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Cell villages and Dirichlet modeling map human cell fitness genetics

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Abstract: The capacity of cells to proliferate and survive is central to development and disease. Assays that measure cell fitness are therefore a cornerstone of biology, but traditional techniques lack donor diversity and have high technical variability that impedes scale and reproducibility. To overcome these barriers, we designed and validated a “cell village”-based fitness screening approach using pooled cultures of 12–39 genetically distinct human neural progenitor cell (NPC) lines. We also developed Townlet to establish a foundational statistical framework based on Dirichlet regression for analyzing proportional data from cell villages. Applying these systems, we identified hyperproliferation in NPCs harboring the autism risk factor chromosome 16p11.2 deletion, mapped common genetic variants near *ZFHX3* associated with NPC proliferation rate, and discovered genetic modifiers of lead (Pb) sensitivity implicating *ARNT2*. Together, these experimental and analytical tools advance a scalable, genetically diverse *in vitro* platform for dissecting human variation in cell fitness and gene-environment interactions.

Keywords: Cell village, Dirichlet model, cell fitness, neurotoxicity

Introduction

Cell proliferation and maintenance are fundamental to life. During early development, cells increase in number over time and differentiate to build tissues and organs through processes governed by genetics and the environment. In humans, differences across individuals in proliferation and viability mechanisms (i.e., cell fitness) contribute to variability in complex traits, such as organ size, regeneration capacity, and resilience to extrinsic stressors¹⁻⁴. Likewise, between-individual variation in the penetrance of deleterious genetic mutations or the susceptibility to harmful environmental exposures can drive differential risk for developmental disorders, tissue degeneration, and cancer⁵⁻⁸. Accurate measurements of cell fitness that take into account many distinct genetic backgrounds and environmental stimuli are therefore essential for elucidating the mechanisms that coordinate cell proliferation and viability under healthy and disease states, as well as for the screening and development of effective therapeutics.

Human pluripotent stem cells (hPSCs) and their derivatives are valuable tools for studying fitness in genetically distinct cells. Conventional hPSC-based approaches utilize an arrayed culture format, in which cells from different individuals (i.e., donors) are plated into separate wells for experimentation and analysis. These designs, however, are often limited by low throughput, small donor sample size, and high well-to-well and batch-to-batch technical variation that can mask biological signals and inhibit reproducibility⁹⁻¹⁴.

To increase the scale, genetic diversity, and number of perturbations in a single experiment's design, we and others recently introduced "cell villages," which consist of tens to hundreds of genetically distinct human cell lines cultured together in shared *in vitro* environments¹⁵⁻¹⁸. This strategy enables quantification of cellular phenotypes across many genetic backgrounds in parallel under uniform culture conditions and reduced technical variation. Villages have been deployed to interrogate between-donor variation in differentiation potential^{19,20}, viral susceptibility¹⁵, and cytokine response²¹. However, villages have not been systematically used to quantify cell proliferation or viability, nor have computational models been developed that consider the unique design and structure of these experiments.

Here, we establish experimental and analytical tools for village-based fitness assays of human neural progenitor cells (NPCs), which are the building blocks of the fetal brain. Many genetic and environmental risk factors for neurodevelopmental disorders alter NPC proliferation and viability²²⁻²⁵. We constructed multiple hPSC-derived NPC villages consisting of 12-39 donors (Tables S1-S2) to quantify and explain between-donor variation in proliferation and sensitivity to environmental stress. Given that the inherent compositional²⁶ (i.e., proportional) data structure of villages invalidates the use of traditional statistical tools like ANOVA, we built a user-friendly hierarchical Dirichlet regression model called Townlet that analyzes village donor proportion data from low coverage DNA sequencing^{17,27} collected over multiple time points and/or perturbations to estimate individual donor fitness.

Using Townlet, we tested several biological hypotheses and addressed common questions about village experimental design, power, reproducibility, and the impact of cell non-autonomous factors. We first devised a statistical simulation framework to evaluate our model's improved performance over more classical statistical models (e.g., linear regression) and to assist in designing future high-powered, reproducible cell village experiments. We then demonstrated how our model can answer biologically relevant questions by: (i) showing that donors harboring neurodevelopmental disorder genetic risk factor chromosome 16p11.2 deletion display NPC hyperproliferation, (ii) deciphering how common genetic variation contributes to between-donor differences in NPC proliferation rates, and (iii) nominating genetic modifiers of sensitivity to the known neurodevelopmental toxicant lead (Pb). Our integrated experimental format elucidates natural human variation in cell fitness while revealing how gene-environment interactions amplify or buffer these effects.

Material and methods

Stem cell lines and maintenance

All protocols were approved by the UCLA Institutional Biosafety Committee (IBC) and the UCLA Human Pluripotent Stem Cell Research Oversight (hPSCRO) Committee. All hPSC methods were carried out in accordance with UCLA IBC and hPSCRO guidelines and regulations. Informed consent was obtained for all cell lines by the labs and/or biobanks that supplied the lines to the Wells lab. All human embryonic stem cells (ESCs) used in this paper are registered in the NIH Human Embryonic Stem Cell Registry. Human induced pluripotent stem cell (iPSC) lines were obtained from CIRM/FujiFilm Cellular Dynamics, the Broad Institute/Harvard Stem Cell Institute, SAMPLED, and the Simons Foundation Autism Research Initiative (SFARI) (Table S2). Lines reprogrammed by the Dolmetsch Lab at Stanford were acquired via SFARI. Isogenic unedited and 16p11.2 deletion lines were generously supplied by Dr. Michael Talkowski (Massachusetts General Hospital). Prior to receipt, all lines were confirmed via genotyping, karyotyping, and *in vitro* differentiation to establish pluripotency. The list of donor lines used for each experiment can be found in Table S1.

All hPSCs were maintained on Geltrex basement membrane matrix (1:100; Life Technologies, A1413301) and fed daily with mTeSR Plus media (Stem Cell Technologies, 100-0276). Upon reaching 80-90% confluency, cells were split by incubating for 10 minutes at 37°C in Accutase (Innovative Cell Technologies, AT104) followed by a 1:1 dilution in mTeSR Plus and centrifugation. Cells were resuspended in mTeSR Plus with ROCK inhibitor Y-27632 (10 μ M; Stemgent, 04-0012) and plated at 1:10 to 1:30 dilution. ROCK inhibitor was removed after 24 hours.

Stem cell-derived neural progenitor cell (NPC) induction and maintenance

Human PSCs were transduced and induced into NPCs as previously described using the SNaP method ^{15,28}. In brief, human stem cells were transduced with TetO-Ngn2-Puromycin and Ubq-rtTA lentiviral constructs. For SNaP generation, transduced hPSCs were plated on Geltrex in mTeSR Plus supplemented with ROCK inhibitor Y-27632 (10 μ M; Stemgent, 04-0012). The next day, cells were fed with Induction Media (Day 1): DMEM/F12 (ThermoFisher, 11320082), Glutamax (1:100; ThermoFisher, 10565018), 20% Glucose (1.5% v/v), N2 Supplement (1:100, ThermoFisher, 17502048), Doxycycline (2 μ g/mL; Sigma-Aldrich, D9891), LDN-193189 (200 nM; Stemgent, 04-0074), SB431542 (10 μ M; Tocris, 1614), and XAV939 (2 μ M; Stemgent, 04-00046). Twenty-four hours later, cells were fed with Selection Media (Day 2): DMEM/F12, Glutamax (1:100), 20% Glucose (1.5% v/v), N2 Supplement (1:100), Doxycycline (2 μ g/mL), puromycin (5 μ g/mL; ThermoFisher, A1113803), LDN-193189 (100 nM), SB431542 (5 μ M), and XAV939 (1 μ M). After 24 hours in Selection Media, cells were replated in NPC maintenance media supplemented with puromycin (5 μ g/mL) and Y-27632 (10 μ M). Starting 12–18 hours post-passaging, cells were maintained in NPC maintenance media without puromycin or Y-27632. Immunostaining was performed to validate NPC identity, defined as $\geq 75\%$ NESTIN+/PAX6+/SOX1+ and $\leq 0.1\%$ OCT4+ cells at Passage 1-3.

NPCs were maintained on Geltrex basement membrane matrix (1:100; Life Technologies, A1413301) and fed daily with NPC maintenance media, which was made fresh every 48 hours: DMEM/F12 (Thermo Fisher Scientific; 11320082), Glutamax (1:50; Thermo Fisher Scientific, 10565018), MEM-NEAA (1:100; Life Technologies, 10370088), Pen/Strep (1:100, Cytiva, SV30010), B27 minus Vitamin A (1:50; Life Technologies, 12587010), N2 Supplement (1:100; Life Technologies, 17502048), recombinant human EGF (10 ng/mL; STEMCELL Technologies, 78006.1), recombinant human basic FGF (10 ng/mL; Thermo Fisher Scientific, PHG0368). Cells were split weekly by incubating for 10 minutes at 37°C in Accutase (Innovative Cell Technologies, AT104) and plating at 120,000 cells/cm². For the first 24 hours after every passage, media was supplemented with ROCK inhibitor Y-27632 (10 μ M; Stemgent, 04-0012). After induction and expansion,

NPCs were banked in Cryostor-10 (Stem Cell Technologies, 07930) for future use. Passage 2-7 NPCs were used for experimentation.

Village Construction

NPCs from multiple donors were thawed, plated, and maintained as independent arrayed cultures for 5-7 days until confluent. Cell lines were frequently imaged to confirm healthy NPC morphology and were passaged at least once prior to village construction. Cell lines showing typical morphology and confluency were included in village pools. We did not exclude hyperproliferating cell lines based on estimates from array cultures given our goal to sample natural variation in growth. To build villages, cells from each donor were dissociated with Accutase, resuspended in NPC maintenance media with ROCK inhibitor Y-27632 (10 μ M), and counted using a Countess II FL (ThermoFisher Scientific). Donors were pooled at equal proportions in a 50 mL Falcon tube and then plated into replicate wells in 6-well plates at 40,000 cells/cm² (with the exception of the Village versus Array experiment; see below).

Census-seq

On harvest days, 250,000-500,000 cells per sample were pelleted and frozen at -80°C. DNA was then extracted from each sample following the standard procedure of the DNeasy Blood & Tissue Kit (Qiagen, 69504). All extracted DNA samples were indexed during library preparation, pooled, and sequenced using an Illumina NovaSeq 6000 SP flow cell (paired end 150 bp reads; at least 0.5X whole genome coverage per sample). Paired-end reads were aligned to the GRCh38 primary assembly reference genome using BWA-MEM (BWA v0.7.17-r1188), and aligned reads were sorted with samtools (v1.15). Donor proportions were estimated from the sorted BAM files using the **CensusSeq** program from Drop-seq Tools (v2.5.4), which infers donor mixture fractions from low-coverage sequencing reads at genotype-informative variants using donor WGS genotype calls as input. Sex chromosomes and mitochondrial variants were excluded from **CensusSeq** inference. All village donors were included when running Census-seq and the compositional data was renormalized when outliers or siblings were removed from the analysis.¹⁷

Village versus array comparisons

Microscopy-based village versus array comparison

Village

We pooled 12 human ESC-derived NPC lines into a village at equal proportions and plated into six technical replicate wells at 22,500 cells/cm². The next day, cells from three of these wells were harvested for DNA extraction and Census-seq analysis in triplicate. The remaining three village wells were independently passaged every 5 days for a duration of 15 days. The passage 1 initial (day 1) and final (day 5) total cell counts were measured with a Countess Automated Cell Counter. These total cell counts were then multiplied by Census-seq's corresponding donor composition estimates to calculate the cells per donor.

Array

Immediately following village construction, the remaining cell suspensions were independently passaged into 20 wells per donor in 96-well plates at a density of 3,333 cells/cm². Half of the wells were stained with Hoechst-33342 (5 μ g/mL) and imaged using a BioTek Cytation 5 (Agilent Technologies; 10x Objective, 3x3 grid) on Day 1; on Day 7, the remaining wells were stained and imaged using the same procedure. CellProfiler²⁹ was used to quantify Hoechst-positive cells for both time points.

Exponential growth rates

The exponential growth equation,

$$n = n_0 \cdot e^{\lambda t}, \quad (1)$$

was used to estimate percent change per day. Because initial and final measurements were not paired by replicate, each final replicate was compared to the mean initial cell count for that donor within the same culture format:

$$p_{d,r} = \frac{\ln \left(\frac{n_{d,r}^{(T)}}{\bar{n}_d^{(0)}} \right)}{t} \cdot 100. \quad (2)$$

The final donor cell count in replicate r is represented by $n_{d,r}^{(T)}$, the mean initial donor cell count across replicates is represented by $\bar{n}_d^{(0)}$, and t is the time elapsed between initial and final measurements. For village samples, final counts were measured on day 5 after passage; for arrayed samples, final counts were measured on day 7. The calculated $p_{d,r}$ values were then averaged across final replicates to obtain each donor's growth rate.

Census-seq-based village versus array comparison

To compare village and arrayed cultures using the same Census-seq measurement modality, 12 human iPSC-derived NPC lines were plated in parallel either as pooled villages or as separately cultured arrayed donor wells. For the village condition, donors were pooled at equal proportions and plated into three replicate wells. For the arrayed condition, each replicate consisted of one plate containing one well per donor. Cells were harvested on day 1 and day 5 after plating.

For Census-seq profiling of arrayed cultures, cells from all 12 donor wells within each replicate plate were dissociated, pooled into a single tube, pelleted, and processed as one arrayed "pseudovillage" sample. Village wells were harvested and processed independently. Genomic DNA was extracted from all day 1 and day 5 samples and donor fractions were estimated by Census-seq as described above.

For each donor, relative growth rate was calculated from raw Census-seq donor fractions as the per-day change in log donor fraction between day 1 and day 5. Because day 1 and day 5 Census-seq samples were not paired by replicate, each day 5 replicate was compared to the mean day 1 fraction for that donor within the same culture format:

$$p_{d,r} = \frac{\ln \left(\frac{Y_{r,d}^{(5)}}{\bar{Y}_d^{(1)}} \right)}{t} \cdot 100. \quad (3)$$

The day 5 donor fraction in replicate r is represented by $Y_{r,d}^{(5)}$, the mean day 1 donor fraction across replicates is represented by $\bar{Y}_d^{(1)}$, and t is the time elapsed between day 1 and day 5. The calculated $p_{d,r}$ values were then averaged across day 5 replicates to obtain each donor's growth rate.

Hierarchical Dirichlet regression donor proliferation model

Townlet models donor proportion data using the Dirichlet distribution, which models a multivariate outcome that sum to one. The following parameterization was implemented:

$$\mathbf{Y}_r^{(t)} \sim \text{Dir}(\boldsymbol{\mu}(r, t) \cdot \exp(\theta(r, t))) \quad (4)$$

The $\mathbf{Y}_r^{(t)}$ vector, of length D (number of donors), is our response variable that contains the compositional data of each donor (d) included in the village estimated from the Census-seq pipeline for a given time point (t) and replicate (r). The Dirichlet distribution requires all components of $\mathbf{Y}_r^{(t)}$ to lie strictly between 0 and 1. To address zeros arising from donor dropouts, we implemented a multiplicative replacement strategy³⁰. Let $\mathbf{Y}_r^{(t)} = (Y_{r,1}^{(t)}, \dots, Y_{r,D}^{(t)})$ be the original data containing zeroes and define k as the number of components equal to zero. We construct a transformed composition $\mathbf{Y}_r^{(t)*}$ as:

$$Y_{r,d}^{(t)*} = \begin{cases} \delta, & \text{if } Y_{r,d}^{(t)} = 0, \\ Y_{r,d}^{(t)}(1 - k\delta), & \text{if } Y_{r,d}^{(t)} > 0, \end{cases}$$

where δ is a small positive constant. This transformation preserves the unit-sum constraint, $\sum_{d=1}^D Y_{r,d}^{(t)*} = 1$, while minimally perturbing the relative structure of the nonzero components.

Estimating biological parameters

The first part of Equation 4 helps us characterize the biological dynamics within the experiment with

$$\mu_d(r, t) = \frac{\exp(\eta_d(r, t))}{\sum_{c=1}^D \exp(\eta_c(r, t))}, \quad (5)$$

$$\eta_d(r, t) = \alpha_{r,d} + x_t \cdot \beta_d, \quad (6)$$

with priors,

$$\begin{aligned} \beta_d &\sim \mathcal{N}(0, \sigma^2), \\ \sigma^2 &\sim \text{Normal}(0, 1). \end{aligned} \quad (7)$$

Here, each donor's replicate specific proportion is modeled by $\mu_d(r, t)$ using a softmax transformation to normalize all model components to unity. The donor specific model in log-space uses a log-linear model on $\eta_d(r, t)$ which has an initial representation, $\alpha_{r,d}$, that is directly given to the model and a temporal proliferation effect, β_d , that is estimated using the time index for each sample provided (X_t , days from t0 or passage number). This generalized linear model logit formulation (Eq. 5) is proportional to exponential growth (Eq. 1) where

$$\mu_d(r, t) = \frac{1}{Z} \exp(\alpha_{r,d}) \cdot \exp(x_t \cdot \beta_d) \quad (8)$$

$$= c_{r,d} \exp(x_t \cdot \beta_d). \quad (9)$$

The proliferation model can optionally incorporate treatments (e.g., chemical, pharmaceutical, environmental, etc.) and donor group covariates with interaction terms (e.g., sex, ancestry, phenotypes, disease status, sex:phenotype, sex:treatment, etc.) to infer significant influences on donor proliferation dynamics. We can extend the model parameterization to include these effects

$$\eta_d(r, t) = \alpha_{r,d} + x_t(\beta_d + \mathbf{z}_d^T \boldsymbol{\tau}) \quad (10)$$

where the treatment effects (τ_d) are partially pooled by donor,

$$\tau_d \sim \text{Cauchy}(0, 2). \quad (11)$$

and the donor group effects (τ_g) are pooled using the following prior,

$$\tau_g \sim \text{Cauchy}(0, 0.5). \quad (12)$$

The Cauchy distributions provide shrinkage of effects towards zero, while still preserving large effect sizes with the heavy tails³¹. The design matrix (z_d) includes donor group identity and treatment dose information per donor. When donor group or treatment effects are included the total donor proliferation effect can be interpreted as $\beta_d + \mathbf{z}_d^T \boldsymbol{\tau}$. All donors are relative to a baseline donor with a median proliferation rate since the prior on β_d is centered at zero. We performed extensive sensitivity analyses on our prior choices, with Figure S5G-H showing the results for parameters τ_g and τ_d .

Accounting for overdispersion

The second component of equation 4 ($\theta(r, t)$) accounts for overdispersion in individual village replicates over time. The following overdispersion parameters quantify technical variation that occurs during cell village experiments (e.g., uneven handling of samples during cell culture, sequencing bias, etc.). Based on observations from real village data (Figure S2C-D), it is expected that the effects of technical variation will accumulate over time and may differ slightly across replicates, therefore, overdispersion is modeled by

$$\theta(r, t) = \theta_0 + x_t \cdot \phi_r, \quad (13)$$

where,

$$\begin{aligned} \theta_0 &\sim N(\mu, 2) \\ \phi &\sim N(0, 2) \\ \phi_r &\sim N(\phi, 0.5). \end{aligned} \quad (14)$$

The amount of technical variation at village initiation is defined as θ_0 and ϕ_r defines the rate of change in variation over time per replicate. The intercept prior mean (μ) is estimated using maximum likelihood under the frequentist model. Lower values of θ imply higher values of technical variation.

Choosing a baseline donor

When selecting a baseline donor for Dirichlet regression, note that all proliferation effects are defined relative to a reference donor fixed at zero. For analyses without donor group covariates, relative proliferation ranks are largely invariant to the choice of baseline, though the scale of posteriors shifts (Figure S3A). Posterior uncertainty, however, is more unstable when extreme (fast, slow or high technical variation) donors are chosen as the baseline, due to the geometry of log-ratio transformations (Figure S3B).²⁶ Townlet automatically estimates the median donor (from the reference group if including group-level covariates) based on the relative rank of donors proportional change, minimizing distortion and improving interpretability of coefficients. When modeling donor group covariates (e.g., sex, genetics), it is especially important to avoid extreme or covariate-associated baselines, as these can shrink or inflate group effects by absorbing signal into the baseline intercept. Simulations confirm that choosing the median grower yields robust inference, while tail references produce unstable results (Figure S3C). Users should inspect nearby donors to verify stability of inference and that Townlet's rough rank estimates result in choosing a median grower with low technical variation that is part of the covariate reference group.

Simulate new cell village experimental designs using our generative Dirichlet model

In silico village composition data can be generated by: (i) fitting experimental data using our Dirichlet regression model and (ii) scaling and sampling from the interpretable fitted parameter posteriors to simulate new data. The following model parameters can be adjusted to simulate different village designs:

- **Donor proliferation effects** (β_d, τ_d, τ_g): The posteriors can be sampled and scaled to create infinite numbers of new *in silico* donors with optional treatment and donor group effects.
- **Initial village composition**($\alpha_{r,d}$): Randomly sample starting donor composition per replicate.
- **Sample time points and replicates** (x_t): The index can be modified to include more replicates and sample time points.
- **Dispersion parameters** (θ_0, ϕ_r): Sample from and scale dispersion parameter posteriors to simulate villages with differing amounts of technical variation.

After setting, sampling and scaling all necessary parameters the mathematical framework from our Dirichlet regression model can be used to simulate new data. First, the α values for each donor are calculated and then these concentration parameters are used to randomly generate new representation data from a Dirichlet distribution. Repeating the simulation over multiple iterations (i.e., thousands of *in silico* experiments) gives us distributions of possible donor representation values under repeated experiments.

Benchmarking Regression Models

Using *in silico* data from our statistical framework, we benchmarked Townlet against more naive analysis approaches. First, we tested the performance of our Dirichlet regression model when only a single donor covariate was included under control conditions (i.e. no treatments). We benchmarked this version of the model against: (i) the exponential growth equation (EGE), (ii) a linear model (LM) and (iii) frequentist Dirichlet regression. These methods were implemented to estimate individual donor growth rates and then t-tests were performed to check for significant donor covariate effects. For Townlet, we used the local false sign rate³² and the estimated effect direction of the posterior to assess if we could recover the simulated donor group effect. Sensitivity and specificity were calculated to compare performance between all four models. In all Figure 3 simulations, the β parameter varied across donors to reflect biological scenarios where multiple loci influence proliferation. In Figure 2B–C, we fixed β across donors to show that Townlet still outperforms other methods under simpler assumptions.

Second, we benchmarked Townlet’s performance when estimating treatment effects per donor (no donor covariates). We compared our model to a more naive analysis approach where first EGE proliferation rates were individually estimated per donor. Then ANOVA followed by estimated marginal means testing was performed to identify donors whose average treatment growth rates significantly differed from the mean across all donors. This is analogous to our treatment effect (τ_d). When simulating the data we set 50% of the donors to have a zero treatment effect and the remaining had a treatment effect drawn from

$$\tau_d \sim \text{Uniform}(0.01, 0.6). \quad (15)$$

After running Townlet and this other approach on the simulated data, we used the false discovery rate to assess performance.

16p11.2 deletion proliferation

We constructed a 23-donor human iPSC-derived NPC village composed of 11 controls and 12 16p11.2 deletion donors. NPCs were thawed and maintained in three wells each of a 12-well plate before pooling,

plating in triplicate at 40k/cm², and passaging every 5 days for a total of 15 days. For each sample, at least 500,000 cells were pelleted prior to DNA extraction for Census-seq.

To validate that intrinsic NPC growth rates for 16p11.2 donors and neurotypical controls were retained in the village, the growth rates of the 23 donors were estimated from arrayed maintenance wells prior to pooling. Brightfield images (10x Objective, 3x3 grid) of these arrayed wells were captured using a Cytation 5 imager on days one and four post-plating (68 hours apart). Per well cell counts at each time point were then measured from these images using Cellpose³³. Exponential growth curves were independently fit for each well across all time points.

CRISPR-engineered isogenic NPCs carrying a 16p11.2 deletion and matched controls were plated at 40,000 cells/cm² in five replicate wells each. Brightfield images (10x Objective, 3x3 grid; Cytation 5) were collected daily for six days. Cell counts were extracted using Cellpose, and exponential growth curves were independently fit for each well across all time points.

Arrayed NPC growth rate validation

To validate the NPC growth rate association identified in villages, we estimated growth rates for 112 hPSC-derived NPC lines grown as separate arrayed cultures. Brightfield images were captured daily for five days (10x objective, 3x3 grid; Cytation 5). Per-well cell counts were measured from these images using Cellpose, and growth rates were estimated independently for each well across days 2-5. Donor-level growth rates were calculated by averaging growth-rate estimates across replicate wells.

Design of 35-donor NPC village

We pooled 39 human ESC-derived NPC donor lines into a village at equal proportions and plated in triplicate at 40,000 cells/cm². Replicate wells were independently maintained and harvested for DNA sequencing at five time points (Day 0, 1, 5, 6, and 10; passaged at Day 5). For quality control, we applied the “Roll Call” algorithm from the Census-seq toolkit to estimate donor abundance at Day 0. One donor was excluded from subsequent analyses due to underrepresentation at this time point (< 0.5%). To limit potential confounding from sibling relatedness in downstream GWAS, we randomly excluded one donor from each of the three sibling pairs before estimating proliferation effects with Townlet and performing GWAS.

GWAS analysis

We conducted genome-wide association analyses using Townlet outputs as the phenotypes and 30X whole-genome sequence (WGS) genotype data from all donor lines in the village, aligned to GRCh38. Analyses were carried out using BCFtools (v1.11), PLINK (v2.0.0), and GEMMA (v0.98.5). We created a variant calling format (VCF) file that included the 35 donors and converted to PLINK binary format. Autosomal variants were retained, and SNPs were filtered to exclude markers with minor allele frequency (MAF) < 5% or genotype missingness > 5%. After filtering, 6,233,757 autosomal SNPs were retained for association testing. To account for population structure and relatedness, we generated a set of autosomal variants pruned by linkage disequilibrium (LD) using the estimated LD within the cohort (window size = 50 SNPs, step size = 5 SNPs, r² threshold = 0.2). This pruned dataset was used to compute the genetic relatedness matrix (GRM) in GEMMA. We performed association analysis across the full set of autosomal SNPs using GEMMA (linear mixed model, option -lmm 4), providing the proliferation effects estimated by Townlet as the primary phenotype alongside GRM and sex as covariates. After association testing, we performed clumping to identify approximately independent signals. Association summary statistics were processed in PLINK using an r² threshold of 0.1 and a 250 kb window. For this clumping step, LD was estimated using the European population reference panel from the 1000 Genomes Project.

Genotype principal components were computed from the LD-pruned autosomal variant set using PLINK. For ancestry visualization, study donor genotypes were intersected with 1000 Genomes reference genotypes

and projected into the 1000 Genomes PC space. Within-cohort genotype PCs and eigenvalues were computed from the 35 retained donors and used for visualization and sensitivity analyses.

To prioritize candidate genes, we extracted the top 100 clumped variants ranked by Wald test p-value. These were mapped to gene symbols using the Ensembl Variant Effect Predictor. We then conducted Gene Ontology (GO) term enrichment via the gprofiler2 R package. Multiple testing correction was applied using the Benjamini–Hochberg false discovery rate method. For GO term permutation testing, we repeated the entire association workflow 1000 times, each time randomly reassigning the Townlet-estimated proliferation effects across donors while keeping the covariates fixed.

We performed additional sensitivity analyses for the index variants in the proliferation and Pb GWASs. To test sensitivity to genotype-PC adjustment, we reran GEMMA models with 0, 1, 2, or 3 within-cohort genotype PCs as additional covariates. For leave-one-donor-out analyses, GWAS models were rerun after excluding one donor at a time, and the index SNP effect estimate, standard error, and p-value were extracted from each fold. To propagate uncertainty in Townlet phenotype estimates, GWAS was repeated using 1000 posterior phenotype draws and the index SNP Wald p-value was extracted from each run. To summarize locus stability, we recorded whether the top Wald association in each leave-one-donor-out fold or posterior-draw GWAS fell within the same plotted locus as the full GWAS index variant. To summarize effect-size stability, we recalculated the in-sample R^2 for the index SNP in each leave-one-donor-out fold and posterior phenotype draw using the same single-variant genotype-phenotype regression used in Figures 5F and 6F. To estimate empirical null distributions, donor phenotypes were randomly permuted relative to genotypes for 1000 genome-wide GWAS permutations, retaining the minimum Wald p-value from each permutation, and for 20 million target-specific permutations in which only the index SNP was tested.

Isoform and transcript annotations were obtained from the UCSC Genome Browser³⁴ using NCBI RefSeq gene models³⁵. Predicted enhancer–gene interactions were taken from GeneHancer³⁶. Chromatin state annotations were obtained from the Roadmap Epigenomics Project 15-core model^{37,38}. DNase-seq and H3K27ac ChIP-seq signal p-value tracks from human ESCs, NPCs, and neurons were downloaded from ENCODE³⁹ and visualized using the WashU Epigenome Browser⁴⁰. Association statistics were visualized with LocusZoom⁴¹.

Lead (Pb) treatment

To prepare a 50 mM lead (Pb) stock solution, 190 mg of lead(II) acetate trihydrate (Sigma, 316512) was dissolved in 10 mL of double-distilled water and subsequently diluted to the desired concentrations using NPC maintenance media. For array culture assays, NPCs derived from the Genea52 ESC line were plated at a density of 60,000 cells/cm² into a 96-well plate. Five different Pb concentrations were tested (0, 1, 5, 10, 30 μ M), each with three replicates. Beginning the day after plating, culture media was replaced daily and supplemented with Pb. After 7 days of continuous exposure, cell viability was measured via CellTiter-Glo[®] 2.0 reagent (Promega, G9241). Luminescence (ATP) readings were recorded for each well using a Cytation 5 imager and normalized to the mean luminescence of the 0 μ M (untreated) control replicates to determine relative viability.

For village experiments, replicate wells were treated daily with one of three Pb concentrations: 0, 3, and 10 μ M. Cells were harvested for DNA extraction and Census-seq in triplicate on Days 4 and 7 post-exposure. The samples underwent 0.5X low-coverage sequencing through Gencove (Long Island City, NY). Libraries were prepared using the Illumina Nextera DNA Flex Library Preparation Kit, then quantified by fluorometry. Quality assessment of the pooled libraries was performed using an Agilent TapeStation. Standard Illumina adapter sequences were employed and sequencing took place on an Illumina NovaSeq 6000 (paired end 150 bp reads; at least 0.5X whole genome coverage per sample).

To calculate donor response as percent viabilities, we first estimated the total number of cells in each replicate by using Cellpose analysis of brightfield images taken with a Cytation 5 imager (10x objective,

5x5 grid). Then, for each donor, we calculated an inferred cell count by multiplying its Census-seq derived proportion by the total cell count in that replicate. Finally, we determined each donor’s viability in a given replicate by dividing its inferred cell count in that replicate by its average cell count in the three untreated control replicates.

Results

Villages are reproducible and capture cell intrinsic proliferation rates

Though cell villages are gaining in popularity, important questions remain regarding this approach, such as the extent to which pooling donors minimizes technical variation and whether cell non-autonomous factors (i.e., between-donor cellular interactions) influence phenotypes. To address these questions and validate villages for *in vitro* cell fitness assays, we measured the NPC proliferation rates of multiple donors cultured in village and array formats and quantified reproducibility across wells, batches, and formats.

First, we pooled hPSC-derived NPCs^{15,28} from 12 donors and plated them into three technical replicate wells (“Batch 1”) to measure well-to-well reproducibility. We then passaged the replicates independently every 5 days for 15 days, harvesting DNA at each passage. We collected 0.5X-coverage whole genome sequencing data from each sample for Census-seq, a computational tool that infers the donor composition of each replicate (Figure 1A-1B; Data S1)¹⁷. Donor proportions were highly correlated between well replicates within a batch (Figure 1C-1D; Spearman and Pearson $r > 0.996$). To test village batch reproducibility, we repeated the experiment on the same 12-donor NPC village constructed 4 days later (“Batch 2”) and found that donor proportion estimates were strongly correlated between Batch 1 and Batch 2 (Figure 1E-1F; $r > 0.939, p < 2.03 \cdot 10^{-17}$). Thus, although donor cell proportions evolve over time, their trajectories are consistent both within and between batches.

Co-culture with non-self cells (i.e., other donors) may influence a donor’s cellular behaviors through cell non-autonomous mechanisms⁴². To test the impact of pooled cultures on donor-level proliferation rates, we plated 12 NPC lines in a standard arrayed format as the reference group and compared their proliferation rates to village-based estimates (Figure 1G). Arrayed exponential proliferation rates for each donor were calculated using an imaging-based approach (Methods); village exponential proliferation rates for each donor were computed by multiplying their Census-seq-derived proportions by total village cell counts measured using an automated hemocytometer. Village and array donor proliferation rates were highly correlated (Figure 1H; $r > 0.895, p < 8.37 \cdot 10^{-5}$), suggesting that donors retain their cell-intrinsic proliferation dynamics in a village with minimal cell non-autonomous influence. Additionally, donor proliferation rates within a batch varied significantly less between village replicates than between arrayed replicates ($p = 5.26 \cdot 10^{-5}$; Figure 1I).

To test whether the reduced technical noise observed in villages reflected the pooled culture format rather than differences in measurement modality (microscopy versus sequencing), we measured proliferation by Census-seq in both arrayed and village cultures (Figure S1A). Twelve donor NPC lines were plated in parallel as (i) pooled villages and (ii) separately cultured wells in array. Cells were harvested from triplicate wells on days 1 and 5 after plating. For Census-seq profiling of the arrayed cultures, cells from all 12 donor wells were dissociated and pooled from each arrayed replicate immediately before DNA extraction to create three replicate “pseudo-villages.” Consistent with our microscopy-based comparison, Census-seq-derived growth rates were strongly concordant between the arrayed and village formats ($r > 0.928, p < 1.36 \cdot 10^{-5}$; Figure S1B-C) while village cultures reduced technical uncertainty in donor-level growth-rate estimates ($p = 3.69 \cdot 10^{-2}$; Figure S1D). These results indicate that villages preserve donor-intrinsic properties observed in arrayed culture while reducing technical variation, enabling scalable fitness measurements across genetically diverse donors in a shared culture environment.

Compositional models accurately estimate donor and group effects in villages

When one donor increases its representation in a village, other donors will decrease proportionally, even in the absence of cell non-autonomous biological influences. Donor proportions are measured using Census-seq, which infers a village’s composition from low-coverage whole genome sequencing¹⁷. However, compositional (proportion data which sums to one) data strongly violates the donor independence assumption of linear models due to the dependence of each dimension on the sum of the others. As such, we built Townlet, a hierarchical Dirichlet regression model that estimates individual donor and group proliferation effects in villages using compositional data measured by Census-seq over multiple time points (Figure 2A, Figure S2A-B). Additionally, Townlet’s time-varying, replicate-specific modeling approach is driven by the observation that uncertainty varies through time and replicates (Figure S2C-D). All effects are relative to a baseline donor (Methods, Figure S3A-C) as log-odds, with positive values indicating faster proliferation and negative values slower than the baseline.

We demonstrated the statistical accuracy of Townlet’s framework through simulation and analysis of the 12 donor NPC villages (Batch 1 & 2). Simulation shows that Townlet improves on the standard linear model’s discordant estimates, even when individual donor proliferation differs subtly (Figure 2B-2C). We inferred log-odds donor proliferation effect from each batch (Figure 2D) and modeled experiment dispersion per replicate (Figure 2E). The proliferation effect (β , Figure 2D and Methods) describes how quickly a donor’s relative abundance changes over time on a log scale. For example if donor 1 has a $\beta_1 = 0.2$ this means that, per unit time, the donor’s abundance grows $\sim 22\%$ faster ($\exp(\beta_1) = 1.221$) relative to a baseline donor with $\beta = 0$. The relatively small variance of effect estimates, despite only being fitted with three replicates, and the strong posterior mean correlation between batches (Figure 2F, Data S2; $r > 0.937, p < 6.99 \cdot 10^{-6}$) confirm the reproducibility of villages and the power of our model to estimate donor proliferation. No conclusive evidence of sex influences on proliferation were found across all batches (Figure S4A). We also compared the per-donor posterior means estimated by Townlet to the arrayed exponential growth rates. There was high correlation (Figure 2G, $r > 0.748, p < 0.007$) even though arrayed estimates were from only two time points, while village estimates were from seven.

We further evaluated Townlet’s ability to recover similar donor proliferation effects from repeat village experiments using predictive modeling. Using posterior predictive checks, thousands of new village experiments with the same design were simulated by sampling from the fitted model. The observed data was then compared to the simulated data to check if it fell into the bounds of the simulation. In both separately modeled batches, $> 95\%$ of the observed data fell within the 95% credible intervals of these new simulated distributions, thereby demonstrating accurate model estimation (Figure S4B-C). Additionally, we fit Batch 1 data and used it to predict the unobserved data from Batch 2 by simulating new villages from the fitted posteriors. Again, $> 65.7\%$ of the real experimental data from Batch 2 fell within these resulting posterior predictive distributions (Figure S4D). These results demonstrate the importance of using a compositional model such as Townlet when assessing village proliferation dynamics.

Townlet has enhanced power to detect factors that influence donor fitness

We performed a statistical power analysis to assess Townlet’s ability to recover meaningful biological signals from *in silico* villages compared to off-the-shelf approaches (e.g., linear, frequentist Dirichlet, and exponential growth models; Methods). Synthetic village data was generated using a simulation framework that we built using Townlet’s hierarchical Dirichlet regression model. Within this framework, we fixed each donor’s proliferation effects and generated new simulated village proportion data. We evaluated two model scenarios where we attempted to recover the simulated: (i) group proliferation effects (e.g. genetics, sex, ancestry, etc.) from villages under control conditions and (ii) treatment-specific donor proliferation effects.

To address issue (i), we ran hundreds of *in silico* experiments where we varied the number of donors, replicates, and sample time points. When trying to recover donor proliferation group effects, Townlet outperformed all other methods—even when effects were small—and it adequately controlled false positive rate while being robust to prior distribution specification choice (Figure 3A-3B; Figure S5A-H). As expected, increasing the number of donors had the strongest effect on sensitivity (Figure 3A); however, increased experiment length (e.g., beyond 3 cell passages) did not increase sensitivity, likely due to faster donors dominating later sampled time points (Figure S5C).

Townlet also performed strongly when evaluating treatment-specific donor proliferation effects (issue (ii)). We simulated a generic treatment experiment where 3 simulated doses were applied to villages in triplicate and sampled over 3 time points. Our modeling approach better controlled false discoveries while maintaining high power and accuracy to detect effects (Figure 3C-3D). These simulations demonstrate the improved statistical power of Townlet’s modeling approach across different village designs (e.g., number of donors, replicates, time points), driven by its ability to model donors jointly under compositional constraints rather than assuming independence. These advantages compound as the number of time points and treatment conditions increases, since the model leverages all observations simultaneously rather than collapsing the data into a limited set of statistical estimates. Finally, through simulation we demonstrated Townlet’s posterior stability even when composition is uncertain as donors approach zero (Figure S6A). With these results in mind, we applied the Townlet model on village-based fitness assays to map the genetics underlying NPC proliferation and vulnerability to environmental stress.

Townlet reveals neurodevelopmental disorder-relevant mechanisms

We sought to determine if our village design and Townlet model could capture disorder-relevant group differences in proliferation. Cortical overgrowth and macrocephaly are commonly observed in individuals diagnosed with autism spectrum disorders (ASD), and there is evidence that hyperproliferative populations of fetal NPCs contribute to these features^{43–46}. For instance, individuals harboring the heterozygous deletion of the 16p11.2 chromosomal region (16pdel) are at higher risk of presenting with ASD, intellectual disability, and enlarged cortical surface area^{47–49}. Some studies have reported increased NPC proliferation in 16pdel models while others have not^{50–54}. The lack of consensus in human *in vitro* 16pdel studies is potentially due to heterogeneity across proband lines, small donor sample sizes ($n = 3-4$ proband lines), and the technical variation of arrayed approaches that can collectively mask the likely subtle, yet disorder-relevant differences in NPC proliferation rates between control and proband populations.

To reconcile these inconsistent observations, we constructed a human induced pluripotent stem cell (hiPSC)-derived NPC village consisting of 11 neurotypical controls and 12 probands that we sampled across 3 passages for Census-seq analysis (Figure 4A). Village well-to-well replicate correlation was high, and again, variance was lower in villages compared to array cultures (Figure S7A-B). Using Townlet, we calculated individual donor proliferation effects and village dispersion (Figure 4B; Figure S7C; Data S3). We detected a significant increase in 16pdel NPC proliferation relative to controls ($lfsr = 0.012$; Figure 4C). When an interaction is added to include sex labels, there is little overlap in the male posteriors of deletion and control (Figure S7D). However, for deletion females, the signal is no longer significant. We caution overinterpretation of the female effect size as due to the availability of donors, we only include three female donors in the 16pdel group. The direction of this effect was consistent when we excluded the fastest 16pdel donor and the $lfsr$ remained low despite the small sample size ($lfsr = 0.098$, Figure S7E-G). Importantly, the individual donor proliferation effects estimated by Townlet correlated well with the individual proliferation rates estimated from arrayed cultures (Figure 4D, $r > 0.765, p < 2.11 \cdot 10^{-5}$). Further, deletion-control comparisons made from the arrayed data confirmed the significantly increased 16pdel NPC proliferation rates ($p = 0.034$, Figure 4E), as did an arrayed growth comparison between a 16p11.2 CRISPR-engineered deletion line and its isogenic control⁵⁵ ($p = 8.21 \cdot 10^{-5}$, Figure 4F). Human iPSC reprogramming technical factors including

facility, method, and cell type starting material did not significantly influence these results (Figure S7H-J), which is consistent with our recent work showing that these considerations have minimal impact on 16pdel hiPSC neuronal differentiation and electrophysiological properties⁵⁶. These results indicate that this rare copy number variant may contribute to macrocephaly through overgrowth of fetal NPC populations.

Common genetic loci associated with NPC proliferation

Complex traits are shaped not only by rare, large-effect mutations like 16p11.2 deletion, but also by common variants with small to modest impact. The genome-wide association study (GWAS) approach is routinely used to identify the genetic loci that have statistical relationships with human traits of interest, such as neuroanatomical features, behavioral tendencies, and risk for brain disorders^{57–59}. Human subject GWASs typically require thousands of participants due to the challenges with accurately quantifying traits and properly accounting for non-genetic factors and confounders (e.g., environmental exposures)^{60–62}. Remarkably, “GWAS-in-a-dish” experiments using arrayed culture designs have detected genome-wide significant loci for viral susceptibility^{15,63}, cell morphology⁶⁴, and drug response⁶⁵ from small sample sizes (~40-300 donors). Here, we leveraged villages and the improved sensitivity and accuracy of Townlet to nominate genomic loci associated with between-donor line differences in NPC proliferation.

We constructed a village of 39 hPSC-derived NPCs, harvested samples in triplicate at five time points (Day 0, 1, 5, 6, and 10; passaged at Day 5; Figure 5A) for Census-seq, and generated a single proliferation measurement per donor using Townlet. We excluded one donor from analysis that showed lower than expected representation at Day 0 (< 0.5%). Further, we detected three sibling pairs in the village via whole genome sequencing, so we randomly removed one donor from each pair to minimize the effects of relatedness on GWAS analysis (Figure S8A). Projection onto 1000 Genomes reference PCs showed that the 35-donor GWAS cohort clustered primarily with European ancestry reference samples, and within-cohort PCA showed no large outlier structure among retained donors (Figure S8B-D). Among the 35 remaining donors, proliferation varied considerably across donors, with effect sizes ranging from -0.506 to 0.598 (Figure 5B). We observed no significant effects of donor sex or NPC passage number (Figures S9A-B).

Using Townlet proliferation effect sizes as the phenotype and whole genome sequencing data collected from each individual donor, we ran a GWAS using a linear mixed model to adjust for population structure and relatedness among individuals⁶⁶. Analysis of 6,233,757 common variants (minor allele frequency > 5%) identified 70 suggestive SNPs that resolved into 21 independent loci ($\lambda_{GC} = 1.010$; $p < 1 \cdot 10^{-5}$; Figure 5C; Data S4). Given the relatively small number of samples compared to large sample size human subject GWASs, we selected the top 100 loci ranked by p-value for downstream analysis. These loci predominantly mapped to non-coding regions of 49 protein-coding genes, many of which are expressed in the human fetal brain and *in vitro* NPCs¹⁵ (Figure S9C). Notable genes include NPC proliferation modifiers *MYTL1*, *BBS2*, and *MSI2*, and gene ontology (GO) terms nervous system development (GO:0007399), regulation of stem cell proliferation (GO:0072091), and cell differentiation (GO:0030154) were significantly enriched after permutation testing (Figure S9D). The mapped gene set was significantly enriched for association with neurodevelopmental disorder risk and tumor suppression^{67–74} (Figure S9E) supporting the clinical importance of dysregulated NPC proliferation. Our analyses confirm that these *in vitro* GWAS loci regulate genes with established roles in human prenatal neurodevelopment.

The strongest proliferation-associated locus harbors 12 SNPs in Chromosome 16q22.3 that are in high linkage disequilibrium (LD; Figure 5D) and map to regulatory regions of the neurodevelopmental disorder-associated gene *ZFHX3*, which is a transcription factor that regulates neuronal differentiation and acts as a cell cycle inhibitor^{75–78}. *ZFHX3* ChIP-seq peaks sit predominantly on promoters of neurodevelopmental genes in human NPCs⁷⁵ and expression is enriched in early prenatal stages in humans and other species when symmetrically dividing NPCs are abundant (Figure 5E). The lead SNP rs34470044 (chr16:73153445T:TT, $p = 1.45 \cdot 10^{-7}$) is in a region upstream of *ZFHX3* that is characterized by high chromatin accessibility

and H3K27ac signals in hPSC-derived NPCs—but not hPSCs or post-mitotic neurons—and therefore shows hallmarks of a cell-type dependent enhancer (Figure 5D). In our sample set, genotype at rs34470044 explains 49.7% of the inter-donor line variation in NPC proliferation with T/T donors showing increased proliferation relative to T/TT donors ($\beta = -0.551$, in-sample $R^2 = 0.497$; Figure 5F). To evaluate this relationship, we calculated arrayed growth rates from a separate cohort of 112 hiPSC-derived NPC lines we generated and confirmed T/T donors proliferated significantly faster than T/TT donors ($p = 4.99 \cdot 10^{-3}$; Figure S9F). We further assessed the robustness of the rs34470044 association and found stable GWAS calibration across genotype-PC specifications, consistent effect direction across leave-one-donor-out and posterior-draw analyses, and stable in-sample R^2 estimates across resampling analyses (Figure S10A-D). Collectively, these results suggest that common genetic variants in *ZFHX3* regulatory regions may contribute to natural variation in cell-intrinsic human NPC proliferation rates.

Between-donor differences in susceptibility to neurotoxicants

We further extended the utility of our village cultures and Townlet model to capture and explain treatment-dependent changes in cell viability across many donors. The World Health Organization ranks the heavy metal lead (Pb) as a top chemical of major public health concern due to its pervasiveness and effects on human health^{79,80}. The fetal brain is particularly sensitive to Pb, and early life exposure shows a continuous dose-response relationship with adverse neurobehavioral outcomes⁸¹. Human subject genetic studies have nominated variants associated with elevated blood Pb levels⁸² and risk of negative cognitive outcomes⁸³, but neither the extent to which individuals differ in Pb sensitivity at the cellular level nor the genetics underlying differential cell-intrinsic risk have been explored.

We first confirmed that our *in vitro* model is susceptible to Pb by exposing a single donor and observing dose-dependent reductions in viability using the CellTiterGlo2.0 reagent (Figure S11A). We then treated the 39-donor NPC village with 0, 3 μ M (high physiological dose^{84,85}), and 10 μ M (supraphysiological dose) Pb concentrations and sampled 4 and 7 days later using Census-seq (Figure 6A)⁸⁶. After excluding the same four donors from downstream analysis for low initial representation and relatedness, we used Townlet to integrate compositional data across all time points and concentrations into a single distribution of effect sizes that reflects Pb sensitivity for each donor relative to a baseline donor (HS420, Figure 6B; Data S5). Donor Pb effect scores ranged from -0.52 (UM14-1) to 0.37 (ESI049), where more positive scores denote increased Pb resistance. Nine donors were significantly more resistant than the baseline control donor ($lfsr < 0.05$), and 11 donors were significantly more vulnerable ($lfsr < 0.05$). No sex or passage number effects were observed (Figure S11B-C). Dose-specific Pb effects calculated from Townlet showed similar between-donor variation (Figure S11D-E). An off-the-shelf, non-Dirichlet approach to calculate donor cell viabilities (Methods) confirmed the considerable between-donor variation in Pb response that ranged from 15.04% (donor: UM14-1) to 79.16% (donor: ESI049) viability one week after exposure (Figure S11F). These analyses reveal surprisingly high heterogeneity in cellular sensitivity to Pb across different genetic backgrounds and highlight the need to account for donor-intrinsic variability when performing toxicological risk assessments.

We performed a GWAS to identify common variants that explain between-donor variation in Pb effect scores ($\lambda_{GC} = 0.995$; Figure 6C). There was suggestive evidence for 13 loci (47 SNPs; $p < 1 \cdot 10^{-5}$; Figure 6C). The top 100 loci mapped to 64 protein-coding genes that were enriched in such GO terms as cellular response to xenobiotic stimulus (GO:0071466) and AIM2 inflammasome complex assembly (GO:0140970; Figure S11G). This gene list also significantly overlapped with risk genes for neurodevelopmental disorders, implying that heavy metal exposure may disturb brain development through similar molecular pathways that underlie these conditions (Figure S11H).

The top locus includes 2 SNPs in perfect LD (rs3833030 and rs3858973, GWAS $p = 2.59 \cdot 10^{-7}$) located in a divergent transcript of the brain-enriched neuroprotective transcription factor *ARNT2* (Figure 6D). Data

from the Genotype-Tissue Expression (GTEx; <https://gtexportal.org/home/>) project⁸⁷ identified rs3833030 as an expression quantitative trait locus (eQTL) for *ARNT2-DT* in human frontal cortex tissue (Figure 6E). In our data, genotype at rs3833030 explains 55.2% of the between-donor line variation in Pb vulnerability ($\beta=-0.250$, in-sample $R^2=0.552$; Figure 6F). This response was dependent on risk allele copy number: donors with zero copies (G/G) were most resistant, followed by heterozygotes (G/GG), and then homozygous carriers (GG/GG). GWAS calibration was again stable across genotype-PC specifications, and rs3833030 showed consistent effect direction and robust in-sample R^2 estimates across leave-one-donor-out analyses and Townlet posterior phenotype draws (Figure S12A-D). These results indicate that natural variation in *ARNT2*-mediated processes may contribute to donor differences in Pb-induced neurotoxicity.

Discussion

In vitro fitness assays are widely used to assess how cells proliferate, survive, and respond to changing environments. Most studies are performed on a few donor lines representing constrained genetic variation due to the limitations of conventional methods, many of which we overcome by taking a village-based approach. The co-culture of donors, however, introduces certain considerations that we address in this work. First, a common concern is the potential influence of non-self on *in vitro* donor line traits. We show that NPC villages reproducibly capture donor-intrinsic proliferation rates, indicating that non-cell autonomous influences are minimal in our assay. Though cell-cell communication is known to govern NPC proliferation,^{88–90} it is likely our cell culture strategy that involves plating NPC villages at 1/3rd normal plating density, passaging cultures before they are confluent, and exchanging media daily to dilute growth altering secreted factors reduces the effects of cell-cell interactions. We acknowledge that non-cell autonomous factors may play a larger role in other contexts and that additional work is needed to determine which other cell types and phenotypes are amenable to village-based assays. Regardless, these findings extend prior work from our group and others showing strong concordance between arrayed and village formats for Zika virus infection susceptibility¹⁵ and gene expression profiling^{16,91}.

Second, we present a Bayesian Dirichlet model named Townlet for time-series cell fitness analysis that models the between-donor dependencies expected in Census-seq data. To address the limited number of replicates common in genomic experiments, Townlet integrates information via multi-dimensional shrinkage across donors, time points, and treatments to provide robust, low-variance estimates.

With these tools, we performed proband-control comparisons to understand the connection between 16p11.2 deletion and macrocephaly. Recent human *in vitro* studies have suggested overgrowth of NPC populations associated with 16pdel; one group reported proband stem cell-derived NPC hyperproliferation ($n = 2$ donors) using cell counting and EdU incorporation assays⁵³, while another found enlarged neural rosettes in ventral organoids generated from a CRISPR-engineered deletion stem cell line⁵⁴. Other studies found no differences in NPC cell cycle dynamics, and instead nominated enlarged soma size, increased dendritic area, and reduced phagocytosis as contributing factors to brain overgrowth in probands^{50,51,53,92,93}. Our village analysis—which represents the largest 16pdel *in vitro* study to date in terms of donor sample size—indicates that overgrowth of early fetal NPC pools contributes to macrocephaly. Previous studies have reported NPC hyperproliferation in human *in vitro* ASD models^{24,94,95}, suggesting this may be a node of biological convergence across genetic risk factors. Our results and others⁹⁶ highlight the utility of villages for future proband-control comparisons.

Our focus on a single cell type, combined with low technical noise and a refined computational model, likely enabled us to nominate strong de novo associations using cell villages. In a 35-donor GWAS of proliferation, we identified a strong suggestive association at rs34470044, which maps to a distal enhancer of *ZFHX3* and experimentally replicated in an independent arrayed cohort. In the rodent brain, *ZFHX3* overexpression results in NPC cell cycle arrest, whereas low nuclear protein abundance is characteristic of proliferating NPCs in the ventricular zone⁷⁶. Our outcomes suggest *ZFHX3* influences naturally occurring

variation in proliferation dynamics across human donors, which could have wider implications on diversity in brain traits like cortical surface area and cognition⁹⁷. Future studies utilizing allele-specific enhancer perturbations are required to validate this genotype-phenotype relationship.

We also nominate genetic modifiers of cell-intrinsic vulnerabilities to environmental exposures, which is an area where human subject GWASs have largely fallen short due to limited cellular resolution and reliance on imprecise exposure estimates (e.g., zip code-level data)^{62,98}. In our study, the most significant Pb-associated locus was located upstream of *ARNT2* (index SNP: rs3833030). ARNT2 forms heterodimers with bHLH-PAS sensor proteins like HIF-1 α to regulate gene expression in response to environmental stimuli such as oxidative stress and hypoxia⁹⁹. Although ARNT2 has not been previously studied in Pb response, our data suggests it may protect cells from the pseudo-hypoxic state and reactive oxygen species production observed in Pb-exposed neural cells¹⁰⁰. Functional validation using CRISPR activation or inhibition of *ARNT2* is needed to evaluate its potential role as a neuroprotective target for mitigating heavy metal cytotoxicity. Overall, our work paves the way for future village-based efforts to dissect cell-intrinsic vulnerabilities to the thousands of chemicals that comprise the human exposome^{101,102}.

These GWAS findings should be interpreted in the context of a 35-donor discovery cohort, where the estimated effect sizes of genetic variants may be falsely inflated (i.e., “Winner’s Curse”)¹⁰³. We therefore evaluated robustness using leave-one-donor-out analyses, Townlet posterior phenotype draws, and phenotype-permutation nulls. These analyses supported the stability of the top proliferation and Pb associations within this dataset and help frame how effect sizes from low-sample-size village GWASs should be interpreted. Village GWASs of donor-level cellular phenotypes also differ from large human-subject GWASs in an important way: phenotypes are measured in controlled *in vitro* environments, where environmental heterogeneity is reduced and measured traits may be closer to the cellular mechanisms through which variants act. This design can make village GWASs a powerful discovery framework, but smaller donor numbers warrant sensitivity analyses, permutation-based calibration, and cautious effect-size interpretation. Larger, more diverse, and independent village cohorts will help clarify expected effect sizes and determine whether candidate associations generalize.

Our study aims to push the boundaries of village experiments and empower future efforts to explain how human genetic variation confers phenotypic diversity in both healthy and disease states. Several additional improvements to our system could facilitate this vision. First, use of cell culture robotics could increase the number of donors and ancestries represented in a village, which would increase statistical power. Second, as villages scale to hundreds of donors, approaches such as combinatorial pooling or meta-analysis of multiple villages will be critical to maintain power and reproducibility, and should be systematically evaluated through both experimentation and simulation¹⁹. Third, more comprehensive simulation frameworks, akin to those used in single-cell transcriptomics¹⁰⁴, could help optimize experimental design by clarifying how parameters such as donor diversity, culture duration, and environmental perturbations influence sensitivity. Finally, extensions of Townlet that enable proper analysis of FACS-based phenotypes, as we recently reported using off-the-shelf tools¹⁵, could increase the number of phenotypes that can be assayed in a village and expand opportunities for discovery.

Data and code availability

Data availability

Raw low-coverage whole genome sequencing data used for Census-seq input can be accessed at the database of Genotypes and Phenotypes (dbGaP) accession number phs004608.v1.p1. The Census-seq readouts used as input to the Townlet model can be found in the Supplementary Data Files. Users can apply for controlled access to previously generated genotype data (VCF files) for the hESC donors (e.g., Proliferation and lead GWAS villages) at <https://duos.broadinstitute.org> (Accession number DUOS-000121). Once approved,

the data can be downloaded from this Terra workspace: https://app.terra.bio/#workspaces/convergneuro-mccarroll-anvil/Broad.ConvergentNeuro.McCarroll_Nehme_hESC_HMB_VillageData. Genotype data for CIRM donor lines (used as 16pdel village controls) can be accessed at dbGAP (accession number phs002032.v2.p1). Genotype data for 16pdel donor 14746.x8 can be accessed at SFARI Base using Data ID DS0000127: <https://base.sfari.org/dataset/DS0000127>. All other 16pdel donor genotypes can be requested from SFARI except for NFID-0176, which can be requested from Olli Pietiläinen (olli.pietilainen@helsinki.fi).

Code availability

Townlet is available to run as an R package and can be installed from <https://github.com/pimentellab/townlet>. Code used for simulations and Townlet analyses can be found at https://github.com/hansonch/townlet_analysis.

Declarations of Interests

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies

During the preparation of this work, the authors used ChatGPT from OpenAI to consolidate text. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Author contributions

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Supplementary Information

Document S1. Figures S1–S12

Table S1: Full list of human pluripotent stem cell lines used for each experiment.

Table S2: Meta-data for human pluripotent stem cell lines.

Data S1: Census-seq outputs for village-based fitness assay validation.
 Data S2: Townlet proliferation effect scores for village-based fitness assay validation.
 Data S3: 16p11.2del village Census-seq and Townlet output.
 Data S4: Proliferation GWAS.
 Data S5: Lead GWAS.

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Figure Legends

Figure 1 Validation of village-based NPC fitness assay (A) Schematic of cell village workflow. Human pluripotent stem cells from 12 donors were induced to NPCs separately in an arrayed format prior to pooling (Passage 0; P0) and plating. Village cells were harvested in triplicate for Census-seq at each of the three passages. (B) Census-seq workflow. DNA from frozen cell pellets is sequenced at low (0.5X) coverage. Census-seq estimates the proportion of each donor's DNA in a sample via expectation-maximization, and growth rates are calculated for each donor based on their changing proportions over time. (C) Census-seq donor proportion estimates from a 12-donor NPC village at P0 and three passages (P1-P3). Individual donor proportions are represented with different colors. (D) Donor proportions from 12-donor NPC village are highly correlated between replicates within a batch (Well 2 vs Well 1, $p < 3.69 \cdot 10^{-39}$, Pearson $r = 0.998$, Spearman $r = 0.997$; Well 3 vs Well 1, $p < 1.63 \cdot 10^{-37}$, Pearson $r = 0.997$, Spearman $r = 0.996$) (E) Batch comparison experimental design. A 12-donor NPC village was constructed (Batch 1). Four days later, the same 12 donors were pooled into a village (Batch 2). (F) Donor proportions are strongly correlated between batches. Each data point represents a donor's mean proportion at a given passage (Pearson $r = 0.941$, Spearman $r = 0.939$, $p < 2.03 \cdot 10^{-17}$; biological $n = 12$ donors; technical $n = 3$ per time point). (G) Village-array comparison. Twelve donor NPC lines were pooled into a village or plated separately in an arrayed format for fluorescent-based cell counting and growth rate analysis. (H) Donor intrinsic growth rates are maintained in a village. Each data point represents a donor's mean exponential growth rate (continuous, % per day) in both a village (Passage 1, Day 1 vs. Day 5) and arrayed (Passage 1, Day 1 vs. Day 7) format. (Pearson $r = 0.909$, Spearman $r = 0.895$, $p < 8.37 \cdot 10^{-5}$; biological $n = 12$ donors; technical $n = 3$ village wells, technical $n = 10$ wells per donor in the array). (I) Villages reduce within-donor variation. Donor growth rates are normalized (for the village, within each well ($n=3$ wells). For the array, within each column ($n=10$ wells/columns per donor)) to a mean of 0 and standard deviation of 1. Each data point represents the standard deviation across replicates of a donor's normalized growth rates (biological $n = 12$ donors; paired t-test $p = 5.26 \cdot 10^{-5}$).

Figure 2 Hierarchical Dirichlet regression for inferring village proliferation and viability (A) Schematic of Townlet. Inputs include village composition data estimated from the Census-seq pipeline and user defined donor group and sample level predictors. Townlet's outputs infer significant differences in donor and group proliferation across all treatments and replicate specific technical variation over time. (B) Simulated 10 donor village composition data where all donors have the same initial growth rate, but the donors at the top (unboxed) around them have an additional positive shared group effect that increases their proliferation. (C) Townlet has higher sensitivity compared to other frequentist models (EGE = exponential growth equation, FreqDir = frequentist Dirichlet, LM = linear model) even under the simple assumptions outlined in b when simulated group effects are small (< 0.2). (D) Proliferation effects estimated from 12 donor villages by Townlet relative to baseline donor RUES1 (dashed line). Split violin shows batch 1 (left) and 2 (right). (E) Overdispersion (technical variation) estimates over time colored by replicate from our 12 donor (Batch 1) experiment. As our model parameter theta decreases, dispersion increases; therefore, in this experiment, replicate technical variation increased consistently over time. (F) Correlation between Batches 1 and 2 donor proliferation effects randomly drawn from posteriors. Dashed lines show high correlation (> 0.93) of donor proliferation expectation values. (G) Townlet's estimated donor proliferation effects correlate strongly with array-based proliferation rates estimated using the exponential growth equation. Imaging-based array growth rates were estimated from passage 1, days 1 and 7 data and Townlet relative proliferation effects were fit from all passage 1-3 sample time points.

Figure 3 Townlet has higher power to detect proliferation effects compared to frequentist approaches (A) Townlet has higher sensitivity, relative to other benchmarked models (FreqDir = frequentist Dirichlet, EGE = exponential growth equation, and LM = linear model), when performing inference on donor group effects (e.g. genetics, sex, ancestry, etc. in control conditions with no additional environmental perturbations; see benchmarking Methods) using *in silico* village data (with $R=3$ replicates, $T=3$ sample time points) and varying donor numbers ($D=1, 50, 100$). Increasing the number of donors in a village has the greatest influence on sensitivity. (B) Specificity is also high across simulated village data with varying numbers of donors. (C) Townlet has a much lower false discovery rate when detecting individual donor treatment effects compared to ANOVA based approaches. (D) Density plot showing correlation between simulated donor treatment effects and those estimated by Townlet. Note that 50% of the donors had their treatment effect simulated as zero and the remaining donor's effects were drawn from a $U(-0.6, 0.6)$.

Figure 4 16p11.2 deletion NPCs are hyperproliferative

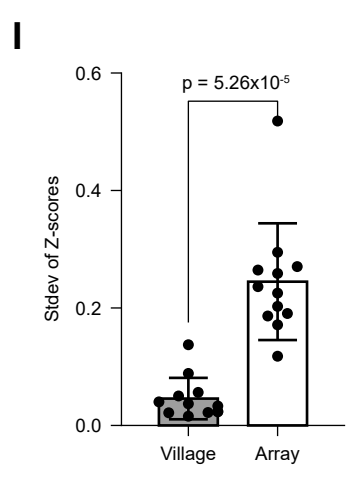
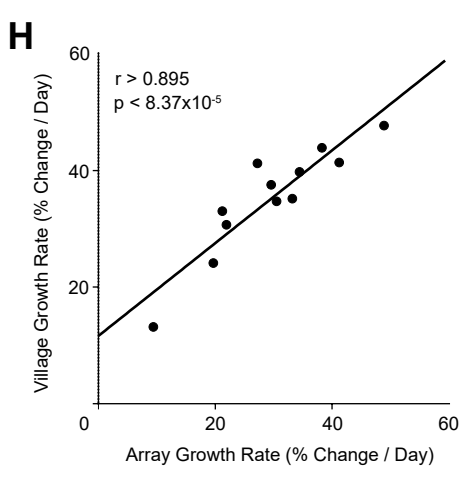
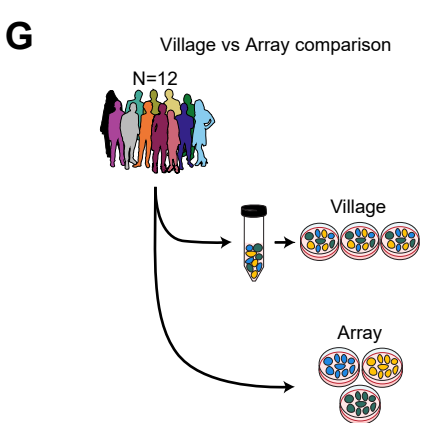
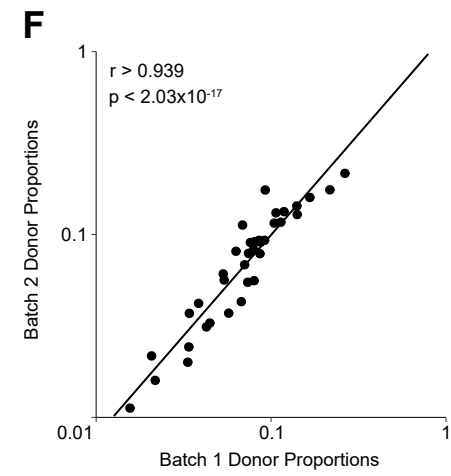
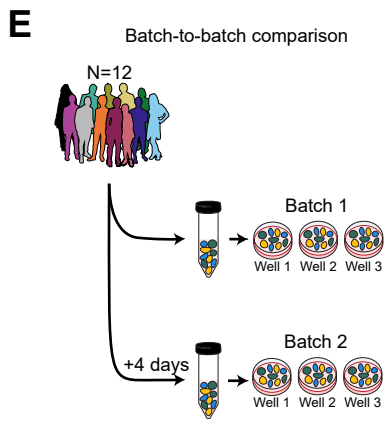
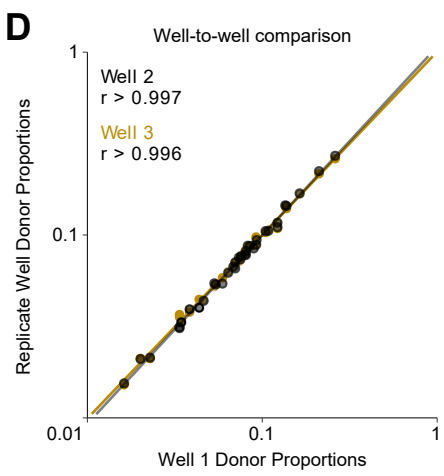
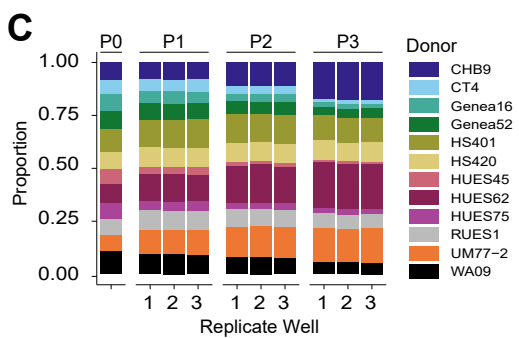
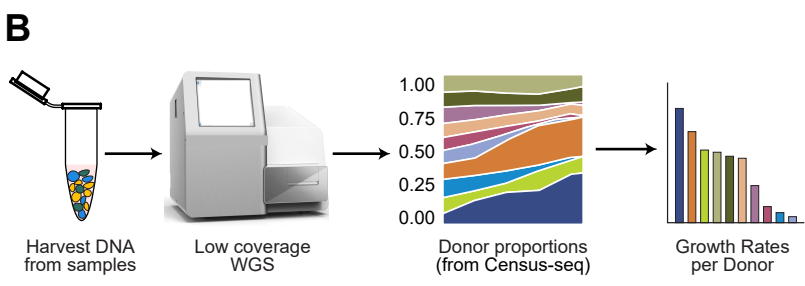
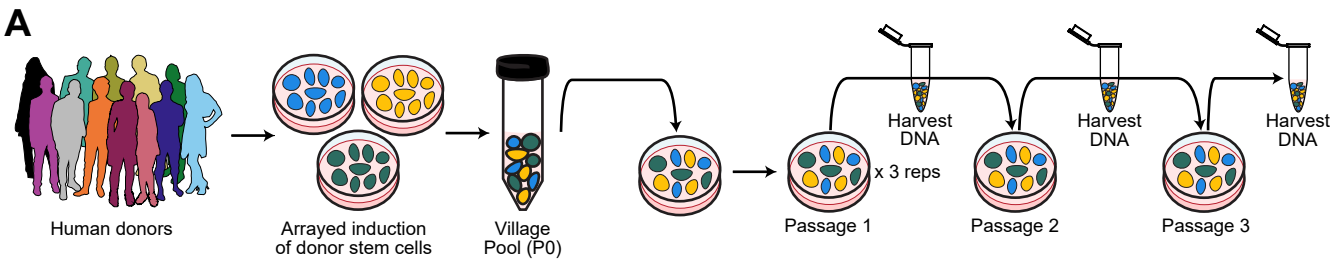
(A) Design of 16p11.2 deletion village. Twelve proband and eleven control NPC lines were pooled and plated in triplicate prior to harvest over three passages. (B) Total donor proliferation effects estimated from Townlet colored by donor deletion status relative to baseline donor CW20069 (represented as dashed line). All posteriors include the mean (point) and 95% credible interval error bars. (C) 16pdel proband group grew significantly faster than controls as shown by fitted posterior distribution being well above zero ($lfsr = 0.012$). (D) Proliferation effects from Townlet correlated strongly with exponential growth rates estimated from arrayed plates prior to pooling ($n = 23$ lines. Pearson $r = 0.765$, Spearman $r = 0.768$). (E) Growth rates estimated from arrays confirm 16pdel hyperproliferation. Mean $\pm$ s.e.m. (Welch's t-test, $p = 0.034$). (F) CRISPR-engineered 16p11.2 deletion line grew significantly faster than isogenic control. Mean $\pm$ s.e.m. ($n = 5$ wells per line; Welch's t-test, $p = 8.21 \cdot 10^{-5}$).

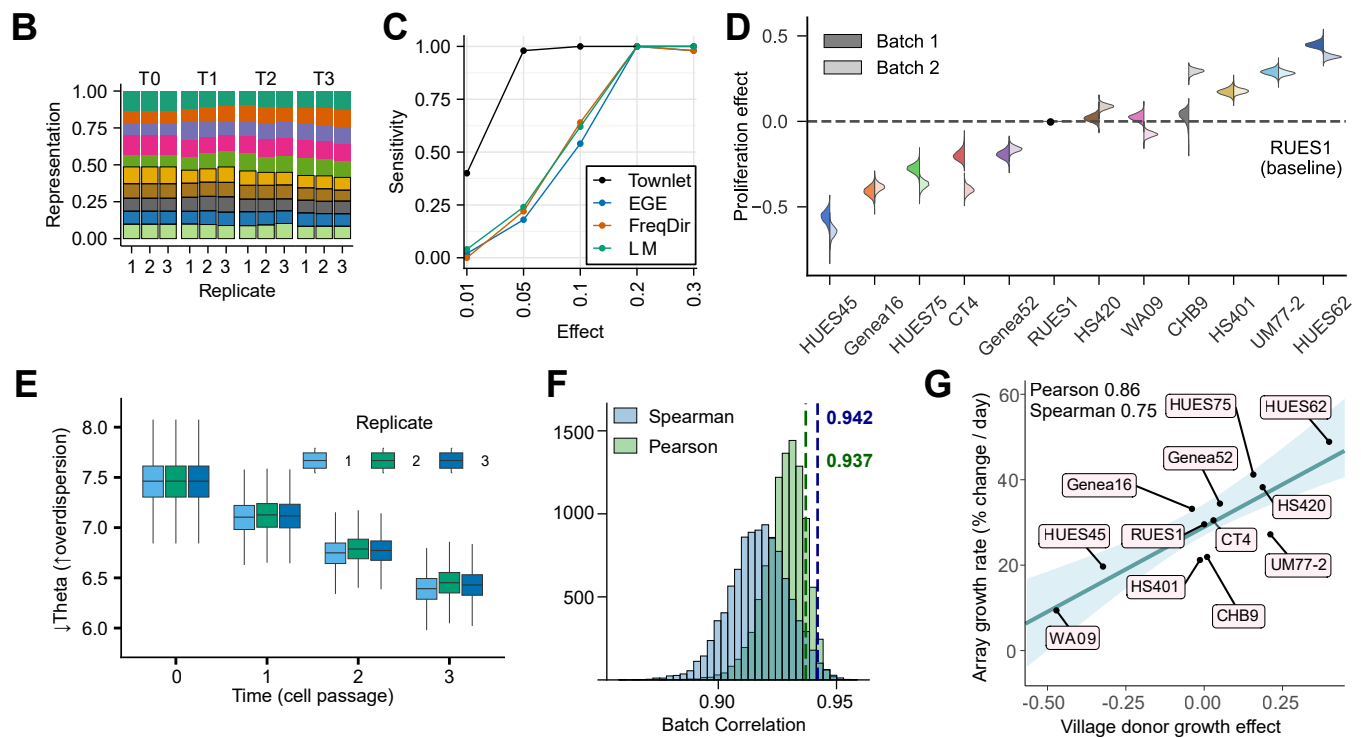
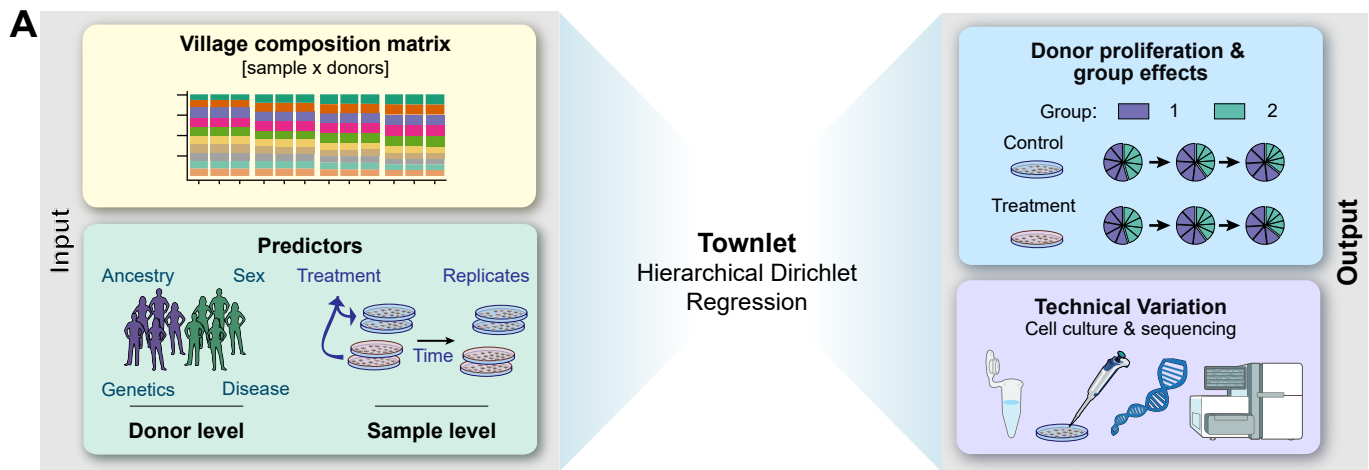
Figure 5 Common variants in *ZFHX3* gene are associated with NPC proliferation

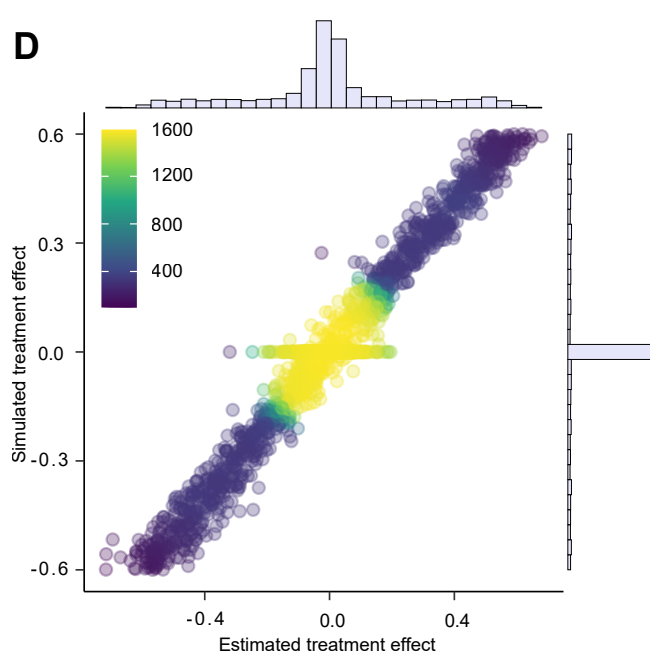
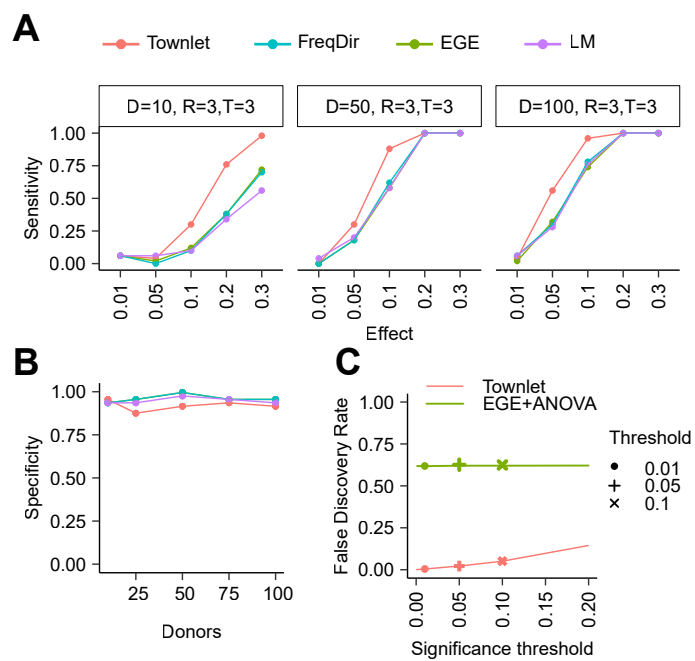
(A) Thirty-nine NPC lines were pooled into a village, plated in triplicate, and sampled on Days 0, 1, 5, 6, and 10. Four donors were bioinformatically removed prior to downstream analysis. (B) Total proliferation effect posteriors estimated for the 35 donors grown in control conditions relative to the baseline donor HUES49 (points = means, error bar = 95% credible intervals). (C) Manhattan plot of genome-wide association scan for donor proliferation effects. Y-axis denotes $-\log_{10}(p\text{-value})$ from GEMMA Wald tests of SNP associations with the proliferation effect posteriors (biological $n=35$). Genome-wide significance threshold ($p < 5 \cdot 10^{-8}$) marked by red dashed line. Blue dashed line shows threshold for suggestive evidence ($p < 1 \cdot 10^{-5}$). (D) Regulatory context at the *ZFHX3* locus. (Top) Genomic locus with RefSeq isoform tracks³⁵. GeneHancer Double Elite interaction region (thin horizontal black line), shown from the UCSC Genome Browser track, indicate high-confidence regulatory element-gene associations (black boxes along black line); the displayed interaction links GH16J073152 to *ZFHX3*³⁶. (Bottom) Enlarged view of the blue-highlighted 25 kb region showing local epigenomic data. Chromatin state annotations from Roadmap Epigenomics Project (<https://egg2.wustl.edu/roadmap/web-portal/>)^{37,38} highlight predicted enhancers (yellow) and weak transcription regions (green) in hPSC-derived NPCs. DNase-seq and H3K27ac signal tracks indicate cell type-specific enhancer activity in NPCs³⁹. GWAS results were plotted using LocusZoom⁴¹, with dot color indicating LD (r^2) to the index SNP rs34470044 (chr16:73153445T:TT, $p = 1.45 \cdot 10^{-7}$; purple diamond, red dashed line). The locus contains 11 SNPs in near-perfect LD with the index SNP (red triangles). ESC = embryonic stem cells; GH = GeneHancer; TSS = transcription start site. (E) Developmental expression ($\log_2$ RPKM, color heat scale) for *ZFHX3*, *MSI2*, and *MYT1L* across 11 human brain stages ranging from 10 post-conception weeks to adulthood. Data from Brainspan (<https://www.brainspan.org/>)¹⁰⁵. (F) Association of rs34470044 donor genotype with proliferation effect. The solid line shows the linear regression with a shaded band indicating the 95% confidence interval (in-sample $R^2 = 0.497$).

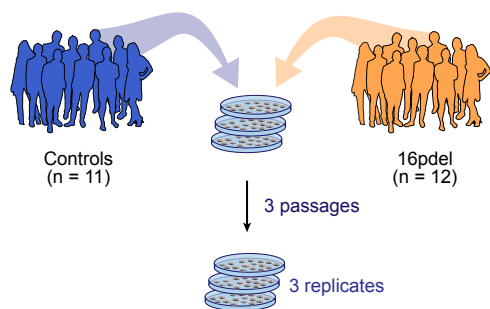
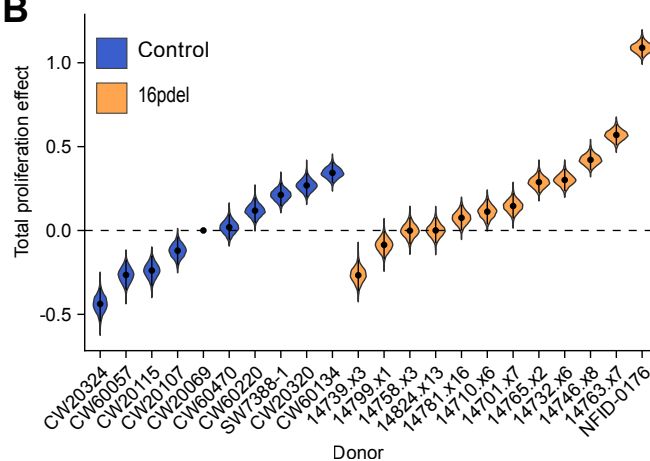
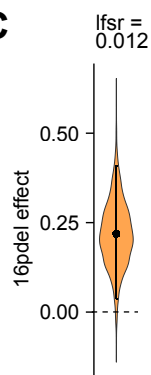
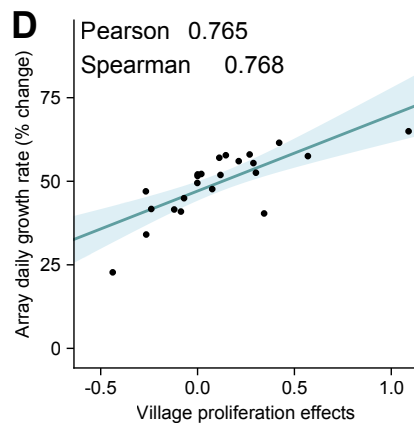
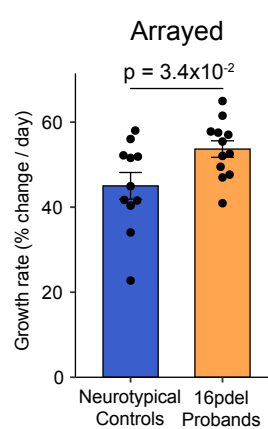
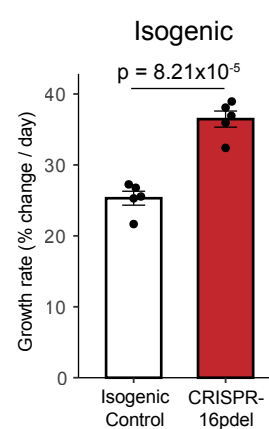
Figure 6 GWAS-in-a-dish analysis of lead (Pb) sensitivity

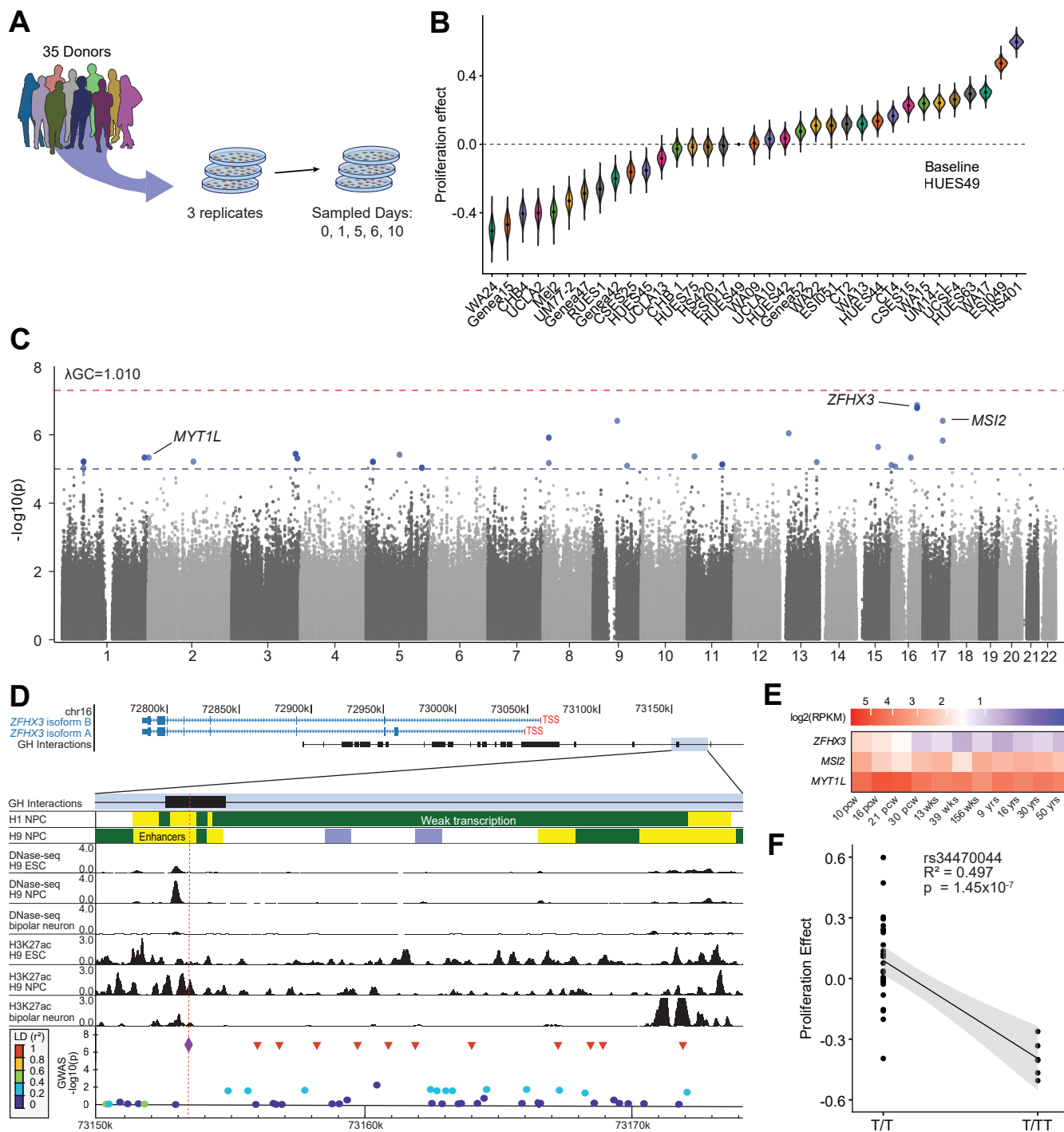
(A) Schematic of Pb exposure experiment. Thirty-nine NPC lines were pooled into a village and plated in triplicate for each dose of Pb treatment (0, 3, or 10 μM). Cells were harvested on Days 4 and 7 post-exposure for Census-seq. Four donors were bioinformatically removed prior to downstream analysis. (B) Donor Pb effects estimated by Townlet across all treatment doses relative to baseline donor HS420. Donors with white diamonds (posterior means) significantly differ from the baseline donor. More positive values denote increased resistance to Pb. (C) Manhattan plot. Y-axis denotes $-\log_{10}(p\text{-value})$ from GEMMA Wald tests of SNP associations with Pb effect posteriors (biological $n = 35$). Red line: Genome-wide significance ($p < 5 \cdot 10^{-8}$). Blue line: Suggestive significance ($p < 1 \cdot 10^{-5}$). The top association maps to a regulatory divergent transcript of *ARNT2*. (D) Regulatory context at the *ARNT2* locus. (Top) Genomic locus with RefSeq isoform tracks³⁵. (Bottom) Enlarged 17 kb region with local epigenomic data. The vertical red dashed lines mark the top SNPs rs3833030 (chr15:80376573G:GG, $p = 2.59 \cdot 10^{-7}$, purple diamond) and rs3858973 ($p = 2.59 \cdot 10^{-7}$, red triangle). rs3833030 resides 125 basepairs downstream of the transcription start site for a noncoding transcript, within the coding region of one *ARNT2-DT* isoform, and within the intron of another *ARNT2-DT* isoform. Roadmap Epigenomics chromatin state predictions are shown^{37,38} (yellow = predicted enhancer, green = weak transcription regions, red = active promoter) in hPSC-derived NPCs. DNase-seq and H3K27ac signal tracks indicate cell type-specific enhancer activity in NPCs³⁹. GWAS results were plotted using LocusZoom⁴¹, with dot color indicating LD (r^2) to rs3833030. (E) rs3833030 is an eQTL for *ARNT2-DT* in human frontal brain cortex. Violin plots show normalized expression grouped by genotype ($p = 1.82 \cdot 10^{-6}$, data from GTEx)⁸⁷. Black boxes indicate expression quartiles and the inner white line marks the median expression per genotype. (F) Association of donor genotype at rs3833030 with Pb effects estimated by Townlet. Solid line shows linear regression with shaded 95% confidence interval (in-sample $R^2 = 0.552$).

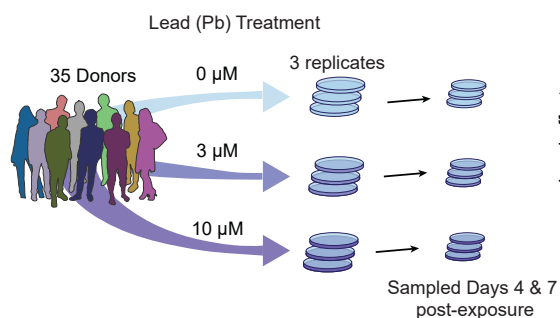
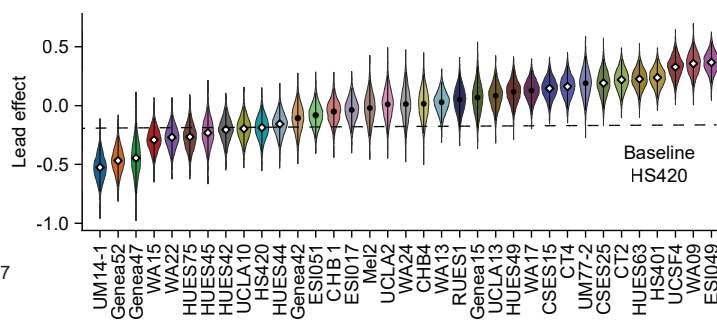
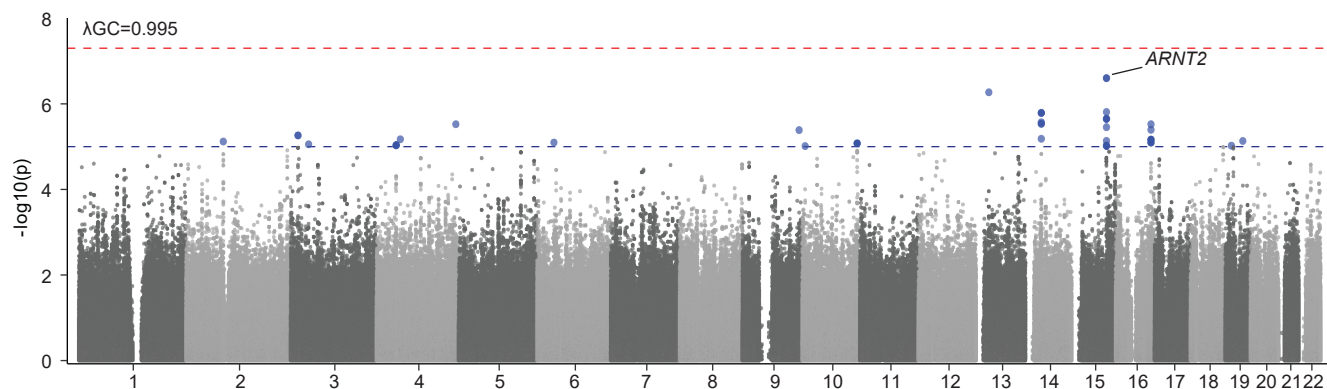
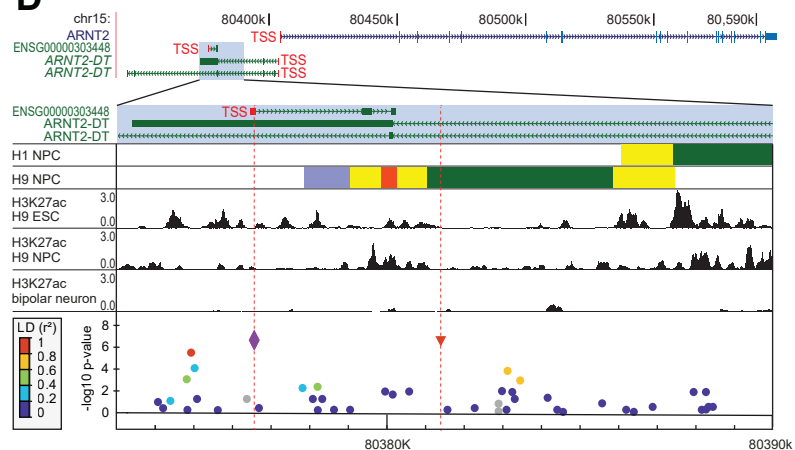
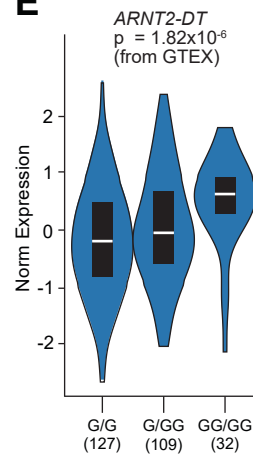
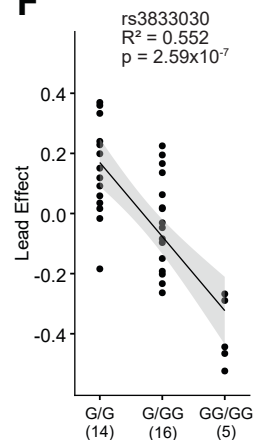






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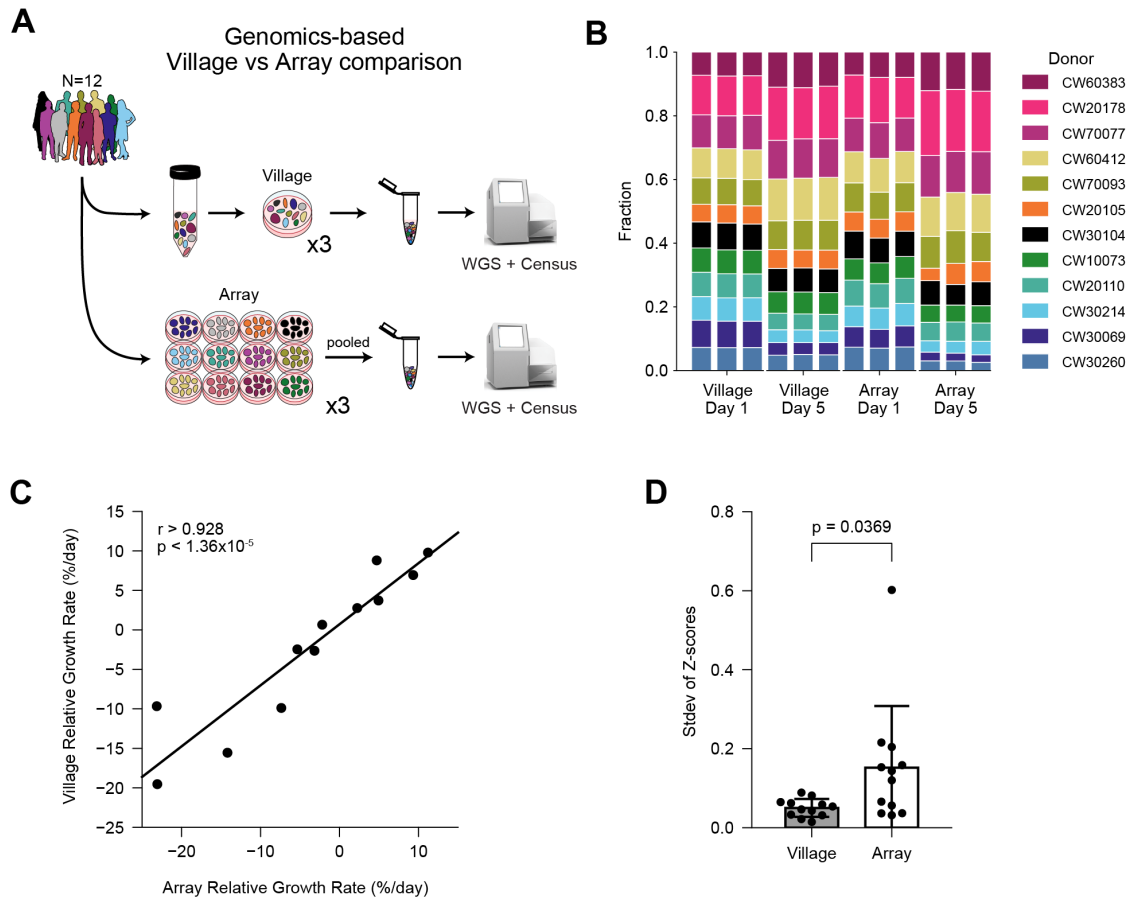


Figure S1 Census-seq comparison of village and arrayed growth

(A) Experimental design comparing the same 12 donors grown as pooled villages or separately in arrayed culture. (B) Census-seq donor fraction estimates across triplicate samples for each culture format and time point. Colors indicate donors. (C) Donor relative growth rates measured in village and arrayed culture formats. Growth rates were calculated as the day 1 to day 5 change in log donor fraction, expressed as %/day. Points represent donors; line shows linear fit (Pearson $r = 0.928$, Spearman $r = 0.930$, $p < 1.36 \cdot 10^{-5}$; biological $n = 12$ donors; technical $n = 3$ village samples and $n = 3$ array pseudovillage samples per time point). (D) Villages reduce uncertainty in donor growth estimates. Donor growth rates are normalized (for the village, within each well ($n=3$ wells)). For the array, within each pseudovillage ($n=3$ plates)) to a mean of 0 and standard deviation of 1. Each data point represents the standard deviation across replicates of a donor's normalized growth rates (biological $n = 12$ donors; paired t-test $p = 3.69 \cdot 10^{-2}$).

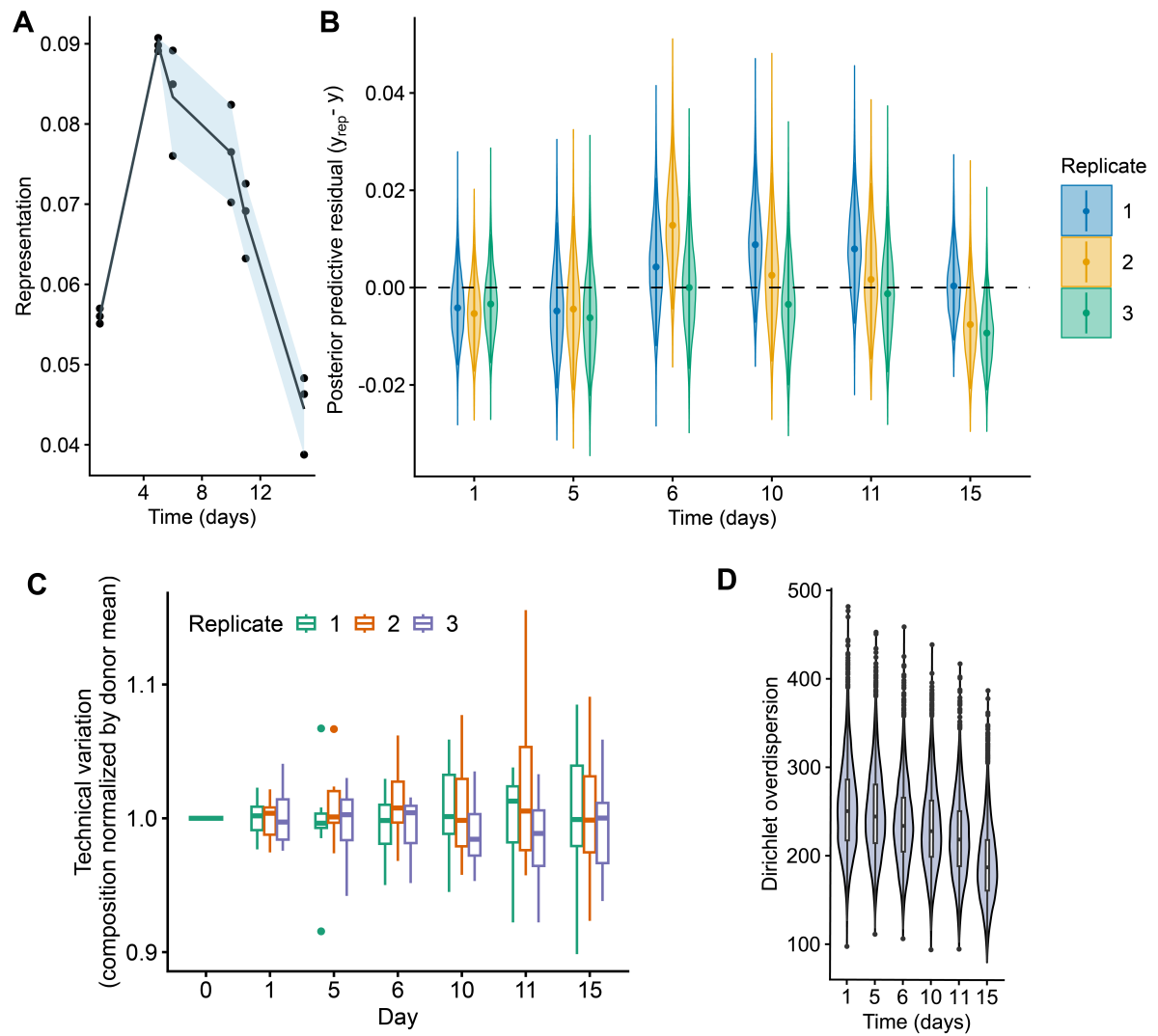


Figure S2 Townlet's statistical assumptions are flexible and data informed

Supplemental analyses further evaluate the statistical assumptions and modeling framework underlying Townlet across village composition experiments. These results demonstrate that the linear model assumptions flexibly captures donor dynamics while appropriately accounting for changing technical variation over time. (A) Townlet can fit village data where donors have nonlinear compositional trajectories. A single average compositional trajectory (line) from donor 14763.x7 from the 16pdel village experiment (Figure 4A) with standard error bars shaded across 6 time points and three replicates (points). (B) Posterior predictive residuals from donor 14763.x7 shown in panel A after fitting full village compositional data with Townlet. Each replicate (color) and time points residual violin show strong overlap with zero (residual mean=point, errorbar=95% credible interval). This demonstrates Townlet's flexibility to successfully fit nonlinear trajectories. (C) Technical variation in village experiments tend to increase over passage time points justifying the use of the linear approximation of dispersion. Village compositional data from the 12 donor batch 3 experiment (Figure 2G) were normalized by grouping by donor and time and then dividing by each donor's mean composition across the three replicates. The boxplots show an increase in the level of technical variation over time, which motivated our implementation of a linear dispersion model. (D) To further assess our linear dispersion assumption, we fit each compositional time point independently (data from panel C) using a simplified hierarchical Dirichlet model with a single dispersion parameter, confirming that technical variation increases over time, as reflected by a decrease in the overdispersion parameter posteriors.

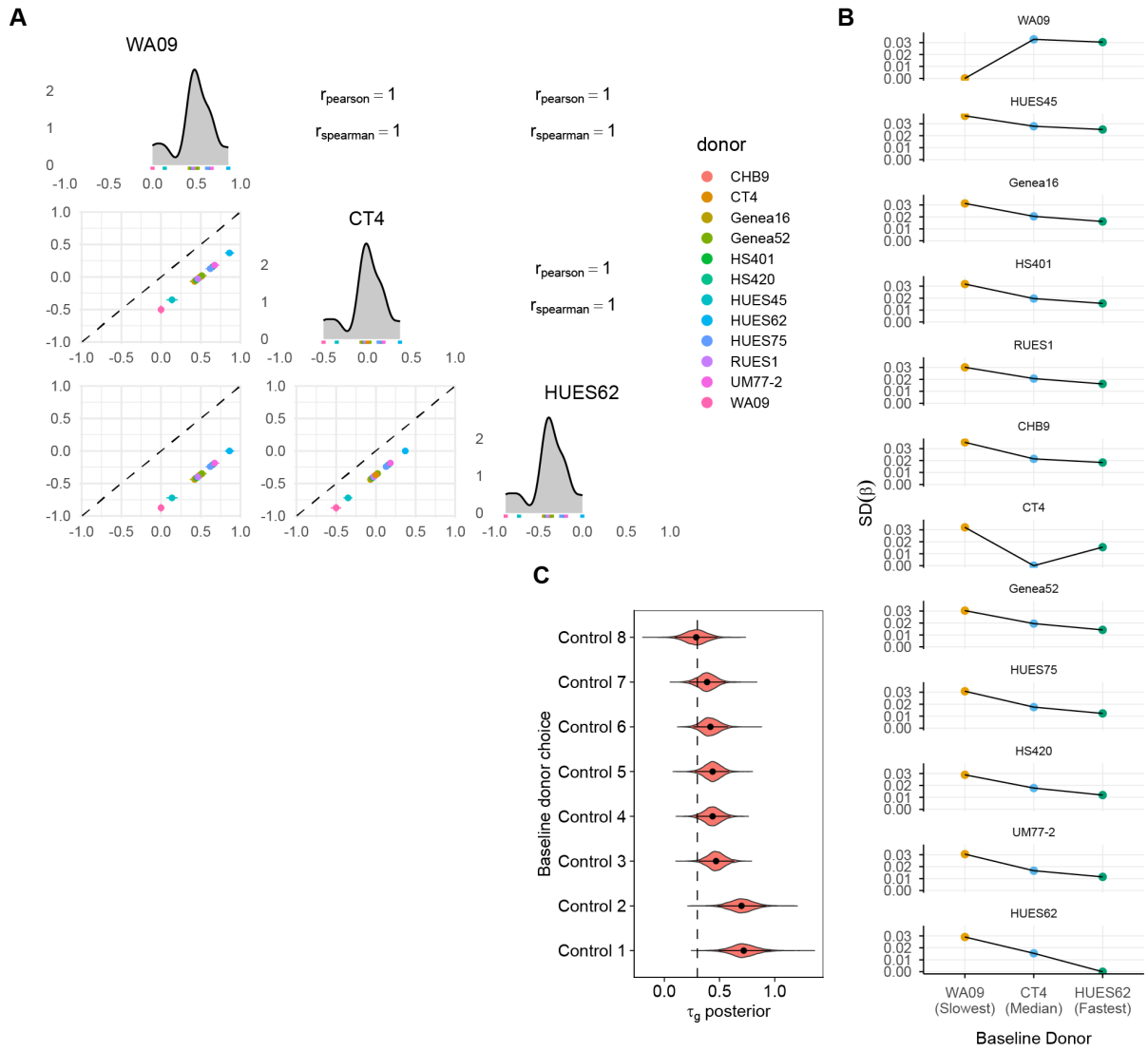


Figure S3 Baseline donor choice influences inference

Supplemental analyses evaluate how baseline donor selection influences parameter estimation and uncertainty within the Townlet modeling framework. These results show that while relative donor rankings remain robust across baseline choices, inference on donor effects is most interpretable and centered when the baseline donor is selected near the median of the control reference population. (A) We ran Townlet on using the compositional data from figure 2G without inference on group effects and found that the relative rank of donor proliferation effects is invariant to the choice of baseline donor. This result was consistent when any of the donors were chosen as the baseline and the correlation between the slowest (WA09), median (CT4) and fastest (HUES62) donors were summarized here. (B) Posterior uncertainty depends on the choice of baseline. Selecting the slowest (WA09) or fastest (HUES62) donor can respectively inflate or shrink the estimated standard deviation relative to the median donor (CT4). We recommend using a median grower as the baseline. (C) Baseline choice is especially consequential when estimating donor group effects. It should be selected from the median of the control reference group; otherwise, effect sizes of τ_g (violin posteriors) may be inflated or attenuated if slower or faster reference donors are used as the baseline. In this plot we fit simulated data with Townlet to estimate group effect (x-axis, τ_g) under different baseline donors (y-axis). The simulated true group effect (dashed line) is strongly overestimated by Townlet when the slowest control donors (1 & 2) are chosen as the baseline, while the other donors are able to capture the true effect within their 95% credible intervals. Control donors 1-8 are ranked from slowest to fastest. Control 3-8 all have similar enough growth rates that they capture the true signal; however, under different simulated data it is possible that Control 7-8 donors could have significantly faster rates than the median leading to inaccurate shrinking of our τ_g effect.

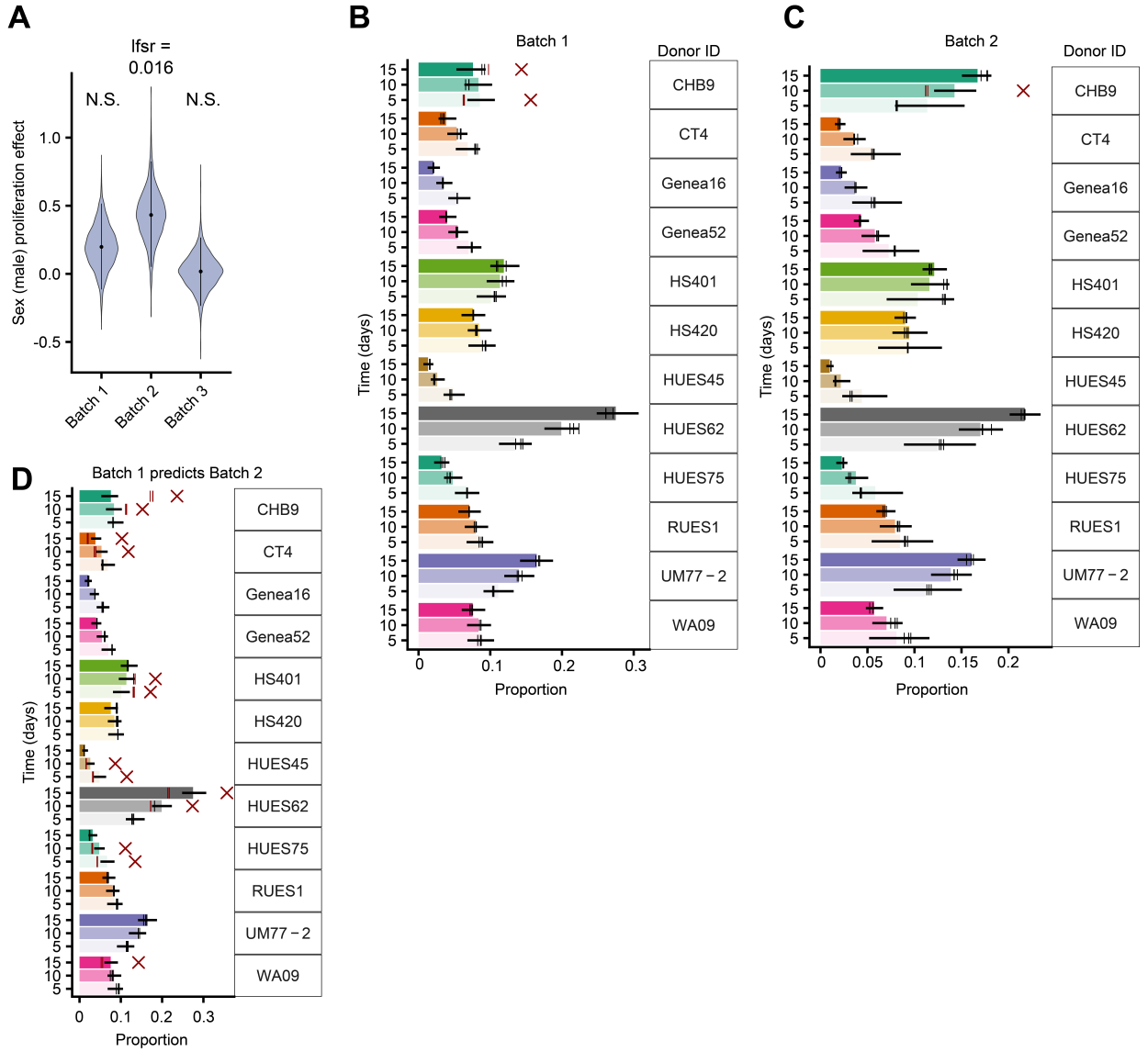


Figure S4 Townlet sex effects and posterior predictive checks

(A) The 12 donor proliferation batch experiments show inconclusive evidence of sex effect with only batch 2 showing significantly faster proliferation in males ($lfsr = 0.016$). (B-C) Posterior predictive checks simulate a distribution of representation values per donor and sample using the model fit (bars represent distribution mean with 95% credible interval error bars). We were able to recover > 95% of the training data (tick marks, black) within these distributions in a, Batch 1 and b, Batch 2. Red Xs denote donors where at least one of the training data points did not fit (tick mark, red) within the posterior predictive check credible intervals. (D) The fitted batch 1 model was used to predict batch 2 outcomes to assess the portability of the inferred donor dynamics and the reproducibility of village experiments. While 65.7% of batch 2 samples fell within the nominal 95% credible intervals, most observations outside these bounds remained close to the predicted intervals, suggesting that Townlet captures the dominant compositional dynamics across independent experiments despite inter-batch variability.

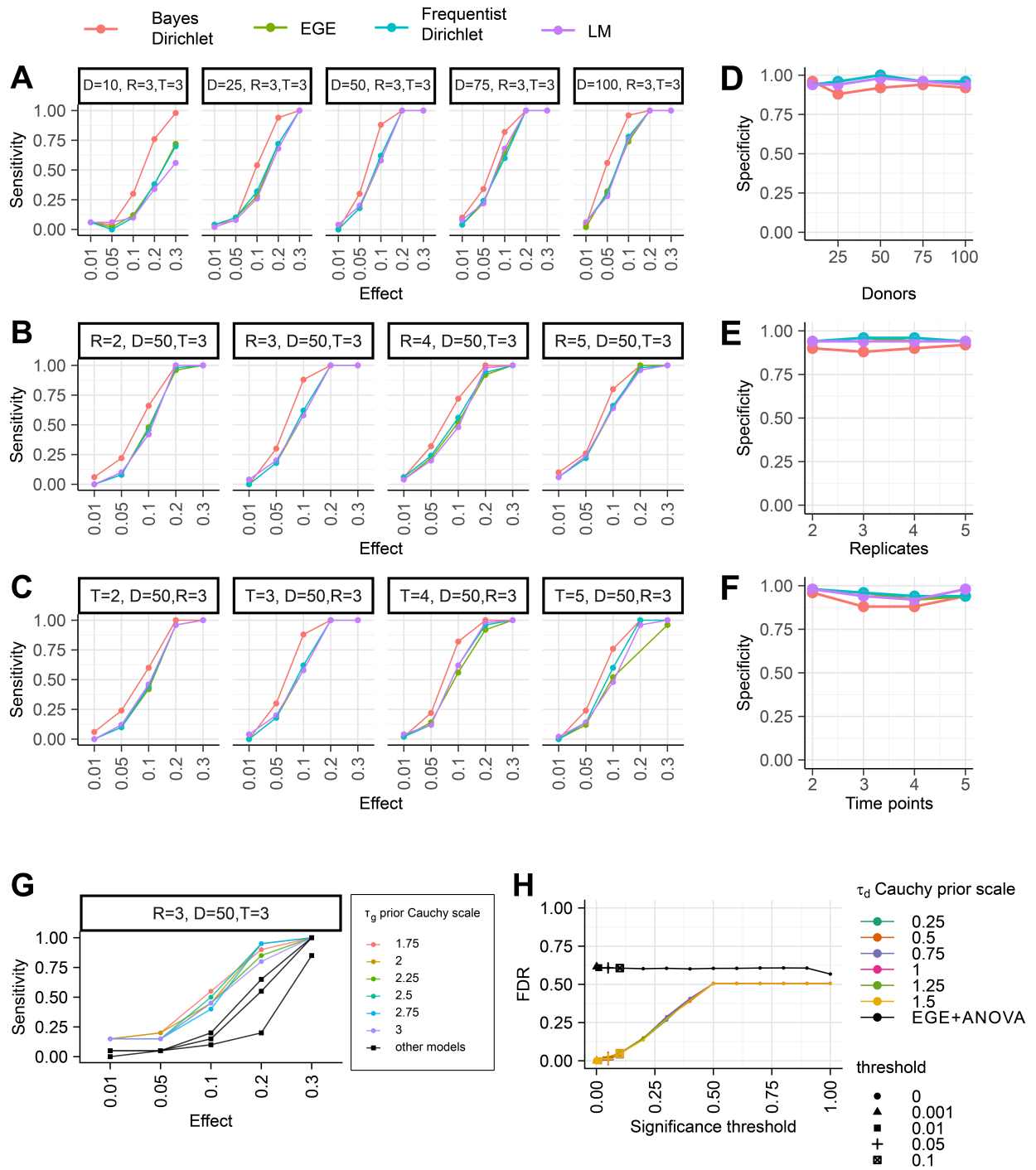


Figure S5 Village design influences statistical power

(A) Increasing the number of donors (D) has the greatest effect on sensitivity when both replicates (R) and sample time points (T) are held constant. (B) Townlet has increased sensitivity, especially when replication is low. (C) Increasing the number of sample time points does not necessarily increase power, likely due to donor takeover and underrepresentation towards the end of the experiment. (D-F) Specificity remains high when the (d) donor, (e) replicate, and (f) sample time point number are varied respectively. (G) Sensitivity is stable under a range of τ_g Cauchy scale prior values. Other models in black are the same frequentist models as in panels A-F. (H) FDR is extremely stable across different τ_d Cauchy scale prior values (all FDR curves overlap for Townlet).

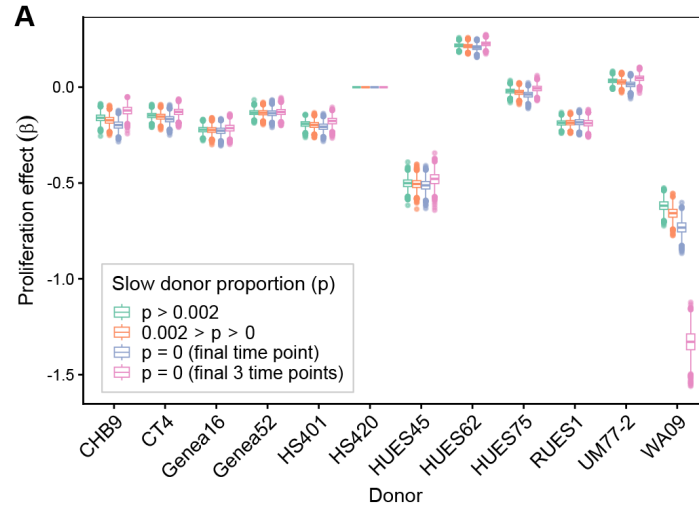


Figure S6 Posterior inference is robust even when donors approach zero

We simulated data where all donors but one were held above a minimum threshold to test how donor drop out influences proliferation effect inference. The last donor's representation was varied at the final time points to check for proliferation posterior stability across all donors. Four different levels of representation (p) were tested: 1) $p > 0.002$ at the final time point (slowest donor set to 0.003), 2) $0.002 > p > 0$ at the final time point, 3) $p=0$ (with multiplicative replacement) at the final time point and 4) $p=0$ (with multiplicative replacement) at the final three time points. (A) These simulations show that all other donor posteriors are stable despite the uncertainty in the slow donor composition when each of these simulated conditions were modeled separately with Townlet. Additionally, as we would expect, the posterior of the slow donor is lower and the uncertainty is drastically increased when they reach zero earlier in the experiment.

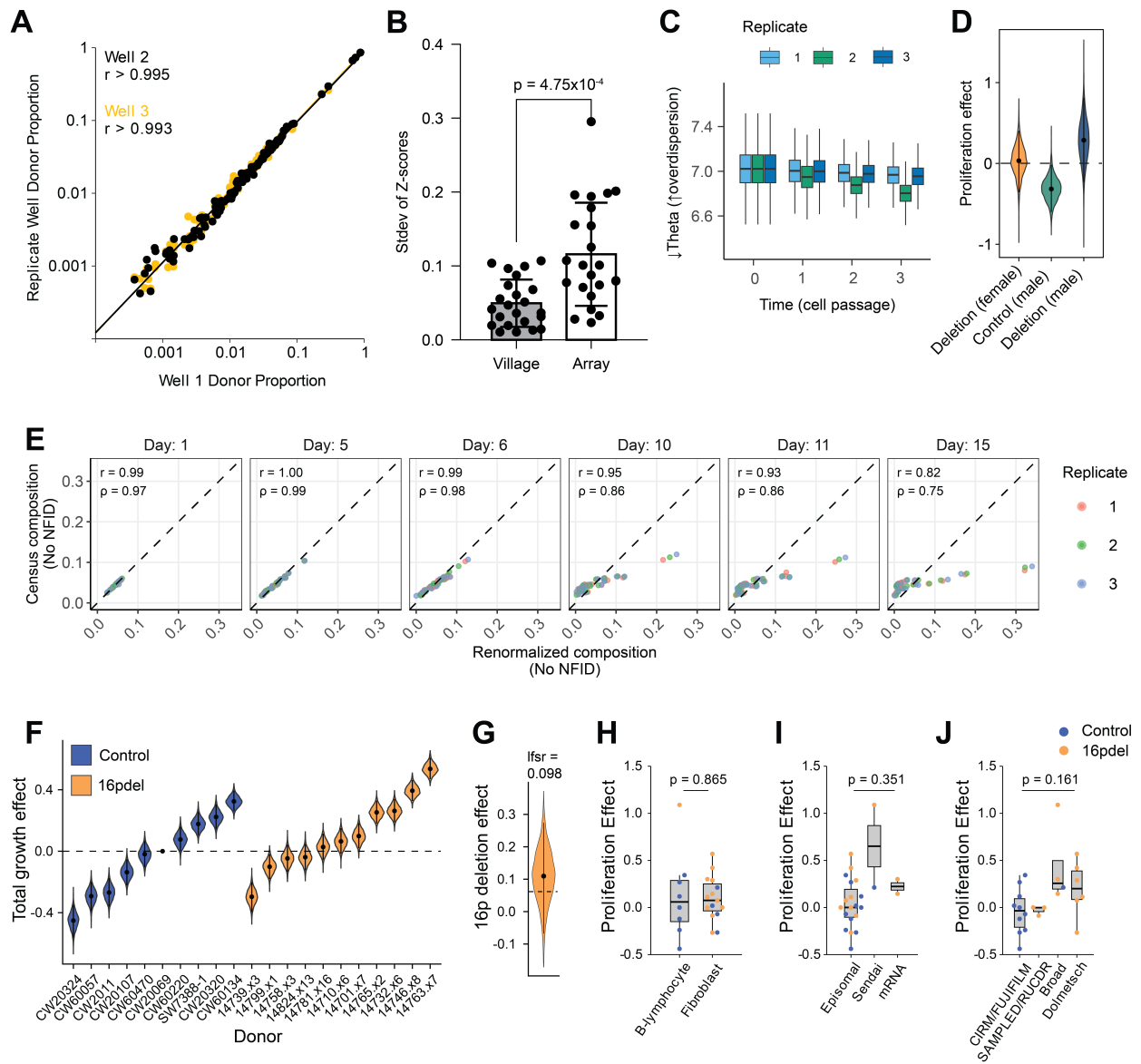


Figure S7 Validation of 16pdel NPC hyperproliferation phenotype (A) Donor proportions from 16pdel village are highly correlated between replicates over all 6 time points (Well 2 vs Well 1, Pearson $r = 0.995$, Spearman $r = 0.995$; Well 3 vs Well 1, Pearson $r = 0.993$, Spearman $r = 0.997$) (B) Donor growth rates are normalized (for the village, within each well ($n=3$ wells). For the array, within each column ($n=3$ wells per donor)) to a mean of 0 and standard deviation of 1. Each data point represents the standard deviation across replicates of a donor's normalized growth rates (biological $n = 23$ donors; paired t -test $p = 4.75 \cdot 10^{-4}$). (C) Technical variation is fairly consistent throughout the 16pdel experiment, but Replicate 2 has slightly more noise detected by Townlet. Decreasing values of theta equals more technical variation. (D) Group proliferation effects relative to reference group Control females (dashed line). Deletion males appear to drive the observed deletion signal ($lfsr = 0.136$); however, we cannot be certain the effect is real due to the sex imbalance among deletion carriers in the village as there were only three female deletion donors. Control males also exhibit slower growth ($lfsr = 0.021$). (E) Donor compositions become unstable when donors are excluded from running the census-seq algorithm (y-axis), compared to running the full donor set, removing outliers and renormalizing post hoc (x-axis). (F) Townlet's individual total donor proliferation effect posteriors relative to the baseline donor (CW20069). These were estimated from the 16pdel village while excluding the fastest deletion donor NFID_0176 (proportion data renormalized). Posterior means (points) and 95% credible intervals (error bars) are shown. (G) Relatively strong 16pdel effect on proliferation is maintained even when omitting the fastest deletion donor from the analysis ($lfsr = 0.098$). (H-J) Townlet donor proliferation effects stratified by reported iPSC tissue of origin (H), reprogramming method (I), and reprogramming facility (J). Points represent individual donors and are colored by deletion status. Proliferation effects were not significantly associated with tissue of origin (Welch's t -test, $p = 0.865$), reprogramming method (Welch one-way ANOVA, $p = 0.351$), or reprogramming facility (Welch one-way ANOVA, $p = 0.161$).

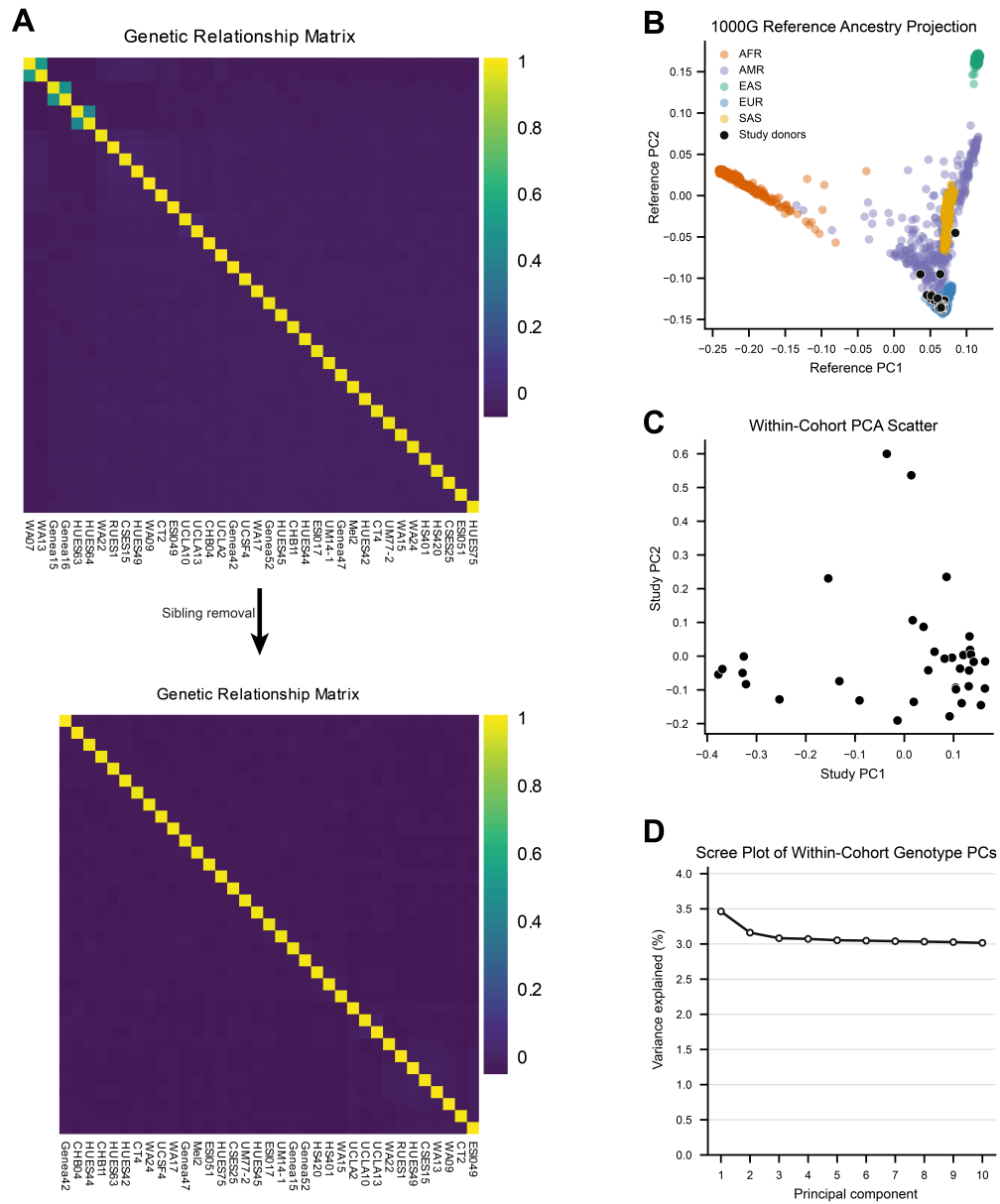


Figure S8 Genotype quality control for 35 donor GWAS (A) Centered kinship coefficients (color viridis scale) estimated from WGS genotypes with GEMMA before and after removal of one donor from each of three sibling pairs ($n = 38$ before removal, $n = 35$ after removal). (B) Projection of 35 study donors onto 1000 Genomes reference principal components. Colored points indicate 1000 Genomes super-populations; black points indicate study donors. (C) Within-cohort PCA of the 35 study donors used for GWAS. (D) Scree plot showing the percentage of genotype variance explained by the first 10 within-cohort genotype PCs.

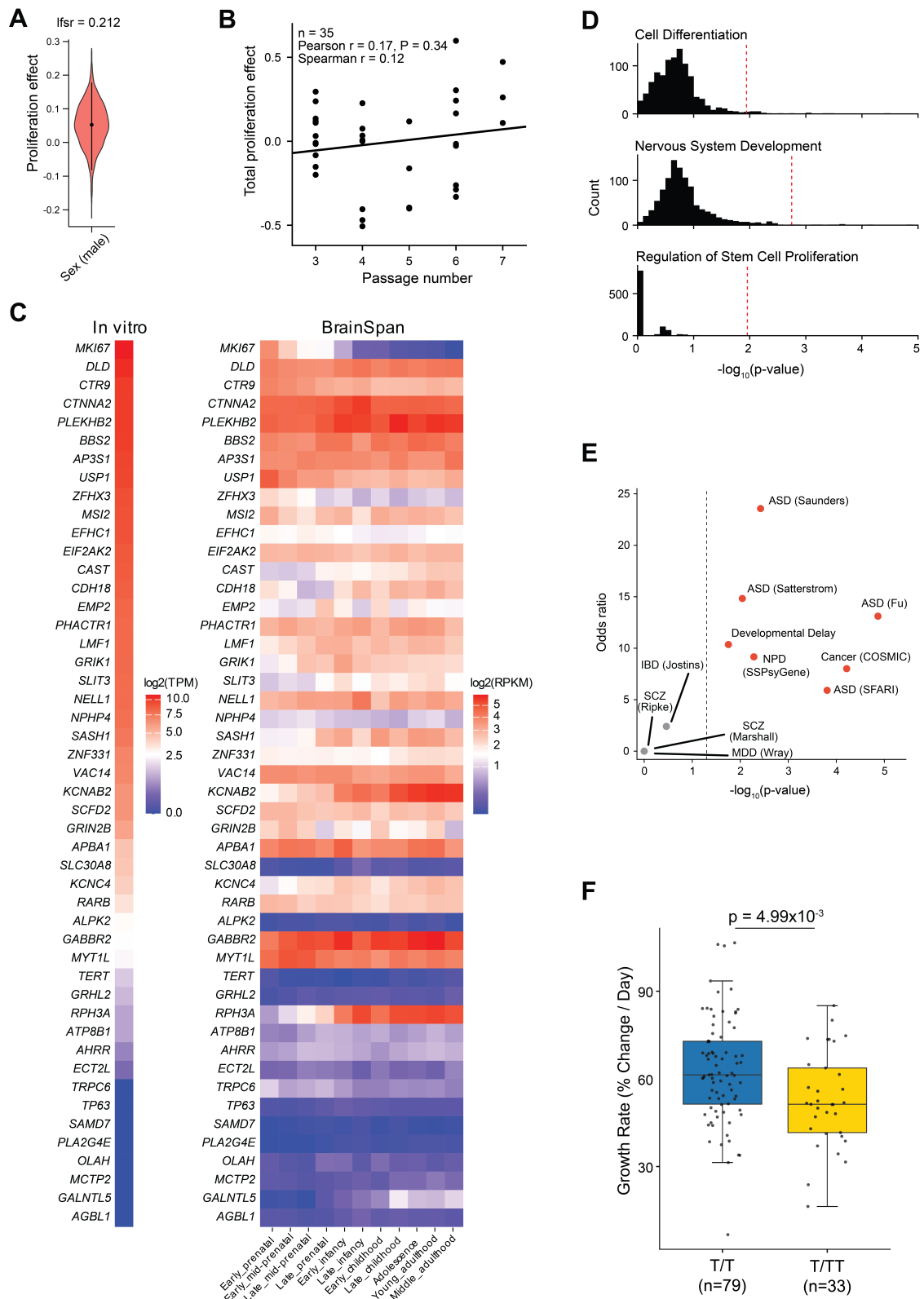


Figure S9 Expression analysis of proliferation GWAS top loci

(A) Donor sex did not significantly impact NPC proliferation effect scores ($lfsr = 0.212$). (B) Passage number of donor NPC line at the time of village construction did not influence proliferation rates ($p = 0.34$). (C) Left, expression data ($\log_2$ TPM, color heat scale) from bulk RNA-seq of *in vitro* SNaP model of human NPCs dataset-text¹⁵. Right, developmental expression ($\log_2$ RPKM, color heat scale) across 11 human brain stages ranging from 10 post-conception weeks to adulthood ($n = 48$ mapped genes with available expression data). Expression data obtained from BrainSpan¹⁰⁵. (D) Distribution of GO term enrichment p-values from random shufflings of donor proliferation effects prior to GWAS ($n = 1000$ permutations). Red dashed lines indicate observed significance in the unshuffled GWAS. (E) Summary of disease gene enrichment analysis. Red dots indicate significant overlap with the 49 mapped protein-coding genes. (F) Arrayed growth rates were computed for an independent cohort of 112 hiPSC-derived NPC lines we induced and quantified using microscopy-based measures of growth. A significant difference in proliferation was observed when grouped by SNP rs34470044 genotype (Welch's t-test, $p = 4.99 \cdot 10^{-3}$).

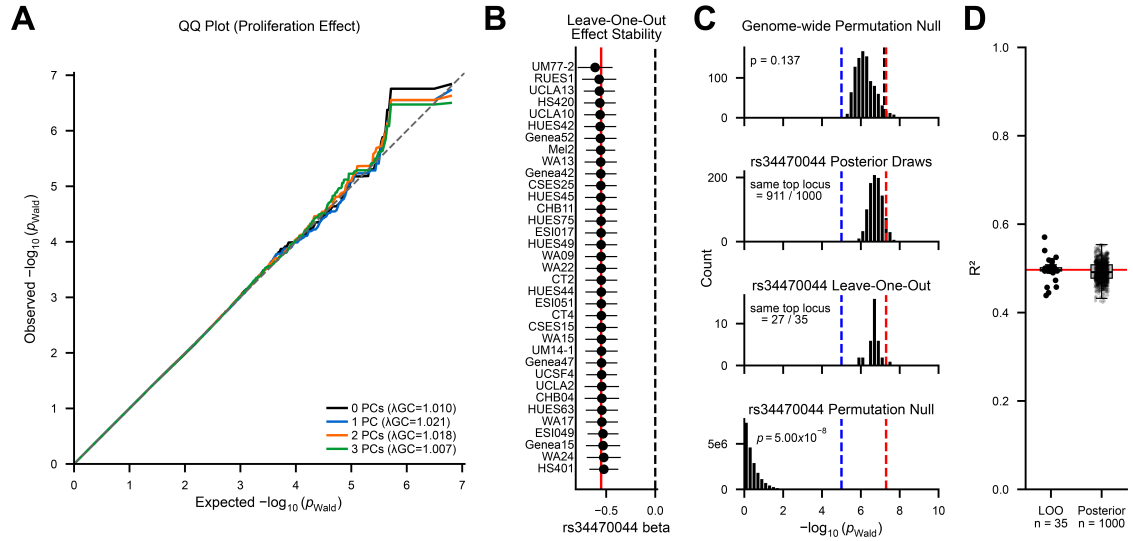


Figure S10 Proliferation GWAS sensitivity analyses

(A) Quantile-quantile plot of GEMMA Wald p-values from proliferation GWAS models including 0, 1, 2, or 3 within-cohort genotype PCs as covariates. Genomic inflation factors (λ_{GC}) are shown in the legend. (B) Leave-one-donor-out sensitivity analysis for rs34470044 effect estimates. Points show the rs34470044 beta after excluding the indicated donor; horizontal lines show 95% confidence intervals. The red vertical line indicates the full-cohort beta and the dashed black line indicates beta = 0. (C) Sensitivity of rs34470044 association significance. Histograms show a genome-wide permutation null generated by randomly reshuffling donor phenotypes relative to genotypes and retaining the minimum GWAS Wald p-value from each permutation ($n = 1000$ permutations), rs34470044 Wald p-values from GWAS using Townlet posterior phenotype draws ($n = 1000$ draws), rs34470044 Wald p-values from leave-one-donor-out folds ($n = 35$ folds), and a target-specific null generated by testing only rs34470044 after random donor phenotype reshuffling ($n = 20$ million permutations). Blue dashed lines indicate $p = 1 \cdot 10^{-5}$, red dashed lines indicate $p = 5 \cdot 10^{-8}$, and the black dashed line indicates the empirical 5% genome-wide threshold from phenotype-permutation minimum p-values ($p = 6.29 \cdot 10^{-8}$). Empirical p-values for the observed rs34470044 association were $p = 0.137$ for the genome-wide minimum-p null and $p < 5.0 \cdot 10^{-8}$ for the target-specific null. Same-top-locus annotations indicate the fraction of posterior-draw GWAS runs (911/1000) or leave-one-donor-out folds (27/35) in which the top association remained in the same plotted rs34470044/*ZFHX3* locus as the full GWAS. (D) Distribution of in-sample R^2 values for the rs34470044 genotype association across leave-one-donor-out folds ($n = 35$; median = 0.497, range = 0.439-0.571) and Townlet posterior phenotype draws ($n = 1000$; median = 0.492, range = 0.423-0.554). R^2 was calculated using the same genotype-phenotype regression as Figure 5F; red horizontal line indicates the full-cohort R^2 . LOO = Leave-One-Out.

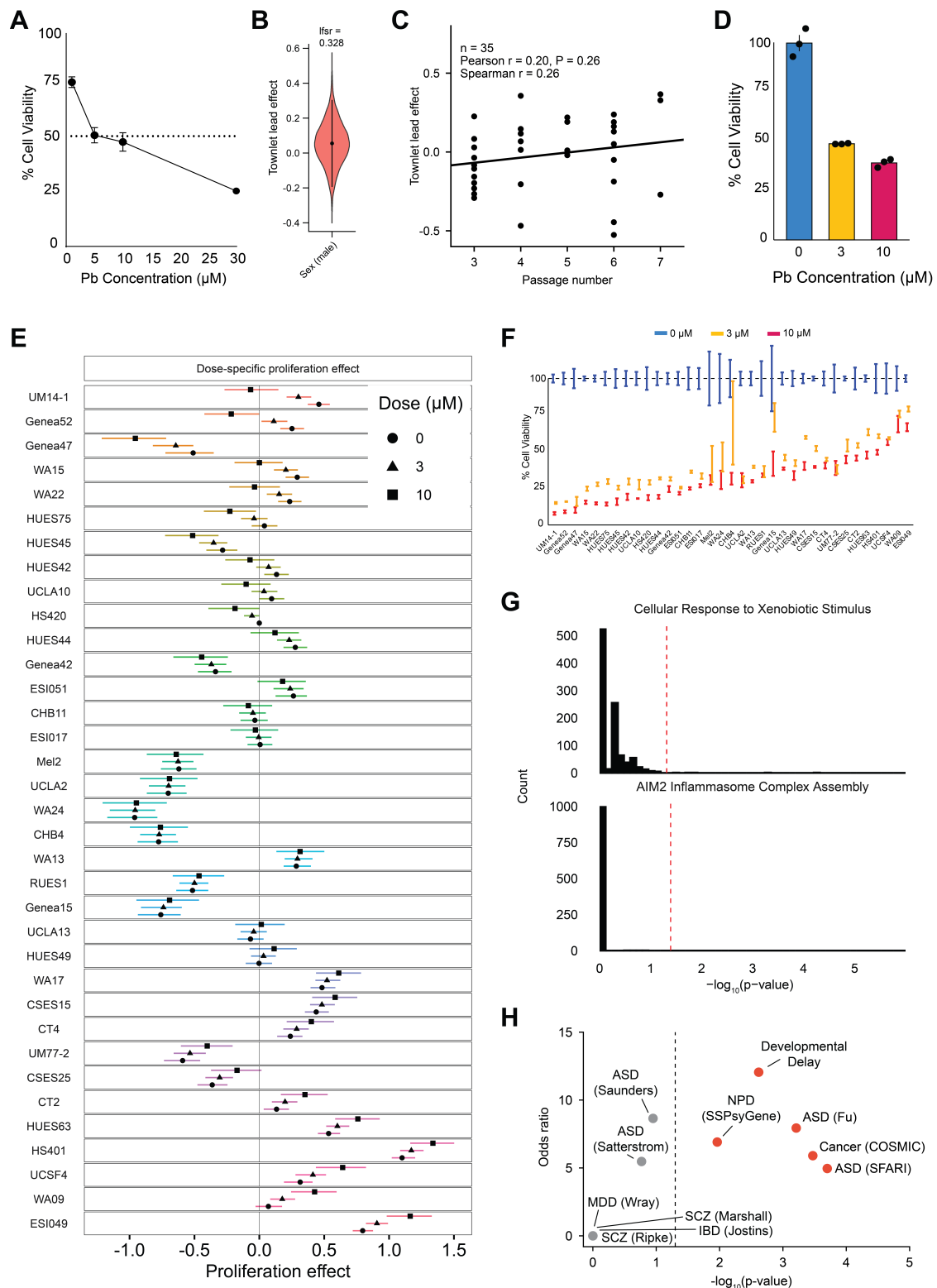


Figure S11 Pb-induced reduction of NPC viability

(A) Pb exposure reduces NPC viability in a dose-dependent manner. NPCs derived from the Genea52 ESC line were exposed daily to Pb (0, 1, 5, 10, 30 μM) for 7 days. Cell viability was quantified with CellTiter-Glo®2.0 and normalized to 0 μM (mean $\pm$ s.d., $n = 3$ wells per condition). (B) No sex differences in Pb-induced changes in viability were detected ($lfsr = 0.328$). (C) Passage number of NPC donor lines at time of pooling did not impact lead sensitivity ($p = 0.26$). (D) Cell viability of NPC village wells exposed daily to Pb (0, 3, 10 μM) for 7 days, estimated from brightfield-derived cell counts and normalized to the mean of 0 μM replicates (mean $\pm$ s.d., $n = 3$ wells per condition). (E) Townlet dose-specific total proliferation effect means with 95% credible interval error bars estimated per donor. (F) Non-Dirichlet donor-specific viabilities after Pb exposure. Donor viabilities were inferred by combining Census-seq donor proportions with replicate brightfield-derived cell counts. For each donor, inferred counts were normalized to the mean of 0 μM replicates (mean $\pm$ s.e.m., $n = 3$ wells per condition). (G) Distribution of GO term enrichment p-values from random shufflings of donor Pb effects prior to GWAS ($n = 1000$ permutations). Red dashed lines indicate observed significance in the unshuffled GWAS. (H) Summary of disease gene enrichment analysis. Red dots indicate significant overlap with the 64 mapped protein-coding genes.

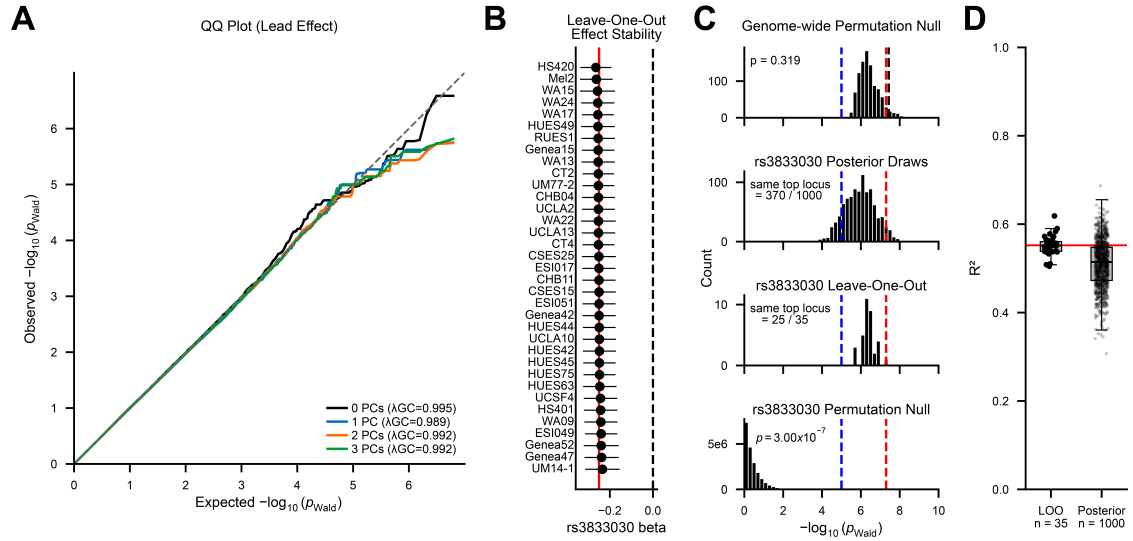


Figure S12 Pb GWAS Sensitivity Analyses

(A) Quantile-quantile plot of GEMMA Wald p-values from Pb sensitivity GWAS models including 0, 1, 2, or 3 within-cohort genotype PCs as covariates. Genomic inflation factors (λ_{GC}) are shown in the legend. (B) Leave-one-donor-out sensitivity analysis for rs3833030 effect estimates. Points show the rs3833030 beta after excluding the indicated donor; horizontal lines show 95% confidence intervals. The red vertical line indicates the full-cohort beta and the dashed black line indicates beta = 0. (C) Sensitivity of rs3833030 association significance. Histograms show a genome-wide permutation null generated by randomly reshuffling donor phenotypes relative to genotypes and retaining the minimum GWAS Wald p-value from each permutation ($n = 1000$ permutations), rs3833030 Wald p-values from GWAS using Townlet posterior phenotype draws ($n = 1000$ draws), rs3833030 Wald p-values from leave-one-donor-out folds ($n = 35$ folds), and a target-specific null generated by testing only rs3833030 after random donor phenotype reshuffling ($n = 20$ million permutations). Blue dashed lines indicate $p = 1 \cdot 10^{-5}$, red dashed lines indicate $p = 5 \cdot 10^{-8}$, and the black dashed line indicates the empirical 5% genome-wide threshold from phenotype-permutation minimum p-values ($p = 3.67 \cdot 10^{-8}$). Empirical p-values for the observed rs3833030 association were $p = 0.319$ for the genome-wide minimum-p null and $p = 3.0 \cdot 10^{-7}$ for the target-specific null. Same-top-locus annotations indicate the fraction of posterior-draw GWAS runs (370/1000) or leave-one-donor-out folds (25/35) in which the top association remained in the same plotted rs3833030/*ARNT2* locus as the full GWAS. (D) Distribution of in-sample R^2 values for the rs3833030 genotype association across leave-one-donor-out folds ($n = 35$; median = 0.552, range = 0.505-0.619) and Townlet posterior phenotype draws ($n = 1000$; median = 0.514, range = 0.307-0.687). R^2 was calculated using the same genotype-phenotype regression as Figure 6F; red horizontal line indicates the full-cohort R^2 . LOO = Leave-One-Out.

Table S1: Cell lines used in this study.

Experiment	# Donors	Cell lines used
Batch 1, Batch 2, Village vs. Array, Townlet simulations	12	hESC: CHB9, CT4, Genea16, Genea52, HS401, HS420, HUES45, HUES62, HUES75, RUES1, UM77-2, WA09
16p11.2 deletion	23	hiPSC: 14739.x3, 14799.x1, 14758.x3, 14824.x13, 14781.x16, 14710.x6, 14701.x7, 14765.x2, 14732.x6, 14746.x8, 14763.x7, NFID_0176, SW7388-1, CW20069, CW20107, CW20115, CW20320, CW20324, CW60014, CW60057, CW60134, CW60220, CW60470
Proliferation and Lead GWAS (pre-filter)	39	hESC: CHB11, CHB4, CSES15, CSES25, CT2, CT4, ESI017, ESI049, ESI051, Genea15, Genea16, Genea42, Genea47, Genea52, HS401, HS420, HUES42, HUES44, HUES45, HUES49, HUES63, HUES64, HUES75, Mel2, RUES1, UCLA10, UCLA13, UCLA2, UCSF4, UM14-1, UM22-2, UM77-2, WA09, WA13, WA15, WA17, WA22, WA24, WA7
Proliferation and Lead GWAS (post-filter)	35	hESC: CHB11, CHB4, CSES15, CSES25, CT2, CT4, ESI017, ESI049, ESI051, Genea15, Genea42, Genea47, Genea52, HS401, HS420, HUES42, HUES44, HUES45, HUES49, HUES64, HUES75, Mel2, RUES1, UCLA10, UCLA13, UCLA2, UCSF4, UM14-1, UM77-2, WA09, WA13, WA15, WA17, WA22, WA24
Proliferation GWAS arrayed validation	114	CW10054, CW20041, CW20049, CW20056, CW20063, CW20069, CW20077, CW20080, CW20098, CW20104, CW20107, CW20110, CW20115, CW20133, CW20137, CW20146, CW20178, CW20183, CW20200, CW20213, CW20239, CW20252, CW20295, CW20300, CW20321, CW20324, CW20325, CW30220, CW30282, CW30290, CW30318, CW30403, CW30408, CW30434, CW30442, CW60054, CW60057, CW60060, CW60097, CW60220, CW60286, CW60304, CW60365, CW60383, CW60389, CW60404, CW60412, CW60428, CW60470, CW60509, 15647.x1, 15648.x2, 15743.x1, 15968.x1, 15653.x1, 15655.x1, 15656.x1, 16020.x1, 16517.x1, 15973.x1, 16064.x1, 15654.x1, 16008.x1, 15439.x1, 16195.x1, 16027.x2, 16382.x1, 16219.x1, 16384.x1, 16323.x1, 16704.x1, 16013.x1, 16283.x1, 15409.x1, 15236.x1, 15265.x1, 15457.x1, 16033.x1, 15508.x1, 15287.x1, 15531.x1, 15516.x1, 15473.x1, 15727.x1, 16529.x1, 15957.x1, 15218.x1, 15272.x1, 15413.x1, 15616.x3, 16392.x1, 15721.x1, 15766.x1, 16258.x1, 16864.x1, 15936.x1, 16243.x1, 15486.x1, 15418.x2, 15912.x1, 16862.x1, 16550.x1, 16179.x1, 16932.x1, 15739.x1, 17234.x2, 17240.x2, 17261.x2, 17354.x2, 17292.x2, 16779.x1, 17331.x2, 16342.x1, 15623.x2

Table S2: Metadata on cell lines used in this study for village experiments.

Donor ID	Age	Sex	Genotype	Type	Ancestry	Village experiment
CT4	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
CHB9	0	male	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations
Genea16	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
Genea52	0	male	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
HS401	0	male	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
HS420	0	male	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
HUES45	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
HUES62	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations
HUES75	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
RUES1	0	male	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
UM77-2	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations
WA09	0	female	CTRL	hESC	EUR	batch 1, batch 2, village_vs_array, townlet simulations, proliferation and lead GWAS pre_filter
CW60014	39	male	CTRL	hiPSC	AMR	16p11.2 deletion
CW60057	41	male	CTRL	hiPSC	EUR	16p11.2 deletion
CW60134	44	female	CTRL	hiPSC	EUR	16p11.2 deletion
CW60220	9	male	CTRL	hiPSC	EUR	16p11.2 deletion
CW60470	31	male	CTRL	hiPSC	EUR	16p11.2 deletion
SW7388-1	76	female	CTRL	hiPSC	EUR	16p11.2 deletion
CW20069	5	male	CTRL	hiPSC	EUR	16p11.2 deletion
CW20107	14	female	CTRL	hiPSC	EUR	16p11.2 deletion
CW20115	13	female	CTRL	hiPSC	AMR	16p11.2 deletion
CW20320	7	female	CTRL	hiPSC	EUR	16p11.2 deletion
CW20324	4	male	CTRL	hiPSC	EUR	16p11.2 deletion
14763.x7	6	male	16pdel	hiPSC	EUR	16p11.2 deletion
14824.x13	14	male	16pdel	hiPSC	EUR	16p11.2 deletion
NFID_0176	N/A	male	16pdel	hiPSC	EUR	16p11.2 deletion
14746.x8	10	female	16pdel	hiPSC	EUR	16p11.2 deletion
14739.x3	9	male	16pdel	hiPSC	EUR	16p11.2 deletion
14765.x2	14	male	16pdel	hiPSC	EUR	16p11.2 deletion
14710.x6	40	male	16pdel	hiPSC	EUR	16p11.2 deletion
14781.x16	10	male	16pdel	hiPSC	EUR	16p11.2 deletion
14758.x3	7	female	16pdel	hiPSC	EUR	16p11.2 deletion
14732.x6	11	female	16pdel	hiPSC	EUR	16p11.2 deletion
14799.x1	14	male	16pdel	hiPSC	EUR	16p11.2 deletion
14701.x7	2	male	16pdel	hiPSC	EUR	16p11.2 deletion
ESI049	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
HUES63	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
CSES15	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UM14-1	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UM22-2	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
WA17	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
WA13	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
WA15	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UCSF4	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
WA22	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
HUES44	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UCLA10	0	male	CTRL	hESC	EUR/AMR	proliferation and lead GWAS pre_filter
HUES49	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
CT2	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
ESI051	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
HUES42	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
HUES64	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
CHB11	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UCLA13	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
ESI017	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
CSES25	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
Genea42	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
Genea47	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UM77	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
Mel2	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
UCLA2	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
Genea15	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
CHB4	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
WA24	0	male	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter
WA7	0	female	CTRL	hESC	EUR	proliferation and lead GWAS pre_filter

Supplemental Data Files

Data S1: Census-seq outputs for village-based fitness assay validation.

Data S2: Townlet proliferation effect scores for village-based fitness assay validation.

Data S3: 16p11.2del village Census-seq and Townlet output.

Data S4: Proliferation GWAS.

Data S5: Lead GWAS.