used as a source of glial cells in all relevant experiments
Wild animals	The study did not involve wild animals
Reporting on sex	Glia were obtained from male Fmr1-/y and Fmr1+/y mice only. Given the more severe phenotypes of male FXS patients, the higher prevalence of FXS in males, and the use of male FXS patient (iPSC) lines (to control for X inactivation), we chose to use male mice for these studies.
Field-collected samples	Not avalable
Ethics oversight	All experimental techniques were approved by the Committee on Animal Care at MIT and all animals were handled in accordance with NIH and MIT guidelines

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

Plants

Seed stocks	N//A
Novel plant genotypes	N/A
Authentication	N/A