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Reporting Summary

Nature Research 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 Research policies, see Authors & Referees 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	Transgenic embryos were imaged with Adobe Photoshop Elements 11. Brain sections were imaged with Nikon NIS Elements acquisition software, version 3.22.15 (Build 738).
Data analysis	Statistical analyses and plot generation were done with R version 3.5.0 (www.r-project.org).

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/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research 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 list of figures that have associated raw data
- A description of any restrictions on data availability

Images of all transgenic whole-mount-stained embryos are included in Supplementary Figure 1. Images of brains sections from knock-in animals and wild-type littermates are in Extended Data Fig. 8, 9, and 10. Human SNVs were obtained from TOPMed, https://bravo.sph.umich.edu/freeze5/hg38/ in June 2020. JASPAR transcription factor motif data was downloaded from http://expdata.cmmt.ubc.ca/JASPAR/downloads/UCSC_tracks/2018/hg19/. Public ChIP-seq data was obtained from https://www.encodeproject.org (mouse embryonic H3K27ac and H3K27me3, mouse and human CTCF) and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124936 (Dlx transcription factors).

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 transgenic embryo sample sizes were determined empirically based on our experience performing >4,000 transgenic enhancer assays (VISTA Enhancer Browser: https://enhancer.lbl.gov/). Activities of reference enhancer alleles were already known from random transgenic assays. For direct comparison to mutant enhancer alleles, we tested all reference alleles anew with targeted transgene assays and made sure to obtain at least two transgenic embryos positive for integration of a transgene into H11 locus, which enhancer activity patterns recapitulated those observed with random transgenic assays. For mutant enhancer alleles we required reproducible patterns to be present in at least three transgenic embryos positive for H11 integration. Number of embryos assayed for each allele are reported in Table S1.
Data exclusions	The embryos genotyped negative for transgene integration into the H11 locus were excluded from further analysis, along with embryos that were not at the correct developmental stage, were heavily damaged during collection or appeared developmentally abnormal.
Replication	In vivo transgenic mouse assays: across all 121 H11 transgenic experiments (distinct allele and developmental stage combinations) reported in the manuscript, ~60% were performed at least twice with the same results observed and for these, data across multiple litters was integrated. The rest of transgenic experiments were performed independently. For 4 enhancers (hs121, hs122, hs123, hs124) effects of mutagenesis was also tested using transgenic mice with random integration and the same results as with the targeted transgenene integration into H11 locus were observed (data not reported). Knock-in lines: for each line, neurological phenotyping was performed on 2 mouse litters with the same results observed.
Randomization	Images of transgenic embryos for each enhancer were pooled together for all alleles of that enhancer and randomized. Enhancer activity patterns for were scored by five independent reviewers blinded to the identity of enhancer allele. The process is described in details in Methods under section: Scoring the Strength of Ultraconserved Enhancer Activity in Transgenic Embryos. For neurological phenotyping wild-type and knock-in littermates were randomized and identified only by numbers with genotype unknown to the investigator during data collection, sample processing and measurement taking.
Blinding	For blinding in scoring of enhancer activity in transgenic embryos, see Randomization. Investigators were blinded to animals' genotype during data collection and measurements for neurological phenotyping of the three knock-in lines generated in this study.

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
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

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

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	All animal work done in this study was reviewed and approved by the Lawrence Berkeley National Laboratory Animal Welfare and Research Committee. Mice were housed in the animal facility, where their conditions were electronically monitored 24/7 with daily visual checks by technicians. Facility was also equipped with automatic alarms. Mice were housed in BioBubble Clean Rooms, soft-walled enclosures powered by 80-100 air changes per hour of HEPA filtration under Light/Dark Cycle of 12:12 starting at 6am, at 22-24.4C, and humidity 30-70%. All animals used in this study were of Mus musculus species and FVB strain. Sex was not determined for transgenic embryos, but cohorts are presumed to include roughly equal numbers of males and females. Transgenic embryos were assayed either at embryonic day 11.5 or 12.5 or 14.5.

	Phenotyping of mice in stable knock-in lines was done for males only due to studied enhancers located on chromosome X. Males were assayed at various ages ranging from postnatal day 32 to 67. Details are in Tables S6 and S7.
Wild animals	The study did not involve wild animals.
Field-collected samples	The study did not involve samples collected from the field.
Ethics oversight	All animal work done in this study was reviewed and approved by the Lawrence Berkeley National Laboratory Animal Welfare and Research Committee.

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