A dynamic *in vitro* model of Down Syndrome neurogenesis with Trisomy 21 gene dosage correction

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ABSTRACT/SUMMARY

Excess gene dosage from human chromosome 21 (chr21) causes Down syndrome (DS), spanning developmental as well as acute phenotypes in terminal cell types. Which phenotypes remain amenable to intervention after development is unknown. To address this question in a model of DS neurogenesis, we generated trisomy 21 (T21) human induced pluripotent stem cells (hiPSCs) alongside otherwise isogenic euploid controls from mosaic DS fibroblasts, and integrated an inducible *XIST* transgene on one chr21 copy. Monoallelic chr21 silencing by *XIST* was near-complete and irreversible in hiPSCs. Differential expression reveals T21 neural lineages and T21 hiPSCs suppress similar translation and mitochondrial pathways, and activate cellular stress responses. When *XIST* is induced before the neural progenitor stage, T21 dosage correction mitigates a pronounced skew towards astrogenesis in differentiation. Because our transgene remained inducible in post-mitotic T21 neurons and astrocytes, we demonstrate *XIST* efficiently represses genes even after terminal differentiation, which will empower exploration of cell type-specific T21 phenotypes that remain responsive to chr21 dosage.

88 **INTRODUCTION**

89 As the most prevalent human viable aneuploidy (trisomy 21, T21 in up to 1:800-1:1000 live
90 births), Down syndrome (DS) poses three significant challenges to understanding and addressing
91 the clinical needs of people living with DS [1]. First, our understanding of the developmental
92 etiology underlying cardiac, hematopoietic and neurological defects in DS is still limited [2]. Ever-
93 more sophisticated rodent DS models faithfully carry most of human chromosome 21 (chr21) [3,4]
94 or traverse syntenic boundaries to represent supernumerary mouse chr21 orthologs [5]. While
95 these rodent models capture many important aspects of DS, human T21 induced pluripotent
96 stem cells (hiPSCs) are serving as an important complementary model for DS development and
97 pre-clinical research [6]. These hiPSC models may prove especially relevant to human-specific
98 aspects of development, for example by reflecting brain region-specific neural cell types [7],
99 intrinsically longer human neuronal maturation [8], and a more rapidly evolving susceptibility to
100 neurodegeneration that may be rooted in human neurodevelopment [9,10].

101 Second, we lack experimental approaches to systematically distinguish irreversible
102 developmental phenotypes from "acute" cellular phenotypes that may remain amenable to
103 postnatal intervention. Supernumerary chr21 genes, or their rodent orthologs, are present
104 throughout differentiation or development of such DS hiPSC and rodent DS models, respectively.
105 With few exceptions [11], we therefore have an incomplete view of which T21 phenotypes depend
106 on ongoing excess dosage of specific chr21 genes in terminally differentiated cells. This question
107 is particularly relevant to DS-associated neurodegeneration [12].

108 Third, cellular stress pathways that are chronically and systemically active in DS [13–18] and likely
109 play an important role in DS neurodegeneration [19], are also shared with human trisomies 13 (T13)
110 and 18 (T18) [20], but not with dosage-compensated sex-chromosomal trisomy. The most plausible
111 origin for such common aneuploidy-associated cellular stress is the cumulative excess expression
112 of the many autosomal genes that code for subunits of dosage-sensitive protein complexes.
113 However, this interpretation has not yet been tested formally in the context of an autosomal
114 trisomy, as it requires distinguishing excess expression from the mere presence of the
115 supernumerary chromosome.

116 A human cellular model with dynamic T21 gene dosage would be instrumental towards
117 addressing these challenges, across a range of DS-affected cell fates and phenotypes. To
118 characterize the impact of T21 in hiPSCs and terminally differentiated cell types, we generated
119 our own dynamic hiPSC model of T21 dosage alongside otherwise isogenic controls from mosaic
120 DS. Pioneering work by the Lawrence lab demonstrated the utility of the long non-coding RNA
121 *XIST*, which endogenously silences the inactive X in females, for T21 dosage correction in DS
122 hiPSCs [21]. *XIST* RNA spreads from its site of transcription across the entire chromosome *in cis*, to
123 directly and indirectly recruit orthogonal repressive chromatin complexes to silence genes [22]. Yet,
124 earlier work in the mouse system indicated that *Xist* could establish gene silencing only during or
125 upon exiting pluripotency, but not after differentiation [23,24], with rare exception [25,26]. Recent work
126 by the Lawrence lab shows that human *XIST* can still silence genes up to the neural progenitor
127 cell (NPC) stage, but technical limitations hindered *XIST* induction in terminal, post-mitotic human
128 neural cells [27].
129 Here, we leverage phased allelic expression analysis to demonstrate that our new inducible
130 T21-*XIST* design enables uniform, near-complete and -irreversible T21 dosage correction in
131 hiPSCs. *XIST* suppresses T21 *trans*-effects, including cellular stress pathways that are also active
132 in T21 neural lineages. T21 silencing prior to the NPC stage rescues T21-altered neuro-astroglial
133 lineage balance that reflects excess gliogenesis previously observed in DS fetal samples [28–32] and
134 rodent models [33–35]. Because our T21-*XIST* system is also the first to effectively induce *XIST* and
135 repress chr21 dosage in post-mitotic neurons and astrocytes, this new model of dynamic T21
136 gene dosage will facilitate identification of cellular pathways and cell types that may present
137 promising targets for intervention after completed development.
138
139 **RESULTS**
140 **T21 dosage correction by *XIST*-mediated monoallelic silencing in DS hiPSCs**
141 To derive T21 hiPSCs alongside isogenic euploid control lines, we reprogrammed dermal
142 fibroblasts from a male individual with mosaic DS [36], and screened the resulting hiPSC clones
143 using copy-number sensitive qPCR [Fig. S1A,B]. Two T21 lines appeared to have a gain on chr17,
144 a frequent karyotypic abnormality primarily observed in human ESCs [37,38], but featured only two

145 centromere 17 signals by fluorescent *in-situ* hybridization (FISH) follow-up [Fig. S1C]. We
146 performed array comparative genomic hybridization (aCGH, CytoSNP-850k) to confirm expected
147 karyotypes for fibroblasts, euploid and T21 hiPSCs, and to resolve segmental (17q) gains in the
148 two corresponding clones, which were excluded thereafter [Fig. S1D]. Differential probe intensity
149 analysis of the remaining T21 and euploid hiPSC lines also revealed a large set of heterozygous
150 SNPs with increased representation in the euploid lines spanning chr21, and a smaller focal set
151 that was lost in euploid lines [Fig. S1E]. These results suggested one chr21 copy from each parent
152 is maintained in euploid lines ($chr21^{M}$ & $chr21^{P}$), whereas T21 hiPSCs carry an additional copy
153 ($chr21^{M2}$), likely a maternal chromatid retained via non-disjunction in meiosis II [39].
154 While T21 is stably maintained in hiPSCs after reprogramming [40–42], sporadic T21 loss has
155 been reported [43,44]. We therefore confirmed the mitotic stability of three chr21 copies by two
156 independent approaches. First, three independent signals were observed in 94.5% of T21 hiPSCs
157 by fluorescent *in-situ* hybridization (FISH, *DSCR8* probe), compared to 99% of euploid hiPSCs with
158 two signals [Fig. S1F]. Second, while targeting a *UBC*-promoter driven doxycycline (dox)-
159 responsive trans-activator (rTTA3G) to the AAVS1 safe harbor in *PPP1R12C* (HSA19), we
160 confirmed that all 32/32 independent colonies arising from single cells maintained T21 by copy-
161 number sensitive qPCR for a *DYRK1A* amplicon [Fig. S1G].
162 We integrated our TET3G-driven *XIST* cDNA transgene on $chr21^{P}$ [Fig. 1A], after re-
163 verifying the karyotypic integrity of rTTA3G clones by aCGH [Fig. S1D]. To this end, we designed
164 Cas9 guides dependent on a $chr21^{P}$-specific protospacer adjacent motif (PAM), targeting an
165 intergenic site well outside of *DYRK1A* to avoid impacting its expression. We proceeded with
166 three independent, PCR-screened T21-*XIST* clones, and tested for *XIST* induction by RNA-FISH,
167 and immuno-fluorescence (IF) staining for ubiquitinated histone 2A lysine 119 (H2AK119ub) and
168 tri-methylated histone 3 lysine 27 (H3K27me3), which follow *XIST/Xist* RNA expression on mouse
169 and human X alike [22]. Of note, because the original fibroblasts were from a male individual with
170 DS [Fig. S1C], there was no endogenous *XIST* expression, and any *XIST*/H2A119ub/H3K27me3
171 cloud must arise from the T21-targeted *XIST* transgene on chr21. Indeed, after cessation of acute
172 dox treatment (3 wk withdrawal, w/d) hiPSCs were negative for *XIST*, underscoring the TET3G
173 system is not leaky and turns off when dox is withdrawn, whereas virtually all T21-*XIST* hiPSCs

174 displayed *XIST* clouds and acquired H2A119ub/H3K27me3 signals in the presence of dox [Fig. 1B].
175 Together, these data illustrate the homogeneity of *XIST* induction, and provide independent
176 confirmation that this third copy of chr21 is faithfully maintained in hiPSCs.

177 We next performed mRNA-seq of euploid, T21 and T21-XIST hiPSCs to confirm high
178 uniformity in pluripotency genes over lineage-specific markers [Fig. S2A], and to quantify T21
179 dosage correction by *XIST* in the presence, absence or upon withdrawal of dox [Fig. 1A,C], which
180 tests whether gene silencing is maintained once established. *XIST* was uniformly expressed in all
181 three T21-XIST clones at 3, 6 and 9 weeks (wks) of dox treatment ("+dox"), and returned to the
182 ("—dox") baseline upon dox withdrawal ["w/d", Fig. 1A,C], consistent with *XIST*-negative FISH in
183 w/d samples [Fig. 1B].

184 To accurately measure chr21 dosage not only by standard differential expression analysis
185 but also chr21 allelic representation, we additionally sequenced euploid and T21 whole genomes
186 with linked-reads to identify and phase variants. In both euploid and T21 hiPSCs, most variants
187 can be grouped in large phase blocks (N50 lengths of 1.8 Mb and 2.9 Mb, respectively), including
188 across chr21, where thousands of heterozygous variants distinguish the *XIST*-bearing ($chr21^{P}$)
189 copy. Allele-specific phasing of mRNA-seq reads indeed reveals that one allele was under-
190 represented with a mean lesser allele frequency (LAF) of one-third for T21 samples. This is in
191 keeping with the presence of a single $chr21^{P}$ copy, and two copies of $chr21^{M/M2}$, whereas the
192 same $chr21^{P}$ allele comprised about half of all allelic reads in the D21 samples [Fig. 2A]. In the
193 absence of dox (-dox), the *XIST*-bearing $chr21^{P}$ copy (T21-XIST) still represented ~one-third of
194 variant-mapping reads, but was largely depleted in the +dox and w/d samples, which were dox-
195 treated (3wks) and collected after another 3 or 6 wks without dox. These data demonstrate that
196 *XIST* silences most genes on the $chr21^{P}$ copy near-completely, and irreversibly, consistent with
197 our expectations based on mouse *Xist* reports (reviewed in [22]).

198 Likewise, differential expression analysis across chr21 reveals that nearly all genes with
199 assessable expression were depleted by ~one-third (log2FC of -0.59 ± 0.15), irrespective of their
200 linear distance from the *XIST* transgene, and maintained that expression level even after 3-6 wks
201 of dox withdrawal [Fig. 2B-D]. Altogether, only a very small group of protein-coding genes
202 retained a LAF > $1/6^{th}$ (*ADAMTS1, APP, H0Y3Z8, IFNAR1/2, MRPS6, ETS2* and *YBEY*), most likely

203 due to mis-phased variants. Indeed, these genes were significantly downregulated by the
204 expected degree ($p \leq 0.1$, log2FC -0.59 ± 0.15), except for *IFNAR2* and the adjacent ORF *H0Y3Z8,*
205 which appeared to escape *XIST*-mediated repression by differential expression (p.adj > 0.1,
206 log2FC > -0.3). To compare this efficacy to previously reported T21-*XIST* systems [21,27], we
207 considered the degree of silencing across all mutually assessed chr21 genes in prior reports,
208 irrespective of adjusted p-value cutoff, as our study included a greater number of samples.
209 Indeed, the distribution log2FC values in our T21-*XIST* indicates more complete gene repression
210 than in previously published datasets [Fig. S2B].

211 Comparing differential expression in hiPSCs after 3 wk and 6wk of dox withdrawal, we
212 find that silencing was maintained in w/d samples, irrespective of the length of original dox
213 treatment [Fig. 2B, Fig. S2C]. We therefore assessed DNA methylation (DNAme) at promoters,
214 which reflects stable gene silencing known to remain intact even upon *Xist* deletion in mouse
215 fibroblasts [45,46]. Excluding likely inactive promoters with high DNAme (>60% methylated),
216 virtually all chr21 promoter-associated CpG probes of the methylEPIC array (Illumina) increased
217 from a median of 7% methylated in the absence of dox (-dox) to 41% methylated after ≥3 wks in
218 +dox [Fig. 2F]. This level is consistent with the presence of a fully methylated alongside two
219 unmethylated alleles (1/3) and is maintained ≥3 wks after dox withdrawal (w/d, 39% methylated),
220 which is also reflected in the absence of reactivated genes by differential and allelic expression
221 [Fig. 2A-E].

222 We next turned to transcriptome-wide effects of T21 and *XIST*-corrected gene dosage.
223 While levels of chr21 genes clearly increased in T21 relative to D21 and decreased in T21-XIST
224 +dox relative to -dox samples, all other chromosomes also responded to chr21 dosage, but
225 roughly balanced up- and down-regulated genes [Fig. 3A]. Consistently, samples with an effective
226 chr21 dosage matching euploid samples (D21, and +dox or w/d T21-XIST) segregated from T21
227 and no-dox T21-XIST samples in PCA [Fig. 3B]. Because our experimental design included the
228 original, non-transgenic D21 and T21 hiPSCs (198-1/2/5) with all treatments (±dox and w/d), we
229 were able to observe that their acute +dox treatment triggered expression changes that
230 correlated to some extent with the T21 dosage effect [Fig. 3C left], which was still significant after
231 excluding chr21 genes from the correlation [Fig. S3A, left]. This was not unexpected, because

232 doxycycline can partially impact mitochondrial translation in eukaryotic cells [47], and
233 mitochondrial pathways are also altered in DS [48–50]. We therefore adjusted for this small dox-
234 specific effect in our analysis (see methods). We then compared T21 over D21 log2FC expression
235 changes with the combined (+dox and w/d) *XIST* correction, which revealed a highly significant
236 and negative Pearson correlation of -0.5 [Fig. 3C, right] that persists even when chr21 genes are
237 excluded [Fig. S3A, right]. These data indicate that differentially expressed genes (DEGs)
238 responding to excess chr21 dosage are also changing in the opposite direction when excess chr21
239 dosage is corrected via *XIST*. This large-scale reversal was also evident in gene-set enrichment
240 analysis (GSEA), with a dominance of anti-correlated terms [Fig. 3D, top left and bottom right
241 quadrant], many of which related to apoptosis, stress, ER, mitochondria and translation terms.
242 Plotting the top three most-concordant terms for each of these categories [Fig. 3E], T21 and
243 dosage-corrected samples were near-uniformly opposed in biological processes previously
244 implicated in DS, including apoptosis [17,51,52], oxidative stress via NRF2 [16,53], mitochondria [18,48–50],
245 and translation [14,54]. Importantly, serine biosynthesis pathways, recently attributed to a general
246 response to aneuploidy [20] were also highly represented, indicating that serine metabolism
247 returns to baseline upon T21 dosage correction, despite the continued presence of the
248 supernumerary chr21 copy.
249
250 **T21 gene dosage effects in mature neural cell types**
251 To assess T21 gene dosage effects in neural lineages, we applied a well-established
252 monolayer differentiation protocol that mimics *in vivo* neural development in the establishment
253 of neurogenic niches (rosettes) and in the production of both mature neurons and astrocytes
254 [55,56]. We chose this approach as nearly all T21 hiPSC-derived neuronal RNA-seq data reported to-
255 date was generated from immature neurons in the absence of astrocytes. We performed mRNA-
256 seq in 10-12 wk neural cultures, obtained 5-6 wks after low-density re-plating of 4 wk NPCs [Fig.
257 S3B]. T21 neural cultures showed elevated chr21 transcript levels with a peak near the expected
258 +0.59 log2FC relative to euploid samples [Fig. 4A,B]. As expected, neural and hiPSC DEGs and
259 GSEA-enriched terms were positively correlated, while neural GSEA terms were largely anti-
260 correlated in direction with terms enriched in XIST-corrected hiPSCs [Fig. 4C-E], which also

261 extended to the three most-concordant terms across all comparisons [Fig. S3B]. Overall, we find
262 *XIST*-mediated T21 dosage correction is reflected in a majority of significantly enriched terms
263 differentiating T21 hiPSCs and neural cultures from their euploid controls [Fig. S4].
264 To assess whether the number of over-represented GSEA terms relating to cell death and
265 apoptosis in T21 neural cultures manifested in differential neuronal survival, we employed a
266 recently developed red genetically-encoded cell death indicator (RGEDI) and tracked two
267 neuronal subtypes by automated longitudinal imaging [Fig. 5A, Fig. S5A-D] [57,58]. Both T21
268 interneurons (hazard ratio of 4.08) and T21 forebrain neurons (hazard ratio of 2.57)
269 demonstrated significantly increased cell death (RGEDI/EGFP ratio > 0.15, Holm-corrected
270 $p<0.01$, linear mixed model) over ~10 days of single-cell tracking, compared to euploid controls
271 [Fig. 5B,C]. Additionally, we also assessed live neurons (RGEDI/EGFP ratio < 0.15) and noted that
272 there was a significant ($p \leq 10^{-8}$) reduction in EGFP signal in T21 compared to euploid controls [Fig.
273 S5E], that mirrored the down-regulation of ribosome and translation-related GSEA terms in both
274 T21 hiPSCs and neural cultures [Fig. 4D, Fig. S4].
275
276 **T21 dosage correction early in neural differentiation rescues neurogenesis**
277 Staining for neuronal MAP2 and astroglial S100B markers, we observed an excess of
278 astrocytes in the T21 relative to euploid neural differentiations. Both neurons and astrocytes
279 derive from NPCs [59], and astrogliosis has been reported in DS models [33,34] and fetal samples [28,30,32],
280 where T21 NPCs prematurely shift from neurogenesis to astrogenesis. To determine whether
281 *XIST*-mediated T21-dosage correction could prevent this lineage skew, we first confirmed that
282 *XIST* expression and H2AK119ub enrichment would persist in 4 wk NPCs, which was the case for
283 well over 75% of NPCs derived across three independent clones, treated with +dox as hiPSCs [Fig.
284 6A]. Indeed, even NPCs derived from untreated T21-*XIST* hiPSCs but receiving dox from the first
285 day of differentiation ("d0") presented with *XIST* and H2AK119ub foci in ≥75% and ≥65% of all
286 cells, respectively. Co-staining for MAP2 and S100B on day28 (4 wk) of neural differentiation
287 revealed a consistent and highly reproducible astrogenic skew in T21 and no-dox T21-XIST
288 samples relative to isogenic euploid control lines across multiple rounds of differentiation [Fig.
289 6B]. Remarkably, neurogenesis was fully rescued in neural differentiations from pre-treated as

290 well as d0+dox treated hiPSCs, across all three independent T21-XIST clones. Because the overall
291 sum of committed cells expressing either marker did not differ significantly between T21 and
292 euploid or T21-XIST dosage-corrected samples, we propose that T21 dosage does not suppress
293 NPC commitment in general (as suggested [27]), but rather advances the shift to astrogenesis,
294 which is consistent with reports in fetal DS and rodent DS models [30,34].
295 To test whether this interpretation is replicated *in vivo,* at the very onset of cortical
296 astrogenesis, we turned to the Ts65Dn model [Fig. S6]. At embryonic day 18.5 (E18.5), the density
297 of S100B+ positive astrocytes in the developing cortex was significantly elevated in Ts65Dn
298 embryos over their wildtype litter mates ($p < 0.05$). In contrast, there was no significant
299 difference in the density of proliferative Ki67^{+} cells in the subventricular zone [Fig. S6], indicating
300 the early cortical astrogenesis in Ts65Dn embryos was not associated with delayed commitment
301 and continued proliferation of neural progenitors, but rather an inherent skew towards
302 astrogenesis also observed in other brain regions [30,34].
303
304 **Postmitotic neural cell types still induce *XIST* and attract repressive chromatin marks**
305 Given the high rate of *XIST* induction in T21-XIST NPCs that only received dox at the onset
306 of differentiation (d0), we next explored whether the *XIST* transgene would remain dox-
307 responsive in terminally differentiated cells [Fig. 7A]. To this end, we forced NPCs (~4 wk neural
308 differentiation) out of the cell cycle into terminal differentiation by inhibiting the Notch signaling
309 pathway for 5 days with gamma-secretase inhibitor DAPT, and added dox a day after completing
310 DAPT treatment (d33). To confirm DAPT treatment was sufficient to deplete mitotic cells, we also
311 labeled cells transiting S-phase with nucleotide analog EdU, and co-stained for this label and *XIST*
312 FISH after two days in +dox media (d35). DAPT-treated NPCs incorporating EdU comprised ≤6%
313 of the neural population on average, which were rarely EdU/*XIST* double-positive (≤26%),
314 whereas *XIST* was induced in ≥40% of all EdU^{-} cells (94% of all cells). We conclude that *XIST* was
315 reliably induced in a sizeable fraction of post-mitotic T21-XIST cells that had terminally
316 differentiated without dox. We next assessed these post-mitotic cells after 3 to 6 weeks of dox
317 treatment post-DAPT exposure ("pD") to provide sufficient time for H2AK119ub enrichment. We
318 included "d0" controls that were treated with the standard 1 µM dox concentration throughout

319 neural diff, as well as low-dose dox "LD" samples, which were treated with 0.01 µM dox up to
320 the DAPT exposure, and standard 1 µM dox thereafter, to test whether "pre-marking" the TRE3G
321 promoter without effective induction through differentiation may render it more responsive in
322 terminal cell types. On average, over half of d0 and LD cells featured prominent XIST clouds,
323 followed by ≥ 40% of pD samples, across both c1 and c7 clones and in three independent rounds
324 of differentiation. As before, H2AK119ub signal lagged slightly behind *XIST*, but consistently
325 marked 27% - 36% of all cells (58-65% of *XIST+* cells), including MAP2+ neurons and S100B+
326 astrocytes [Fig. 7C,D].
327
328 ***XIST* induction in terminal neurons and astrocytes corrects T21 gene dosage**
329 We next performed single-nuclei RNA-seq, to determine whether such *XIST* induction and
330 H2AK119ub accumulation in these terminally differentiated neural cell types reflect T21 dosage
331 correction [Fig. 8]. Transgenic neural differentiations treated with dox from d0 or pD were
332 collected alongside untreated T21-XIST c7 cells ("T21"), and two euploid lines (198-1/2 or
333 "D21a/b"). Nuclei distributed across four broad clusters, of which three corresponded to the
334 expected NPC, neuronal and astroglial populations [Fig. 8A, S7A]. Cell cycle analysis classified the
335 smaller NPC cluster as S and G2/M phase cells, while all other clusters scored as G1, consistent
336 with terminally differentiated G0 cells [Fig. 8A, S7A]. We labeled the first three clusters according
337 to their predominant cell type using cell type markers from [60], and the fourth as "Other" given
338 lower scores returned by sctype [61]. Due to its proximity to higher-scoring oligodendroglial
339 progenitor cells (OPC), this fourth cluster probably reflects OPC-derived lineages that
340 predominantly arose from one of the euploid lines (D21a). Crucially, *XIST* induction in T21-XIST
341 neural differentiations (d0 & pD) was detected in all clusters with 74-94% of d0+dox treated and
342 24-58% pD+dox treated cells [Fig. S7A], in agreement with our FISH and IF data [Fig. 7].
343 To determine whether *XIST* induction repressed T21 gene dosage, we assessed three
344 complementary metrics. First, we phased single-nuclei RNA-seq reads and summed allele-specific
345 counts across all chr21 genes to calculate the cumulative paternal allele representation, thereby
346 mitigating the effect of transcriptional bursts in individual nuclei [Fig. 8B]. As expected, euploid
347 cells have a significantly higher $chr21^P$ allele fraction compared to T21 cells (0.46 vs. 0.32, Wilcox

348 $p = 1.6x10^{-11}$), whereas T21-XIST day0+dox treated cells have a significantly lower chr21^{P} allele
349 fraction (0.14, Wilcox $p < 2.2x10^{-16}$). Crucially, while *XIST*-negative pD (pD-X^{-}) cells show little
350 reduction in this *XIST*-carrying paternal allele, *XIST*-positive pD cells (pD-X^{+}) reveal significant
351 repression of this allele (0.19, Wilcox p-value = $1.3x10^{-12}$). Separating cells by cell type group [Fig.
352 S7B] reveals *XIST+* d0 and pD neurons repress the transgene-bearing chr21^{P} copy most robustly,
353 with paternal allele fractions of 0.15 and 0.17 (Wilcox $p<10^{-4}$), respectively.

354 Second, we compared the normalized expression for each gene on chr21 to the genome-
355 wide mean without chr21. We then determined the per-cell cumulative distribution of all chr21
356 genes, as well as the per-gene cumulative distribution by cell type group [Fig. S7C, D], and
357 aggregated these ratios across all neurons, astrocytes and NPCs [Fig. 8C,D]. Indeed, euploid and
358 T21-nodox medians fell near the expected 1.0x and 1.5x ratios relative to the genome-wide
359 mean, indicating chr21 genes are not inherently dosage-compensated. Likewise, *XIST*-negative
360 pD nuclei failed to differ significantly from the T21-nodox sample (1.5x and 1.3x for the per-cell
361 and per-gene ratio, respectively). *XIST*+ pD nuclei however displayed near-euploid chr21 dosage
362 medians (ratios of 1.1x and 1.0x, Wilcox p-values $\leq 2.4x10^{-7}$), very close to d0 nuclei [Fig. 8C,D].
363 Separated by cell type group, neurons again demonstrated near-complete dosage compensation
364 in *XIST+* pD nuclei with median ratios of 0.97x and 0.92x on a per-cell and per-gene basis,
365 respectively [Fig. 8C,D]. Assessing both metrics across full distributions for each sample, the *XIST*-
366 negative pD distribution tracked closely with T21-nodox cells, remaining well within its 95%
367 confidence interval. In contrast, the *XIST+* pD and d0 distributions shifted to the left significantly
368 (dashed lines, Kolmogorov-Smirnov, K.S. test p-values) towards the euploid distributions for all
369 cell type groups, irrespective whether normalized expression ratios were averaged over cells or
370 genes [Fig. S7C, D]. Aggregating these distributions across astrocytes, neurons and NPCs
371 illustrates *XIST+* d0 and pD nuclei tracked on top of each other, with their 50% percentile ratios
372 near-identical to euploid nuclei [Fig. S7E,F]. In sum, these data indicate that *XIST* corrects T21
373 gene dosage in post-mitotic cell types via robust repression its chr21^{P} copy, especially in neurons,
374 whereas NPCs mitigate *XIST's* impact to some extent, likely due to continued passage through M
375 phase.

376 As our third metric for T21 dosage correction, we performed differential expression
377 analysis on variance-stabilized data [62], and compared effectively trisomic (*XIST*-negative pD and
378 nodox) nuclei to euploid (D21a/b), *XIST+* pD and d0 nuclei for each cell type. Indeed, generally
379 lower expression of chr21 in euploid relative to trisomic nuclei was also observed for *XIST+* pD
380 and d0 nuclei (Fig. S7G). Due to greater dispersion inherent in single-nuclei RNA-seq, the
381 moderated effect sizes (avg.log2FC) reflected in variance-stabilized data were smaller than in our
382 bulk RNA-seq (Fig. 2,4) but enabled comparing euploid to *XIST*-corrected T21 gene dosage.
383 Interestingly, *XIST* repressed chr21 genes to the near-euploid median, in all except 'Other' cell
384 types, which showed a small but significant residual excess expression (Wilcox p = 0.0007, Fig.
385 S8A). While the linear distance to the *XIST* transgene integration site did not correlate with mean
386 log2(FC) values in any of the d0+dox cell types, there was a distance effect in pD+dox neurons
387 and NPCs [Fig. S8B,C].
388 Importantly, differential expression of non-chr21 genes in *XIST*$^{+}$ d0 and pD nuclei
389 correlated strongly with log2(FC) values in euploid relative to effectively trisomic nuclei across all
390 cell type groups [Fig. 8E], with Pearson coefficients ranging from 0.25-0.46 in all except *XIST*$^{+}$ pD
391 NPCs. GSEA also reflected such transcriptome-wide reversion with strong NES correlations
392 between *XIST+* d0, pD and euploid conditions in MsigDB Hallmark and canonical pathways that
393 were significantly enriched (q-value ≤ 0.1) in euploid relative to effectively trisomic nuclei [Fig.
394 8F]. Again, the correlation in NPCs was lowest across all cell type groups, yet still significant
395 ($R=0.19$, $p=8.2\times10^{-7}$). Importantly, the vast majority of MsigDB Hallmark and canonical pathways
396 significantly enriched in any pairwise comparison (combined Fisher's method $p\leq1\times10^{-4}$) followed
397 the direction of change in the remaining third comparison [Fig. S8D]. In sum, these results
398 indicate that the degree of chr21 dosage correction in *XIST+* d0 and pD nuclei is sufficient to
399 revert T21 cells towards the euploid expression profile. We conclude that this new T21-*XIST*
400 transgene corrects chr21 dosage even in terminal and post-mitotic cell types, which will facilitate
401 detailed exploration of cellular and transcriptional T21 hallmarks that remain reversible after
402 completed development.
403
404

405 **DISCUSSION**

406 The relevance of the inducible T21-XIST system presented herein ranges from DS biology
407 and cellular responses to aneuploidy in general, to fundamental questions relating to human
408 *XIST*-mediated gene silencing and escape. In hiPSCs, our T21-XIST system is induced with near-
409 uniformity [Fig. 1] and corrects T21 dosage to euploid levels across chr21 [Fig. 2], which we
410 demonstrate by inclusion of isogenic euploid control hiPSCs lines we derived in parallel from a
411 male donor with mosaic T21 [36]. Leveraging phased sequence variants from linked-read WGS, we
412 can assign mRNA-seq reads allele-specifically to show that chr21 genes derive half of their
413 expression from the chr21^{P} allele in euploid (D21) cells, and one-third in T21 cells, but nearly
414 extinguish expression from this *XIST*-bearing copy after dox induction [Fig. 2].

415 *XIST*-mediated T21 dosage correction extends to genes responding to chr21 dosage on all
416 chromosomes, reflecting a transcriptome-wide reversion of gene expression that moves dox-
417 treated T21-XIST hiPSCs towards their isogenic euploid controls [Fig. 3]. The balanced
418 experimental design and stability of T21 silencing after dox withdrawal (w/d) enabled us to adjust
419 for dox effects that can inhibit mitochondrial translation [47] and selenocysteine incorporation [63].
420 GSEA reflects the large-scale reversion of T21-responsive cellular components, pathways and
421 functions [Fig. 3], including boosted expression of serine biosynthesis genes. These genes are
422 notable as dermal fibroblast of DS, as well as Patau (T13) and Edwards (T18) syndromes, depend
423 on serine for proliferation [20]. As such genes return to baseline upon correction of T21 dosage [Fig.
424 3], despite maintenance of the chr21 copy number, metabolic demand for serine, while common
425 to all three viable human autosomal aneuploidies, results from expression of the supernumerary
426 chromosome rather than its presence. We also find resurgent expression of ribosomal protein
427 genes and translation factors in T21-dosage corrected hiPSCs [Fig. 3], which may be due to a
428 resetting of the integrating stress response [14]. Indeed, cellular stress pathways and apoptosis are
429 dampened in response to T21 dosage correction, which is consistent with the previously reported
430 excess of apoptosis [17,51,52], mitochondrial dysfunction [18,48–50] and oxidative stress [16,53] in DS.

431 In T21-driven expression of neural cultures, we find many of the same terms are enriched
432 by GSEA and concordant with T21 hiPSCs [Fig. 4]. In keeping with a recent DS meta-analysis [64],
433 the interferon response [Fig. S4], as well as mitochondrial pathways and apoptotic terms [Fig.

434 S3], are particularly exacerbated in T21 neural cultures. Consistent with these results, we
435 demonstrate impaired survival of T21 forebrain neurons and interneurons relative to isogenic
436 euploid controls [Fig. 5], as well as reduced translation [Fig. S5]. Because these enriched terms
437 and related genes responded to *XIST*-mediated T21 dosage correction in hiPSCs, they likely reflect
438 cellular phenotypes that depend on ongoing excess chr21 expression, and may therefore remain
439 amenable to T21 dosage correction after completed development.

440 During early neural differentiation, we observe that T21 NPCs produce an excess of
441 astrocytes at the expense of neurons [Fig. 6], which is consistent with astrogliosis noted in fetal
442 DS [28,30,32]. Because *XIST*-mediated T21 silencing remains inducible during differentiation and
443 persists into the NPC stage, neurogenesis is fully rescued when T21-dosage is corrected [Fig. 6].
444 This is consistent with a prior report using T21-*XIST* dosage correction [27], but we can attribute
445 this neurogenic rescue to suppression of a conserved T21 astrogenic bias, rather than slowed
446 commitment of T21 NPCs [Fig. 6]. We replicate this observation at the onset of cortical
447 astrogenesis in Ts65Dn embryos (E18.5), which indeed show a significant increase in S100B+ cells
448 relative to wildtype embryos, while the density of Ki67+ neural progenitors remains unchanged
449 [Fig. S6]. Several chr21 genes have been suggested to play a role in this astrogenic lineage bias,
450 including amyloid precursor *APP, S100B*, and *DYRK1A* [30,34]. In addition, it is possible that cellular
451 stress pathways impact NPC lineage decisions, as seen in rodent models of neurogenesis [65]. For
452 example, oxidative stress is elevated in DS hiPSC-derived neurons and NPCs [40,66] and also
453 implicates these and other chr21 genes [16,30,53].

454 We also examined T21-*XIST* hiPSC-derived mature neural lineages after forcing NPCs to
455 terminally differentiate and thus commit to terminal neural cell fates via notch-inhibitor DAPT
456 [Fig. 7A,B]. This more mature (~9-12wk), post-mitotic neural population is composed of
457 terminally differentiated neurons and astrocytes, as well as residual neural progenitors and some
458 oligodendroglial lineages [Fig. 8, S6]. Remarkably, a large fraction of this terminal post-mitotic
459 population can induce *XIST* and attract repressive H2AK119ub on chr21 in response to dox [Fig.
460 7], regardless of whether they were never exposed to dox prior to cessation of the DAPT
461 treatment (post-DAPT, "pDAPT/pD"), or were treated with only a low ("LD", 1% of the standard

462 1 µM) dox concentration prior to DAPT treatment. In contrast, a previously reported T21-*XIST*
463 system could not be induced at all anymore in post-mitotic monolayer neural cultures [27].
464 Our results indicate our T21-*XIST* system represents a major improvement, which we
465 attribute to technical characteristics of transgene expression. First, our system uses a *UBC-rTTA*
466 construct integrated at the AAVS "safe-harbor" locus, in contrast to prior studies that used EF1a
467 [21] or CAG promoters [27] at this locus or re-delivered a high-copy *EF1α-rTTA* construct via piggyBac
468 to NPCs [67]. Second, there may be differences in these human *XIST* cDNA constructs on account
469 of sequence variation or splicing. And third, we integrated our *XIST* cDNA transgene outside of
470 *DYRK1A*, to minimize its impact on *DYRK1A* prior to dox treatment, but also to avoid attracting
471 gene-body associated DNAme from overlapping transcription, which serves to suppress
472 intragenic transcription start sites [68]. We suspect this last aspect to have contributed most, but
473 cannot rule out a confluence of AAVS1 regulation and/or rtTA promoter selection to play a role
474 as well.
475 We assessed whether *XIST* induction in terminal, post-mitotic neural cell types ("pD") was
476 as effective in correcting T21 dosage as it was during differentiation ("d0") using three
477 independent analytic approaches: chr21^{P} allele representation, normalized expression across
478 chr21, and differential expression across the transcriptome [Fig. 8, S6, S7]. The chr21^{P} allele is
479 effectively repressed to nearly the same degree in *XIST+* pD as in d0 cells, especially in neurons.
480 [Fig. 8B,S6]. Likewise, both the median sum of normalized chr21 expression by cell, as well as the
481 entire distribution of cells, shift toward the euploid mean ratio [Fig. 8, S6]. The same significant
482 shift is observed when aggregating the mean ratio across cells for individual genes [Fig. 8, S6].
483 While some residual cycling NPCs retain excess expression in a subset of chr21 genes far (~20
484 Mbp) from the *XIST* integration site [Fig. S7], both normalized expression and allele-specific
485 metrics indicate *XIST* represses T21 dosage across all cell type groups, being most effective in
486 post-mitotic neurons. The transcriptome-wide analysis mirrors these results: differential
487 expression in *XIST*$^{+}$ d0 and pD cells correlates significantly with that of euploid cells when
488 compared to non-induced T21 cells [Fig. 8E]. Likewise, canonical pathways significantly enriched
489 by GSEA in *XIST*$^{+}$ d0 and pD cells match and follow the direction of change observed in the euploid
490 to T21 comparison [Fig. 8, S7]. In sum, these data demonstrate for the very first time that post-

491 mitotic terminal neural cell types remain responsive to chr21 gene dosage. This motivates several
492 avenues for future investigation, for example to dissect neuron-intrinsic vs. extrinsic phenotypes
493 [69] that will be relevant for developing cell-type specific interventions [70], or to identify pathways
494 that remain amenable to novel therapeutic approaches [14].

495 Regarding *XIST* biology, our results suggest human neural lineages remain competent to
496 initiate *XIST*-mediated heterochromatin deposition even non-cycling cells, provided *XIST* can be
497 induced. Similar developmental plasticity in initiating [25,26], or re-initiating [71,72] *XIST*-mediated
498 repression was previously reported only for proliferative hematopoietic cell lineages. Our work
499 therefore suggests *XIST* may be able to re-establish X chromosome inactivation even outside
500 pluripotency and hematopoiesis, which is relevant to reported X chromosome conformational
501 changes in the female brain across the estrous cycle [73].

502 Additionally, prior reports of *XIST* induction in human HT1080 fibrosarcoma cells indicate
503 that spreading of autosomal gene silencing remains reversible [74] and remains only effective near
504 its site of integration [75]. Both of these observations may be impacted in part by *XIST* impacting
505 haploinsufficient genes, which are not uncommon on mammalian autosomes, making whole or
506 partial trisomy a valuable research tool to better understands the determinants of *XIST* spreading
507 [76]. In hiPSCs, our T21-XIST system repressed virtually every chr21-linked gene without
508 measurable impact of its linear distance from the *XIST* integration site across a large span of
509 expression values, and in irreversible fashion [Fig. 2]. Interestingly, the human inactive X in
510 pluripotent stem cells frequently undergoes progressive reactivation following loss of *XIST*
511 expression [77], the order of which we recently showed was determined in part by the distance to
512 the nearest escapee gene [78]. While our T21-*XIST* dox withdrawal was limited to 3-6 weeks, we
513 found little evidence of reactivation over this period, indicating that DNA methylation served to
514 maintain T21 silencing [Fig. 2]. Whether this remarkable epigenetic stability was due to the
515 paucity of chr21 genes escaping *XIST* to seed potential reactivation, or the levels of *XIST* prior to
516 dox withdrawal, and to which degree these two aspects are related, represent additional areas
517 for future investigation.
518
519

520 **FIGURE LEGENDS**

521

522 **Figure 1: T21 hiPSCs induce *XIST* transgene and deposit heterochromatin on one chr21 allele**

523 (A) Schematic of cell lines and sample collection timepoints and dox treatment conditions.
524 Samples were collected after 3-9 wks of +dox or no-dox treatment, or 3 wk of +dox and 3 or 6
525 wks of dox withdrawal. A subset of samples was treated with dox for 2mo+ prior to
526 collection/withdrawal.

527 (B) Representative images (left) for XIST-FISH, and H2AK119ub and H3K27me3 IF, and image
528 quantification (right). Control panel of XIST-negative cells after 3wk of dox w/d for XIST-FISH is
529 included on the left.

530 (C) *XIST* expression in T21-XIST samples from mRNA-seq (VST, variance-stabilized
531 transformed counts, approaching a log-transformation).

532

533 **Figure 2: *XIST*-mediated silencing extends across entire chr21^{P} and attracts DNAme**

534 (A) Representation of M/M2 and P alleles of all protein-coding chr21 genes across dox
535 conditions and genotypes. Thick black line at 0.5 marks expected biallelic expression in euploid
536 (D21) cells, and dashed thin black line expected 1/3rd representation for chr21^{P} allele in T21 cells.

537 (B) Heatmap showing VST counts normalized to the mean count in T21 samples (left panels)
538 for all expressed genes on chr21.

539 (C) Distribution of log2(FC) (log2-foldChange) relative to no-dox T21 samples, for all
540 differentially expressed genes (DEGs, p.adj≤0.1). "Combined" represents the differential
541 expression condition used in subsequent figures. Blue and green shaded bands correspond to
542 ±10% and ±0.15 variation from a log2(FC) of -0.59 (dashed vertical line, log2FC expected for
543 complete T21 dosage correction).

544 (D) Log2(FC) values across chr21 genes (arranged by position) compared to T21 samples.
545 Bubble colors shaded as indicated in the legend above, bubble size reflects base mean
546 expression.

547 (E) As in (B), displaying only genes that maintain a LAF ≥ 1/6th in allelic T21 mRNA-seq in +dox
548 or w/d conditions.

549 (F) Distribution of DNAme values from promoter-associated methylEPIC probes on chr21
550 genes in T21-XIST no-dox, +dox, and w/d. Bimodal distribution medians of probes > and ≤ 60%
551 methylated displayed on their respective peaks.
552
553 **Figure 3: Transcriptome-wide response to T21 gene dosage and correction via *XIST***
554 (A) Fraction of genes differentially expressed in response to T21 (top, relative to euploid) and
555 T21-XIST dosage correction (bottom, relative to T21 and no-dox) by direction (increasing,
556 decreasing, or unchanged, ~0) and by chromosome.
557 (B) PCA of all mRNA-seq samples, batch-corrected for timepoint and dox status. Open
558 symbols for original euploid (198-1/2) and T21 (198-5) lines, filled symbols for T21-XIST transgenic
559 lines (c1/4/7). Colors indicate effective chr21 dosage as indicated in Figures 1 and 2 (w/d colored
560 separately to distinguish from +dox T21-XIST samples).
561 (C) Overlap of differential expression between hiPSC T21/D21 (y-axis) over the dox effect
562 (left, non-transgene +dox/no-dox) or T21-XIST dosage correction (right, T21XIST+dox &
563 withdrawal / T21). Adjusted p-values combined by Fisher's method (C.padj) for all DEGs, and
564 colored by -log10(C.padj) provided C.padj ≤0.1. Linear regression plotted with the Spearman
565 correlation coefficient and p-value for all genes.
566 (D) Overlap of enriched GSEA terms comparing normalized enrichment scores (NES) for hiPSC
567 T21/D21 over dosage-corrected T21-XIST/T21. Bubbles correspond to the -log10(combined
568 pvalue by Fisher's method). Colors denote chosen categories (all with combined pvalue ≤ 0.2).
569 (E) Individual significantly-enriched terms (combined p≤0.1) from each of the categories in
570 (D), selected by concordance measure (Euclidean distance from origin in D). Colors denote NES,
571 bubble size the individual -log10(p.adj).
572
573 **Figure 4: Concordant T21 dosage imbalance and transcriptomic response after neural**
574 **differentiation**
575 (A) Heatmap showing VST counts normalized to the mean count in T21 neural samples (left
576 panels) for all expressed genes on chr21.

577 (B) Distribution of log2(fold-change) from chr21 genes comparing neural T21 to D21. Dashed
578 black line denotes expected log2(FC) of +0.59 (includes all chr21 genes, irrespective of p.adj).
579 (C) Overlap of differential expression between hiPSC T21/D21 (y-axis) over neural T21/D21
580 samples (x-axis). Adjusted p-values were combined and colored as in Fig. 3C, with linear
581 regression line (dashed, with Spearman coefficient and p-value for all genes with c.padj ≤ 0.1).
582 (D) Overlap of enriched GSEA terms comparing normalized enrichment scores (NES) for hiPSC
583 T21/D21 (y-axis) over neural T21/D21. Bubble plots corresponds to the -log10(combined pvalue
584 by Fisher's method). Colors denote chosen categories (all with combined pvalue ≤ 0.2).
585 (E) As in (D) but combining neural T21/D21 GSEA (x-axis) with GSEA of dosage-corrected T21-
586 XIST hiPSCs (hiPSC rescue) on the y-axis.
587
588 **Figure 5: Increased cell death of T21 relative to euploid interneurons and forebrain neurons**
589 (A) Representative sequence of a TS21 interneuron labeled with RGEDI-P2a-EGFP dying
590 over the course of imaging and a control neuron surviving during the course of imaging
591 (scale/width is 10 µm).
592 (B) Plot of the cumulative rate of death of TS21 and control interneurons. Step functions
593 correspond to the mean cumulative death rate across wells per line and the faint lines
594 represent each well replicate (n = 6 wells each, HR = 4.08, Linear mixed model with Holm
595 correction, ** *p* <0.01). C) as in B) for the cumulative rate of death of TS21 and control
596 forebrain neurons (n = 6 wells each, HR = 2.57, Linear mixed model with Holm correction, ** *p*
597 <0.01).
598
599 **Figure 6: Early astrogenesis at neurogenic expense is suppressed by T21 dosage correction prior**
600 **to NPC stage**
601 (A) Representative images (left) and quantification (right) of H2AK119ub (IF) and XIST (FISH)
602 signal after neural differentiation from dox treated cells. Dox treatment initiated while cells were
603 hiPSCs (left top), or on the first day of differentiation ("d0", left bottom).
604 (B) Relative lineage commitment to astrocytes (S100b) or neurons (MAP2) in euploid, T21,
605 and T21-XIST cells under dox treatment. Symbols (Wilcox p-value level, or NS for p > 0.05) above

606 each boxplot denote significance of median difference from the corresponding euploid (198-1/2)
607 condition, whereas symbols below each boxplot compare to T21 (198-5) lineage proportions.
608 (C) Representative images at d28 of neural differentiation in euploid (left column), trisomic
609 (center column) and rescued (right column) labeled for neuronal MAP2 (red), astroglial S100B
610 (green) and H2AK119ub accumulating on chr21 (white). Insets (labeled 1,2) display H2AK119ub
611 foci in both astrocytes and neurons.
612
613 **Figure 7: Post-mitotic neurons and astrocytes can induce *XIST* and deposit H2AK119ub after**
614 **terminal lineage commitment**
615 (A) Sample and timeline schematic of XIST induction during (d0+dox) and after terminal
616 neural differentiation post DAPT treatment (pD+dox). Cell cycle exit of NPCs is assessed by EdU-
617 labeling of S-phase transiting cells, with co-detection of *XIST* by FISH after 2 days in dox media.
618 Chr21 Barr body formation (H2AK119Ub) and lineage commitment (MAP2/S100B) are assessed
619 by IF in 8 wk and 10-12 wk cultures.
620 (B) Representative *XIST* (red) and EdU (green) image and quantification of d36 neural
621 differentiation following 4d of DAPT and 2d of dox treatment. Less than 6% of cells were EdU+
622 (green), EdU+XIST+ cells represented less than 2% of total cells.
623 (C,D) Representative images (C) and quantification (D) of H2AK119ub (yellow) IF and XIST-FISH
624 (red) signal after neural differentiation from different dox treatments. Bottom pDAPT panel
625 shows H2AK119ub IF (white) co-stained with MAP2 (red) and S100B (green).
626
627 **Figure 8: Effective monoallelic repression of chr21 genes by *XIST* induced in terminal neurons**
628 **and astrocytes**
629 (A) Single-nuclei RNA-seq UMAP of terminal 10-12 wk neural cultures (as outlined in Fig. 7A).
630 Integrated UMAP colored by original clusters, granular and grouped cell types, cell cycle stage,
631 XIST count categories and samples.
632 (B) Paternal allele fraction by cell, aggregated over all chr21 genes. Cells are split by sample
633 (D21a/b, T21XIST-nodox, d0+dox, pD+dox) and *XIST* status (0, >1 counts denoted “X-“ and “X+”),
634 except for euploid lines (absent XIST transgene, grey). Median paternal allele fraction and

635 significance of difference in medians relative to T21-nodox cells (Wilcox p-value) are denoted
636 below and above boxplot, respectively.
637 (C) Normalized gene expression ratio (chr21 over genome mean excluding chr21) by cell,
638 aggregated over all chr21 genes. Cells are split as in B (excluding cell type group "Other"), with
639 median expression ratio and difference in medians relative to T21-nodox cells (Wilcox p-value)
640 denoted above and below boxplot, respectively.
641 (D) Normalized expression ratio by chr21 gene, aggregated over cells. Mean ratios per gene
642 calculated from cells are split as in C. Respective median expression ratios and difference in
643 medians relative to T21-nodox cells (Wilcox p-value) denoted above and below boxplots.
644 (E) Differential expression of non-chr21 genes relative to XIST-negative postDAPT and T21-
645 nodox cells. Sample-averaged log2(FC) values in euploid cells (x-axis) correlate with XIST-positive
646 d0 and pDAPT log2(FC) values (y-axis), faceted by cell type group. Colors and transparency denote
647 respectively, ROC power and ROC power of the euploid relative to T21 comparison. Regression
648 line (black), Pearson coefficient and p-value (Fisher-transformed) denoted on each plot.
649 (F) GSEA results of differential expression comparisons depicted in (E), correlating
650 normalized enrichment scores (NES) and plotted as in (E). Plotted MSigDB gene sets limited to
651 Hallmark and Canonical pathways subsets significant (q-value ≤ 0.1) in T21/euploid comparison
652 (x-axis). Colors and transparency denote respectively, -log10-transformed q-values of each
653 comparison and the euploid to T21 comparison. Regression line (grey), Pearson coefficient and
654 p-value (Fisher-transformed) denoted on each plot.
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664 **SUPPLEMENTARY FIGURE LEGENDS**

665 **Figure S1**

666 (A) Taqman copy number (CN) assay for 21q22 (*DYRK1A*) in GM00260-derived hiPSC clones.

667 (B) Taqman CN assay for 17q25 (*SEC14L1*) in GM00260-derived hiPSC clones.

668 (C) DNA SureFISH for chr21q, and FastFISH chrX, chr8, chr12, and chr17 centromeres in 198-
669 6/8 with counts and images denoting the dominant staining pattern.

670 (D) Summary of CytoSNP850k calls across GM00260 and its reprogrammed hiPSC clones. Calls
671 marked with a red 'x' were common across all lines, including donor fibroblasts. Call marked with
672 a small 'x' were line-specific, except for T21 (denoted by a capital 'X').

673 (E) Absolute difference in allele (theta) frequency of CytoSNP850k probes comparing T21 and
674 euploid clones (D21), colored by T21-specific base calls (red/blue) and hetero/homozygosity
675 (grey and yellow, respectively).

676 (F) DNA FISH against DSCR8 (Vysis, LSI21) in interphase and metaphase spreads of validated
677 hiPSC clones, with counts and images denoting the dominant staining pattern.

678 (G) Taqman CN assay for 21q22 (*DYRK1A*) across all AAVS-rTTA subclones of T21 198-5 hiPSCs.

679

680 **Figure S2**

681 (A) Germ layer-specific and pluripotency marker expression across the euploid (198-1/2), T21
682 (198-5), and transgenic T21-XIST (c1/4/7) hiPSC lines.

683 (B) Change in log2-transformed expression relative to corresponding T21 samples in this and
684 the two referenced T21-XIST studies. Mean difference of chr21 gene expression comparing w/d
685 T21-XIST samples and T21 samples. Line type denotes the length of the initial dox treatment, and
686 color the duration of dox w/d.

687 (C) Mean difference of chr21 gene expression between w/d T21-XIST and T21 samples. Line
688 type denotes the length of the initial dox treatment, and color the duration of dox w/d.

689

690 **Figure S3**

691 (A) Same as Fig. 3C but excluding chr21 genes: overlap of differential expression between
692 hiPSC T21/D21 (y-axis) over the dox effect (top, non-transgene +dox/no-dox) or T21-XIST dosage

693 correction (T21XIST+dox & withdrawal / T21). Adjusted p-values were combined (C.padj,
694 calculated using Fisher's method), and colored by -log10(C.padj) provided C.padj ≤0.1 (or grey for
695 C.padj > 0.1). Linear regression is plotted along with the Spearman correlation coefficient and p-
696 value for all non-chr21 genes.
697 (B) Individual significantly-enriched terms (combined p ≤ 0.1) from each of the categories in
698 Fig. 4(D,E), selected by a concordance measure (euclidean distance from origin in D). Colors
699 denote NES, bubble size the individual -log10(p.adj).
700
701 **Figure S4**
702 Comparison of terms over-represented in GSEA across hiPSC T21, neural T21, T21 dosage
703 correction ("rescue"), and the dox-effect in non-transgenic hiPSCs. Terms from Hallmark, KEGG,
704 and WikiPathways (WP) are shown. All terms are sorted by hiPSC T21 NES (normalized
705 enrichment score from GSEA), with a p.adj thresholds of p ≤ 0.1 for hiPSC T21, and additionally
706 p.adj ≤ 0.2 for the three additional panels (neural T21, T21 rescue, and dox-effect).
707
708 **Figure S5**
709 (A) Representative images of TS21 interneuron and euploid control cultures transduced
710 with hSyn1:RGEDI-P2a-EGFP lentivirus. Scale bar = 100 µm.
711 (B) Quantification of the GEDI ratio (mean RGEDI/mean EGFP) across each interneuron per
712 time point (T21 n= 8810, D21/euploid control n= 15,999). Live/dead threshold of 0.15 (red line)
713 (C) as in (B), but across each forebrain neuron per time point (T21 n= 30,735, D21/euploid
714 control n= 24,950).
715 (D) Number of cells tracked by segmentation in independent wells of T21 and D21 neurons
716 across all timepoints.
717 (E) Fluorescence intensity signal of RGEDI, EGFP and RGEDI/EGFP ratios in live (RGEDI/EGFP
718 ratio < 0.15) T21 and euploid control neurons ($p \leq 1x10^8$, linear mixed model with Holm
719 correction).
720 (F) Anti-GFAP (red) and DAPI (blue) staining of cultures from (A) showing abnormal glia
721 morphology. Scale bar = 300 µm.

722 **Figure S6**

723 (A) S100B$^+$ cell density and representative images of E18.5 wildtype and Ts65dn cortical
724 slices (100 µm scale bars). Two-tailed, independent samples Student's t-test $p \leq 0.05$ (*).

725 (B) Ki67$^+$ cell density and representative images of E18.5 wildtype and Ts65dn cortical slices
726 (60 µm scale bars). Two-tailed, independent samples Student's t-test $p > 0.05$ (n.s.).

727

728 **Figure S7**

729 (A) Sample proportions (% nuclei) of assigned cell types, cell cycle stage and *XIST* status
730 faceted by cell type group.

731 (B) Paternal allele fraction by cell, aggregated over all chr21 genes, and faceted by cell type
732 group. Cells split by sample (D21a/b, T21XIST-nodox, d0+dox, pD+dox) and *XIST* status (0, >1
733 counts denoted "X-" and "X+"), except for euploid lines (absent XIST transgene, grey). Median
734 paternal allele fraction and significance of difference in medians relative to T21-nodox cells
735 (Wilcox p-value) are denoted below and above boxplot, respectively.

736 (C) Left: Normalized gene expression ratio (chr21 over genome mean excluding chr21) by cell,
737 aggregated over all chr21 genes, and faceted by cell type group. Cells are split as in B, with median
738 expression ratio and difference in medians relative to T21-nodox cells (Wilcox p-value) denoted
739 above and below boxplot, respectively. Right: empirical cumulative distributions (eCDF) of
740 corresponding panels on the left, colored by sample and XIST-positive cells represented by
741 dashed lines. T21-nodox 95% confidence interval denoted by transparent ribbon (purple).
742 Sample-colored p-values (Kolmogorov-Smirnov, KS-test) denotes significance of distribution of
743 shift from the T21-nodox sample. Grey lines indicate the 50th percentile cell (horizonal), and chr21
744 to genome-wide mean ratio (vertical 1.0 and 1.5).

745 (D) Left: Normalized expression ratio by chr21 gene, aggregated over cells, and faceted by
746 cell type group. Mean ratios per gene calculated from cells are split as in C. Respective median
747 expression ratios and difference in medians relative to T21-nodox cells (Wilcox p-value) denoted
748 above and below boxplots. Right: eCDF of corresponding panels of the left, with lines colored as
749 in C. T21-nodox confidence-interval, sample-colored p-values (KS-test) and grey vertical lines s in
750 C, with the horizontal grey line indicating the 50th percentile chr21 gene.

751 (E,F) Cumulative distributions aggregating across cell type groups (right panels in C & D),
752 excluding the "Other" group. Lines, sample-colored p-values (KS-test), grey horizontal and
753 vertical lines as in C and D, respectively.
754 (G) Differential expression of chr21 genes relative to XIST-negative postDAPT and T21-nodox
755 cells. Average log2(FC) values in euploid cells (x-axis) correlate with XIST-positive d0 and pDAPT
756 log2(FC) values (y-axis), faceted by cell type group. Colors and transparency denote respectively,
757 ROC power and ROC power of the euploid relative to T21 comparison. Regression line (black),
758 pearson coefficient and p-value (Fisher-transformed) denoted on each plot.
759
760 **Figure S8**
761 (A) SC-transformed sample-averaged log2(FC) values relative to XIST-negative T21-nodox
762 and pDAPT cells faceted by cell type group. Median sample-averaged log2(FC) values of euploid
763 (D21a/b), and XIST-positive d0+dox and pD+dox samples, and significance of median differences
764 (Wilcox p-value) from T21 to euploid comparison denoted below and above corresponding
765 boxplot.
766 (B) Loess-smoothened SC-transformed log2FC values of differential expression comparisons
767 (as in A, with 95% confidence intervals) across chr21 coordinates of transcriptional start sites
768 (TSS, in Mbp).
769 (C) SC-transformed sample-averaged log2(FC) values of d0+dox and pD+dox comparisons
770 (colored as in A,B) plotted over the TSS distance from the *XIST* transgene integration site, faceted
771 by cell type group. Regression lines, 95% confidence interval ribbons, pearson coefficient (R), and
772 p-value (fisher-transformed R) indicated on each plot, black horizontal line denotes no change.
773 (D) Normalized enrichment scores (NES, x-axis) of GSEA against Hallmark and Canonical
774 pathways MSigDB subsets in euploid (center) and XIST-positive d0+dox and pD+dox cells. Plotted
775 categories (y-axis) limited to significant enrichment across at two comparisons (pairwise Fisher's
776 method combined p-value ≤ 0.005). Colors and transparency denote respectively, NES sign and -
777 log10-transformed q-values (capped at 3, q <= 0.001).
778
779

780 **MATERIALS & METHODS**

781

782 **Reprogramming, hiPSC validation and culture**

783 Human male fibroblast line (GM00260) mosaic for trisomy 21 (T21) was obtained from the Coriell
784 Institute (Camden, NJ) and reprogrammed by the University of Connecticut's Cell and Genome
785 Engineering Core, using the CytoTune hiPSC 2.0 Sendai Reprogramming kit (Thermo Fisher
786 Scientific, Waltham, MA). Genomic DNA from hiPSC clones was screened via duplex Taqman Copy
787 Number (CN) Assay (*DYRK1A* - Hs01748441_cn and RNaseP, Thermo Fisher Scientific) to select
788 euploid and T21 lines, followed by additional screening for Chr17q Taqman CN assay (*SEC14L1*,
789 Hs05476397_cn), and the hPSC Genetic Analysis Kit (Stemcell Technologies, Vancouver, Canada).
790 Interphase and M phase nuclei were also screened via FASTFISH (WiCell, Madison, WI) using Chr
791 20q (BCL2L1) and centromere enumeration probes for Chr 8, 12, 17, and X, alongside a SureFISH
792 assay for Chr 21q (DSCR8). Selected hiPSC lines were further screened by aCGH (Infinium
793 BeadChip CytoSNP 850K assay, Illumina, San Diego, CA) at the Center for Genome Innovation,
794 Institute for Systems Genomics at the University of Connecticut.
795 T21 and euploid hiPSCs were cultured on mitotically inactive mouse embryonic fibroblasts (mEFs)
796 in standard hiPSC media in 5% CO_2 at 37°C (80% DMEM/F12, 20% Knockout Serum Replacement,
797 1% Glutamax, 1% Non-Essential Amino Acids, 0.1% ß-Mercaptoethanol, and 8ng/mL bFGF,
798 Thermo Fisher Scientific), and passaged weekly by manual disruption of 6-10 representative
799 colonies. For hiPSC cultures requiring doxycycline induction, 2 µM doxycycline was added to the
800 culture media.

801

802 **Neural differentiation**

803 T21 and euploid hiPSCs were passaged manually onto mEFs and in hiPSC medium. On day 2 post-
804 passage, cultures were switched into N2B27 neural differentiation medium (Neurobasal medium
805 (Gibco), 2% B27 (Gibco), 1% N2(DMEM:F12 (Gibco), 25ug/ml insulin (Sigma), 100ug/ml holo-
806 transferrin, 100 µM putrescine, 20nM progesterone, 30nM selenite), 1% ITS-A (Gibco), 1%
807 Penicillin-Streptomycin (Gibco), and 1% Glutamax (Gibco), supplemented with noggin at 500
808 ng/ml (R&D Systems). Cultures were fed and supplemented with Noggin every other day through

809 day 10. For neural differentiation with doxycycline induction, 1 µM doxycycline, or 0.01 µM for
810 low-dose cultures, was added to the culture media. Cultures were fed N2B27 until the
811 appearance of mature rosette structures (D14-D16). Rosette-stage cultures were passaged
812 manually 1:1 onto poly-d-lysine (PDL) and laminin-coated tissue culture plates and grown to
813 confluence. Cells were cultured until d23 and passaged enzymatically with Accutase (Invitrogen)
814 onto PDL and laminin-coated substrates at 1.9x10^4 cells/cm^2- 2.2x10^4 cells/cm^2. At d25,
815 cultures were switched to Neural Differentiation medium (NDM) (Neurobasal medium (Gibco),
816 2% B27 (Gibco), 1% non-essential amino acids (NEAA; Gibco), 1% Penicillin-Streptomycin (Gibco)
817 2mM Glutamax (Gibco) 10 ng/ml brain-derived neurotrophic factor (BDNF; Peprotech), 10ng/ml
818 glial-derived neurotrophic factor (GDNF, Peprotech), 1 µM cyclic AMP (cAMP, Sigma), and 200
819 µM ascorbic acid (AA, Sigma). Cells were cultured in NDM until assay endpoint. For DAPT-treated
820 cultures, DAPT (Tocris Bioscience) was added for 5 days at 10 µM. For EdU labeling, Edu was
821 added at 5 µM for 24 hours, and detected using the Click-iT EdU Imaging Kit (Invitrogen).
822 Doxycycline (1 µM) was added to EdU-labeled, pDAPT cultures at the time of EdU
823 addition/labeling.
824
825 **Nucleic acid purification**
826 Genomic DNA was purified from fresh or frozen pelleted cells using three different methods
827 depending on application. For colony PCR screening, genomic DNA was extracted via standard
828 high-salt chloroform extraction. For standard PCR, and array hybridization, cell pellets were
829 resuspended in either cell lysis buffer 1 (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 10 mM EDTA, pH
830 8.0; 1% SDS; 200 µg/ml Proteinase K), or cell lysis buffer 2 (10 mM Tris-HCl, pH 8.0; 200 mM NaCl;
831 10 mM EDTA, pH 8.0, 0.5% Sarcosyl; 1 mg/mL Proteinase K) and incubated at 55°C for 3 hours to
832 overnight with optional RNAse A, 10 µg/mL treatment following at 37C for 5 minutes. Genomic
833 DNA was isolated using Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen, Waltham,
834 MA), precipitated with either isopropanol or 300 mM sodium acetate with 2 volumes of 100%
835 chilled ethanol and 15 minutes at -80C, washed with 70% ethanol, and resuspended in TE buffer.
836 For linked-read WGS, high molecular weight (HMW) genomic DNA was prepared from fresh or
837 frozen hiPSC cell pellets, following the protocol listed in the Chromium Genome Reagent Kits v2

838 User Guide (10x Genomics, Pleasanton, CA), using the MagAttract HMW Kit (Qiagen,
839 Germantown, MD) with minor modifications. Plasmid and fosmid DNA was purified with either
840 the Zyppy Plasmid Miniprep kit or ZymoPURE II Plasmid Midiprep kit (Zymo Research, Irvine, CA).
841 Following restriction digests or PCR, DNA purification was performed with the Monarch PCR &
842 DNA Cleanup kit (New England Biolabs, Ipswich, MA), or the DNA Clean & Concentrator Kit (Zymo
843 Research). For RNA isolation, the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) was used,
844 followed by cDNA synthesis using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad
845 Laboratories, Hercules, CA).
846
847 **Quantitative RT-PCR**
848 Gene expression was measured via qRT-PCR using the iTaq Universal SYBR Green Supermix (Bio-
849 Rad Laboratories) and the ΔCt method. Primer specificity was confirmed by melting curve
850 analysis, and relative expression was calculated as %GAPDH ($100/2^{\Delta Ct}$). Taqman CN assays were
851 normalized to 198-1 genomic DNA. HOT FIREPol Probe Universal PCR Mix Plus - no ROX (Solis
852 BioDyne, Tartu, Estonia) was used per manufacturer's instructions, using 4 ng of high-quality
853 genomic DNA template in 20 µl reactions. The hPSC Genetic Analysis Kit (Stemcell Technologies)
854 was used following the manufacturer's instructions. All qPCR reactions were performed in
855 triplicate in a Bio-Rad CFX 96 Real-Time System.
856
857 **Plasmid construction**
858 For PCR amplifications, either Herculase II polymerase (Agilent, Santa Clara, CA), Platinum Taq
859 High Fidelity (Thermo Fisher Scientific), Advantage 2 polymerase (TaKaRa Bio, Shiga, Japan), or
860 Q5 Hi-Fidelity DNA Polymerase (New England Biolabs) was used. For cloning reactions except the
861 large *XIST* insert steps, ligations were performed with T4 DNA ligase, 2,000,000 units/mL (New
862 England Biolabs) and DH10B homemade chemically competent cells were used at 37°C. For all
863 cloning with XIST cDNA fragments aside from ligation of oligo not followed by transformation
864 (which used the same T4 DNA ligase), the TaKaRa DNA Ligation Kit LONG (TaKaRa Bio) was used
865 along with NEB Stable Competent E. coli (New England Biolabs) with bacterial incubations at 25-
866 30°C to mitigate instability in initial steps observed at 37°C.

867 To clone the XIST transgene cDNA in a temporary pUC19 vector, a 10 kb fragment containing
868 most of XIST exon 1 was cut from a fosmid (WI2-935P22, BACPAC Genomics) with SacII and PshAI.
869 Linker oligos were used to clone the 10 kb SacII and PshAI fragment into the KpnI and XbaI sites
870 of pUC19, respectively. The 5'-most end of *XIST* exon 1 was PCR amplified, concurrently adding a
871 5' PmeI restriction site. The resulting PCR product was digested with PmeI and BtgZI and cloned
872 in between the PmeI/BtgZI restriction sites of the XIST exon 1-containg plasmid. The 3' end of
873 exon 1 through exon 6 was amplified from cDNA of Wi-38 cells (AG07217, Coriell Institute), while
874 adding a 3' SalI restriction site. The resulting PCR was digested with PshAI and SalI and cloned
875 between the PshAI and SalI sites of the plasmid from the prior step to complete the XIST cDNA
876 construct. The *UBC* promoter from the FUGW construct [79] (Addgene plasmid #14883) was cloned
877 into AAVS1-Neo-M2rtTA [80] (Addgene plasmid #60843). The *UBC* promoter was PCR-amplified
878 with flanking PvuII and FseI sites, and cloned into the AleI and FseI sites on the AAVS1-neo-
879 M2rtTA plasmid. This rtTA-advanced ORF was replaced with rtTA3G from the pCMV-Tet-3G
880 plasmid (TaKaRa Bio), by XbaI and XmaI digestion and cloning of three separate fragments to
881 create the final rtTA vector AAVS1-NEO-UBrtTA-3G.
882 To generate the final targeting vector, pTRE3G (TaKaRa Bio) was used as the backbone, following
883 excision of an XbaI fragment to reduce the size of the vector via removal of the SV40 small intron,
884 NLS, and SV40 polyA sequences. The 5' homology arm for chr21 was generated by PCR with EcoRI
885 sites on each primer using the genomic DNA of the destination cell line as the template. This PCR
886 product was ligated into the EcoRI sites of the pTRE3G vector to make pTRE3G-5P. Next a fusion
887 PCR product was generated, harboring a BGH polyA amplified from pl452 [81] and the 3' homology
888 arm using overlapping PCR from the genomic DNA of the destination cell line. This PCR product
889 was cloned into the PstI and BamHI sites of the pTRE3G-5P to make pTRE3G-5P3P. Next a loxP-
890 flanked PGK-puro-TK fragment was removed from vector pl452-puroTK by XhoI digest. The
891 fragment was cloned into the XhoI sites of the vector pTRE3G-5P3P. This pl452-puroTK vector
892 was derived from plasmids pl452 and plasmid pLCA.66/2272 [82] (Addgene plasmid #22733) by
893 removing the PGK-puroTK cassette with AgeI and BbsI and cloning that fragment into pl452 using
894 the same enzymes. To add the *XIST* cDNA construct, this CMV-puroTK-TRE3G vector was digested
895 with MluI and SbfI, and a phosphorylated linker oligo was ligated to purified, digested backbone,

896 creating a SacI-compatible overhang. Following purification, this vector was ligated to the SbfI-
897 SacI fragment containing the *XIST* cDNA construct, to create the TRE3G-XIST cDNA targeting
898 vector.
899
900 **hiPSC targeting and screening**
901 Targeting was performed using a Lonza Nucleofector 4D (Lonza, Basel, Switzerland) with primary
902 cell kit P3 and program CD-150. AAVS1-Neo-ubrtTA was targeted to chr21 of the T21 cell
903 line 198-5. Two ug of each AAVS1-targeting TALEN [83] was nucleofected along with 6 ug of AAVS1
904 integration vector. Cells were plated on mitotically inactivated DR4 MEFs (ATCC, Manassas,
905 Virginia) post-nucleofection. After 96 hours, cells were cultured with G418 at a concentration of
906 25 ug/ml. Selection was done for a total of 6 days. Resulting colonies were isolated and screened
907 for targeting. cDNAs from targeted clones were tested for expression of rtTA by RT-qPCR. AAVS1
908 TALEN-L (Addgene plasmid #59025) and AAVS1 TALEN-R (Addgene plasmid #59026) were used
909 for targeting the UBrtTA. Integrants were screened using (AAVS1 scr F/Neo R and AAVS1 3'scr
910 R/rtTA F). For targeting XIST transgene via CRISPR, guides designed to target the chr21P copy
911 without cutting the chr21$^{M/M2}$ alleles were cloned into PX459V2 (Addgene plasmid #48139) at
912 the BbsI site by annealing and ligating overlapping oligos. Two ug each this CRISPR guide plasmid
913 was used along with 6 ug XIST cDNA targeting vector. Cells were plated on DR4 MEFs and after
914 96 hours, puromycin was added to the culture at a concentration of 0.5 ug/ml for a total of 4
915 days. Resulting colonies were isolated and genotyped for targeting by PCR. Clones were verified
916 by sequencing across the target to verify the loss of the targeted allele.
917
918 **Immunofluorescence and *XIST* RNA-FISH**
919 For *XIST* RNA FISH, cells were permeabilized with 0.5% Triton-X and 2mM ribonucleoside vanadyl
920 complex (RVC) in CSK Buffer (10mM PIPES pH 6.8, 100mM NaCl, 3mM MgCl2, 300nM sucrose),
921 fixed in 4% PFA in PBS, then dehydrated through a series of ethanol washes (70%,80%, 90%, and
922 100%). Digoxigenin-labeled XIST probe hybridization was performed overnight in a humidity
923 chamber at 42°C. Anti-digoxigenin antibody (ThermoFisher; 700772) was hybridized for 1 hour at
924 RT. Secondary antibody hybridization (Alexa Fluor; Invitrogen; 1:500) followed for 1 hour at RT.

925 Nuclear DNA was counter-stained with Hoechst 33342 dye. Slides were imaged with an EVOS FL
926 Auto2 system (Thermo Fisher Scientific).
927 For immunofluorescence cells were grown in Nunc Lab-Tek II glass chamberslides coated with
928 poly-d-lysine and laminin (for neural differentiation) or permanox chamberslides with MEFs
929 (iPSCs). Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for
930 10 minutes at room temperature (RT), rinsed with PBS 3 times for 5 minutes and then
931 permeabilized in 1% Triton-X in PBS for another 10 minutes. Samples were blocked in blocking
932 buffer containing PBS, 0.1% Triton-X, 5% BSA and 2% normal goat serum (MilliporeSigma). Cells
933 were placed in antibodies diluted in blocking buffer overnight at 4°C in a humidity chamber.
934 Primary antibodies were used as follows: MAP2 (Abcam; ab5392; 1:10,000), S100B (Sigma; s2532;
935 1:1000), H2AK119ub (Cell Signaling; 8240, 1:1600). Following washes in 0.1% Triton-X in PBS, 3
936 times for 5 minutes, cells were labeled with Alexa Fluor secondary antibodies at 1:500 for 1 hour
937 at RT. Nuclei were counterstained with Hoechst 33342and slides preserved in Prolong Gold
938 Antifade Mountant (Thermo Fisher Scientific). IF and FISH automated image quantification was
939 performed using a custom CellProfiler pipeline [84].
940
941 **Cytogenomics, DNA methylation, Linked-read WGS and mRNA sequencing**
942 For cytogenomic analysis, genomic DNA was labeled and hybridized to the Infinium BeadChip
943 CytoSNP 850K array (Illumina, San Diego, CA) according to the manufacturer's protocols. For
944 DNAme analysis, genomic DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo
945 Research, Irvine, CA), then labeled and hybridized using the Infinium Methylation EPIC BeadChip
946 Kit (Illumina) following standard protocol of each manufacturer. Both types of arrays were
947 scanned on a NextSeq 550 system (Illumina).
948 HMW genomic DNA was sequenced to ~30x coverage on a NovaSeq 6000 (Illumina), following
949 library preparation on the 10X Genomics Linked-Read platform at the Center for Genome
950 Innovation, Institute for Systems Genomics at the University of Connecticut. For standard mRNA-
951 seq, libraries were prepared using the Illumina stranded mRNA Kit and 100bp paired-ends reads
952 were sequenced to an average depth pf 40 million reads/replicate on the NovaSeq 6000 (Illumina,
953 San Diego, CA).

954 **Single-nuclei RNA sequencing**

955 Neural cultures were treated with TrypLE (Life Technologies) for 4 minutes at 37C, and cells
956 counted with Trypan blue on the Countess Automated Cell Counter. Nuclei isolation was
957 performed with the Nuclei Isolation Kit: Nuclei EZ Prep (Sigma-Aldrich) with some modifications.
958 Briefly, cells were centrifuged at 1330 rpm for 3.5 minutes, media aspirated, and resuspended in
959 Nuclei PURE Lysis Buffer containing 1M DTT, 10% Triton X-100 and RNAsin. Nuclei were
960 centrifuged at 500g and 4C for 5 minutes in a swing-bucket rotor centrifuge, and lysis buffer was
961 aspirated. Nuclei resuspended in Nuclei PURE Sucrose Cushion buffer and RNAsin were pelleted
962 at 500g and 4C for 5 minutes, resuspended again in 25% iodixanol solution (280ul of Nuclei PURE
963 Sucrose Cushion buffer containing RNAsin + 200ul of 60% iodixanol), and under-layered by
964 corresponding 30% and 35% iodixanol solutions. Nuclei traverse the 25% and 30% layers of this
965 density gradient upon centrifugation (3166g at 4C for 30 minutes), and were collected from the
966 30% to 35% interface in volume of 300 µl. Resuspended in PURE Storage Buffer (1ml) and (5ul)
967 of RNAsin, nuclei were pelleted at 3166g for 15 minutes at 4C, resuspended in remaining 100ul,
968 and counted again. Isolated nuclei were stored at -80 until fixation. Nuclei fixation was performed
969 according to the Evercode Fixation v2 protocol (Parse Biosciences). Nuclei were counted after
970 fixation and barcoded using the Evercode WT mini v2, following the manufacturer's instructions.
971 Sublibraries were quantified using the Agilent Tapestation, pooled in proportion to their
972 respective nuclei counts, and sequenced on MiSeq v2 and NextSeq 550 v2.5 mid-output kits
973 (Illumina).

974

975 **Expression and DNA methylation analysis**

976 The FASTQ files were quality controlled using FASTQC, reads were filtered and adapter-trimmed
977 was done using BBDuk [85]. Expression quantification was performed using Salmon [86], and
978 differential expression analysis was performed using DESeq2 [87]. Gene set enrichments were
979 performed using the GSEA function from the R package clusterProfiler [88] on the following gene
980 sets: Biocarta, GO (Biological Processes, Cellular Components, and Molecular Functions), KEGG,
981 Pathway Interaction Database, Reactome, and WikiPathways. For DNAme analysis, the
982 MethylEPIC IDAT files were processed using the R package minfi and its preprocessIllumina

983 function to obtain methylation β-values [89]. Promoter probes were defined as having one of the
984 following designations in the array manifest file: TSS1500, TSS200, or 5_UTR.
985
986 **Phased allelic RNA-seq analysis**
987 The 10X Genomics Long Ranger pipeline was used to align reads, and call and phase SNPs, indels,
988 and structural variants from linked-read WGS reads. The processed FASTQs from above were
989 aligned using HISAT2 to the hg38 human reference genome [90]. Relevant chr21 SNPs were
990 extracted from the Long Ranger output and used to get haplotype gene expression counts from
991 aligned RNA-seq bam files using the phASER software package [91]. While this provided reliable
992 intragenic phasing, for intergenic phasing on chr21 we relied on the imbalanced haplotype gene
993 expression in the T21, compared to its isogenic euploid controls. The higher expressed haplotype
994 was designated as the major (duplicated or $chr21^{M/M2}$) haplotype and the other as the minor
995 haplotype ($chr21^{P}$).
996
997 **Single-nuclei RNA-seq analysis**
998 Fastq files were processed using the split-pipe pipeline (Parse Biosciences), and imported to
999 Seurat [92]. We excluded nuclei with over 10% reads mapping to the mitochondrial genome, fewer
1000 than 2500 mapped reads, fewer than 1500 genes detected, or over 7500 genes detected.
1001 Following normalization and cell cycle scoring, the data were SC-transformed [62] while regressing
1002 out the mitochondrial read fraction, and samples integrated. UMAPs were projected using the
1003 first 30 principal components, and clusters labeled under default settings. Cell types were
1004 identified and grouped using markers from [60] and sctype [61]. Allelic counts from single nuclei were
1005 phased as described above, and aggregated across all chr21 genes to mitigate the impact of
1006 transcriptional bursts. Chr21 dosage by cell (aggregating across chr21 genes), or by gene
1007 (aggregating across cells) was performed on counts-per-million (cpm) normalized expression. For
1008 differential expression analysis, Seurat functions PrepSCTFindMarkers and FindMarkers using the
1009 ROC test were applied to compare euploid, *XIST*$^{+}$ d0 and pD cells to *XIST*$^{-}$ T21 cells by cell type
1010 group. GSEA was performed as for bulk RNA-seq while limiting gene terms to Hallmark and
1011 canonical pathways (KEGG, Reactome, wikipathways, PID, biocarta) of MSigDB.

1012 **Animals**

1013 Ts(1716)65Dn (Ts65Dn; strain #005252) mice were obtained through crossings of a B6EiC3Sn a/A-
1014 Ts (1716)65Dn (Ts65Dn) female to F1 hybrid B6C3F1/J males purchased from The Jackson
1015 Laboratory. Genotyping was performed by amplifying genomic DNA obtained from the mouse
1016 tail as described in [20]. The colony of Ts65Dn mice was maintained in the Animal Facilities in the
1017 Barcelona Biomedical Research Park (PRBB, Barcelona, Spain). Mice received chow and water ad
1018 libitum in controlled laboratory conditions with temperature maintained at 22 ± 1oC and
1019 humidity at 55 ± 10% on a 12h light/dark cycle (lights off 20:00h). The control group consisted of
1020 wild-type (WT) euploid littermates. All experimental procedures were approved by the local
1021 ethical committee (Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure
1022 number MDS-12-1464P3), and met the guidelines of the local (law 32/2007) and European
1023 regulations (EU directive n° 86/609, EU decree 2001-486) and the Standards for the use of
1024 Laboratory Animals n° A5388-01 (NIH).

1025

1026 **Slice immunohistochemistry, imaging and analysis**

1027 Breeding pairs were established so that vaginal plugs could be checked twice daily. The presence
1028 of a vaginal plug was designated as E0.5. A 10% weight gain at E10 was used to confirm
1029 pregnancy. Male and female embryos were collected from pregnant trisomic dams at E18.5,
1030 chilled, decapitated immediately and processed for fluorescent immunohistochemical staining.
1031 Fetal heads were immersed in Paraformaldehyde 4% (PFA) (Sigma Aldrich) and the brain was
1032 dissected 24 hours later and immersed again in PFA o/n. Brains were cryoprotected by immersion
1033 in 30% sucrose solution and embedded in OCT for posterior histological processing. Embryos
1034 heads were embedded in optimal cutting temperature compound (OCT) and embedded tissue
1035 was frozen rapidly and either stored at − 80 °C or immediately sectioned into 20-µm thick frozen
1036 sections using a cryostat (Leica CM3050S). Histological sections were placed in Superfrost® slides
1037 and placed at -20ºC. We processed two sections at three different coordinates (+1.81/2.52/3.15
1038 from Bregma). Slices were fixed with methanol and incubated with blocking buffer (Tris-Buffered
1039 Saline, TBS, with 0,5% Triton-X 100 and 6% normal donkey serum). Primary antibodies were
1040 diluted in TBS ++ (TBS with 0,1% Triton-X 100 and 6% normal donkey serum): anti-S100b (Rabbit,

1041 1:1000; Synaptic systems, #287003) and anti-Ki67 (Rabbit, 1:500; Abcam, ab15580). Slices were
1042 incubated with primary antibodies overnight. After three washes with TBS, sections were
1043 incubated with the following secondary antibodies diluted in TBS ++: goat anti-rabbit (1:500;
1044 Alexa 594, Invitrogen A11012) and goat anti-rabbit (1:500; Alexa 488, A-11008) . Slices were
1045 washed with TBS three times and incubated ten minutes with Hoechst 33342. Finally, sections
1046 were mounted on glass slides using an aqueous mounting media, Mowiol.
1047 To quantify the density of S100B cells six sections per animal were imaged using a Leica
1048 TCS SP5 inverted scanning laser microscope (Leica Microsystems). 15 - 16-µm thick z-stacks (512
1049 x 512 resolution, 8 bits) of each region of interest (ROI) in the cortex of embryos, were acquired
1050 using a 20X objective (N.A = 0.8). After imaging, labeled cells were manually counted using ImageJ
1051 (NIH, Bethesda). Cell counting was performed using the Cell Counter plugin on ImageJ software
1052 (NIH, Bethesda) in a maximum projection of all z-stacks (1 µm step size). Cell counts from the
1053 cortex were processed first by image, then by coordinate (two images were taken from each
1054 coordinate), then by animal and finally by genotype. To quantify the density of Ki67 cells three
1055 sections per animal were imaged in the same form than S100B but imaging the ventricular zone
1056 of the cortex, the area with higher proliferation. An area of 100 μm^2 was delimited to normalize
1057 across sections and due to the high density of cells in this area. Outliers in data sets from both
1058 euploid and trisomic mice were statistically determined using a calculation of interquartile range
1059 (IQR). All data points outside of the IQR fences were excluded from analysis without bias. All
1060 variables were assessed with a two-tailed, independent samples Student's t test, and passed both
1061 the Shapiro-Wilks normality test and an equal variance test.
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DECLARATION OF INTERESTS

No competing interests

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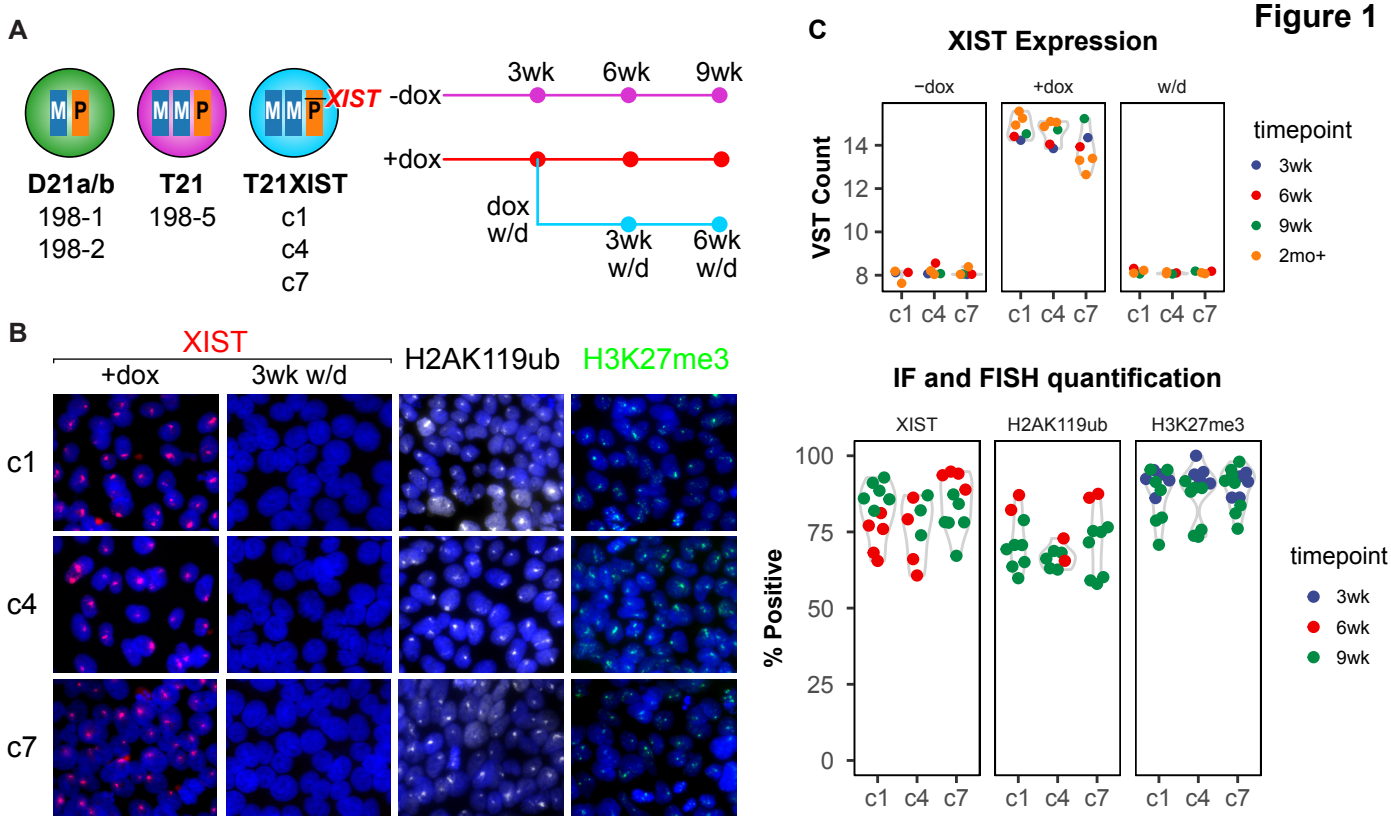
1362

Figure 1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6wk
w/d
B
XIST
+dox
3wk w/d
H2AK119ub
H3K27me3
c1
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IF and FISH quantification
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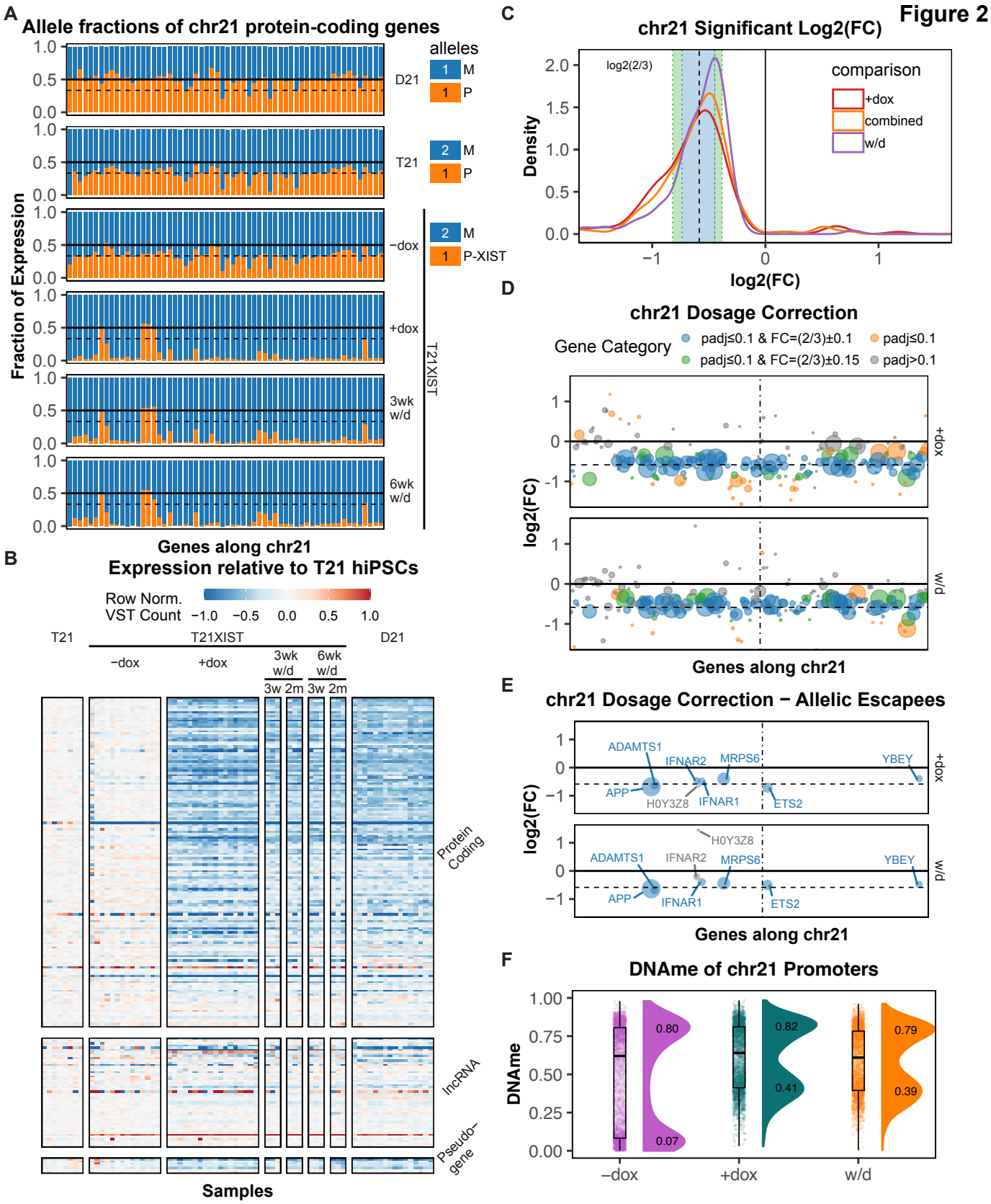
Figure 2
A
Allele fractions of chr21 protein-coding genes
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+dox
3wk w/d
6wk w/d
M
P
P-XIST
T21XIST
Fraction of Expression
Genes along chr21
B
Expression relative to T21 hiPSCs
Row Norm. VST Count
T21
T21XIST
D21
−dox
+dox
3wk w/d
6wk w/d
3w 2m 3w 2m
Protein Coding
lncRNA
Pseudo-gene
Samples
C
chr21 Significant Log2(FC)
log2(2/3)
comparison
+dox
combined
w/d
Density
log2(FC)
D
chr21 Dosage Correction
Gene Category
padj≤0.1 & FC=(2/3)±0.1
padj≤0.1
padj≤0.1 & FC=(2/3)±0.15
padj>0.1
log2(FC)
Genes along chr21
E
chr21 Dosage Correction − Allelic Escapees
ADAMTS1
IFNAR2
MRPS6
YBEY
APP
H0Y3Z8
IFNAR1
ETS2
log2(FC)
Genes along chr21
F
DNAme of chr21 Promoters
DNAme
0.80
0.07
0.82
0.41
0.79
0.39
−dox
+dox
w/d

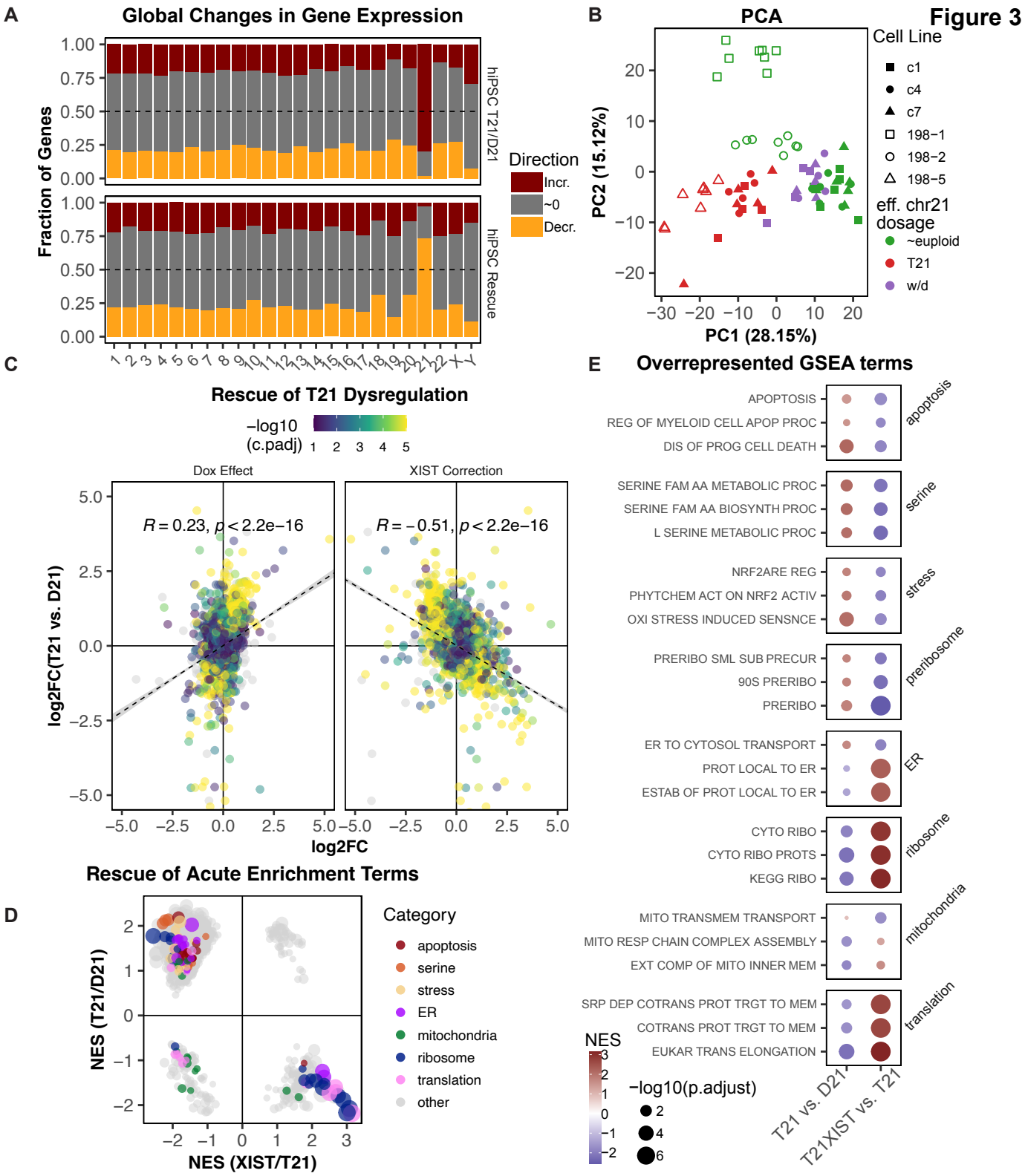


Figure 3
A
Global Changes in Gene Expression
Fraction of Genes
hiPSC T21/D21
hiPSC Rescue
Direction
Incr.
~0
Decr.
B
PCA
PC2 (15.12%)
PC1 (28.15%)
Cell Line
c1
c4
c7
198−1
198−2
198−5
eff. chr21 dosage
~euploid
T21
w/d
C
Rescue of T21 Dysregulation
−log10 (c.padj)
Dox Effect
XIST Correction
R = 0.23, p < 2.2e−16
R = −0.51, p < 2.2e−16
log2FC(T21 vs. D21)
log2FC
D
Rescue of Acute Enrichment Terms
NES (T21/D21)
NES (XIST/T21)
Category
apoptosis
serine
stress
ER
mitochondria
ribosome
translation
other
E
Overrepresented GSEA terms
APOPTOSIS
REG OF MYELOID CELL APOP PROC
DIS OF PROG CELL DEATH
apoptosis
SERINE FAM AA METABOLIC PROC
SERINE FAM AA BIOSYNTH PROC
L SERINE METABOLIC PROC
serine
NRF2ARE REG
PHYTCHEM ACT ON NRF2 ACTIV
OXI STRESS INDUCED SENSNCE
stress
PRERIBO SML SUB PRECUR
90S PRERIBO
PRERIBO
preribosome
ER TO CYTOSOL TRANSPORT
PROT LOCAL TO ER
ESTAB OF PROT LOCAL TO ER
ER
CYTO RIBO
CYTO RIBO PROTS
KEGG RIBO
ribosome
MITO TRANSMEM TRANSPORT
MITO RESP CHAIN COMPLEX ASSEMBLY
EXT COMP OF MITO INNER MEM
mitochondria
SRP DEP COTRANS PROT TRGT TO MEM
COTRANS PROT TRGT TO MEM
EUKAR TRANS ELONGATION
translation
NES
−log10(p.adjust)
T21 vs. D21
T21XIST vs. T21

Figure 4

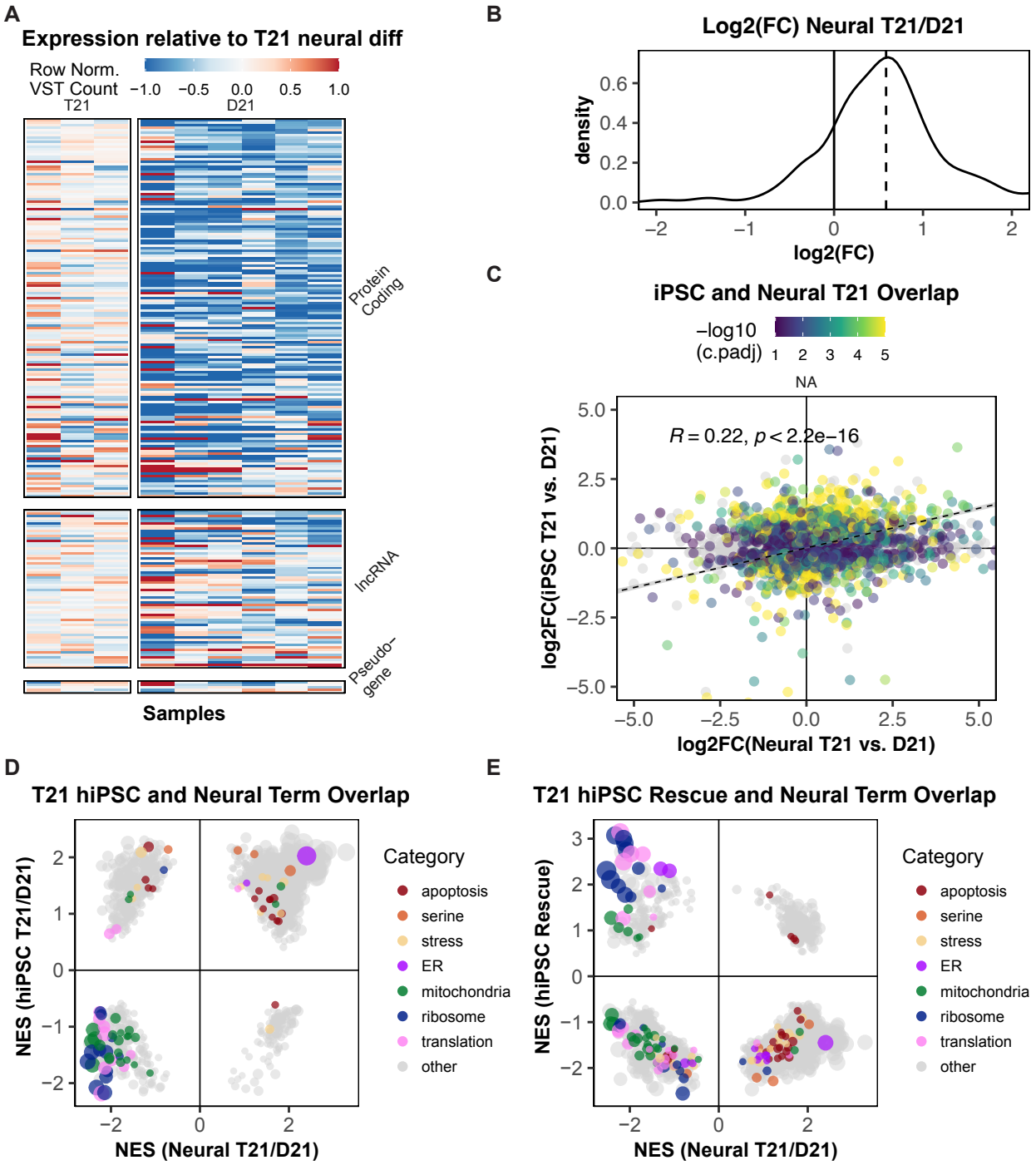
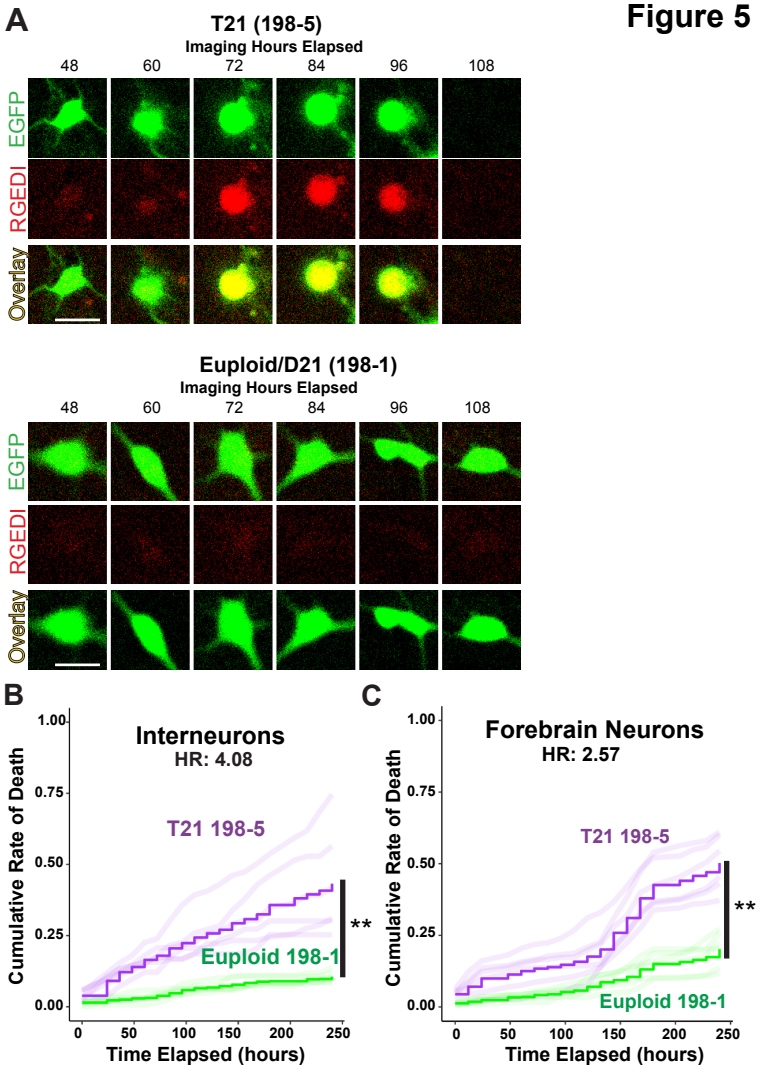

A
Expression relative to T21 neural diff
Row Norm.
VST Count
−1.0 −0.5 0.0 0.5 1.0
T21
D21
Protein Coding
lncRNA
Pseudo-gene
Samples
B
Log2(FC) Neural T21/D21
density
log2(FC)
C
iPSC and Neural T21 Overlap
−log10 (c.padj)
1 2 3 4 5
NA
R = 0.22, p < 2.2e−16
log2FC(iPSC T21 vs. D21)
log2FC(Neural T21 vs. D21)
D
T21 hiPSC and Neural Term Overlap
NES (hiPSC T21/D21)
NES (Neural T21/D21)
Category
apoptosis
serine
stress
ER
mitochondria
ribosome
translation
other
E
T21 hiPSC Rescue and Neural Term Overlap
NES (hiPSC Rescue)
NES (Neural T21/D21)
Category
apoptosis
serine
stress
ER
mitochondria
ribosome
translation
other

Figure 5


A
T21 (198-5)
Imaging Hours Elapsed
48
60
72
84
96
108
EGFP
RGEDI
Overlay
Euploid/D21 (198-1)
Imaging Hours Elapsed
48
60
72
84
96
108
EGFP
RGEDI
Overlay
B
Interneurons
HR: 4.08
T21 198-5
Euploid 198-1
**
Cumulative Rate of Death
1.00
0.75
0.50
0.25
0.00
0
50
100
150
200
250
Time Elapsed (hours)
C
Forebrain Neurons
HR: 2.57
T21 198-5
Euploid 198-1
**
Cumulative Rate of Death
1.00
0.75
0.50
0.25
0.00
0
50
100
150
200
250
Time Elapsed (hours)

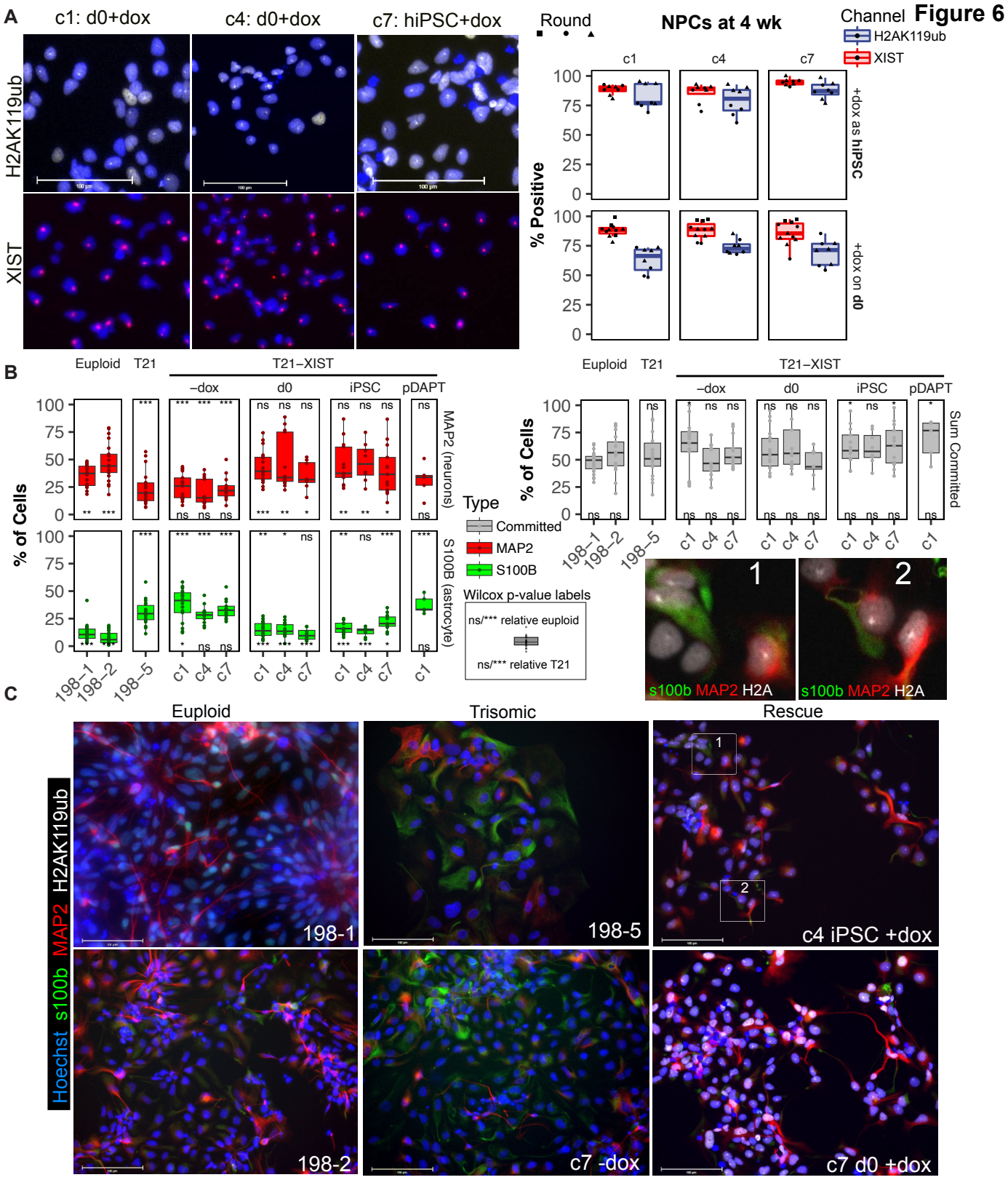
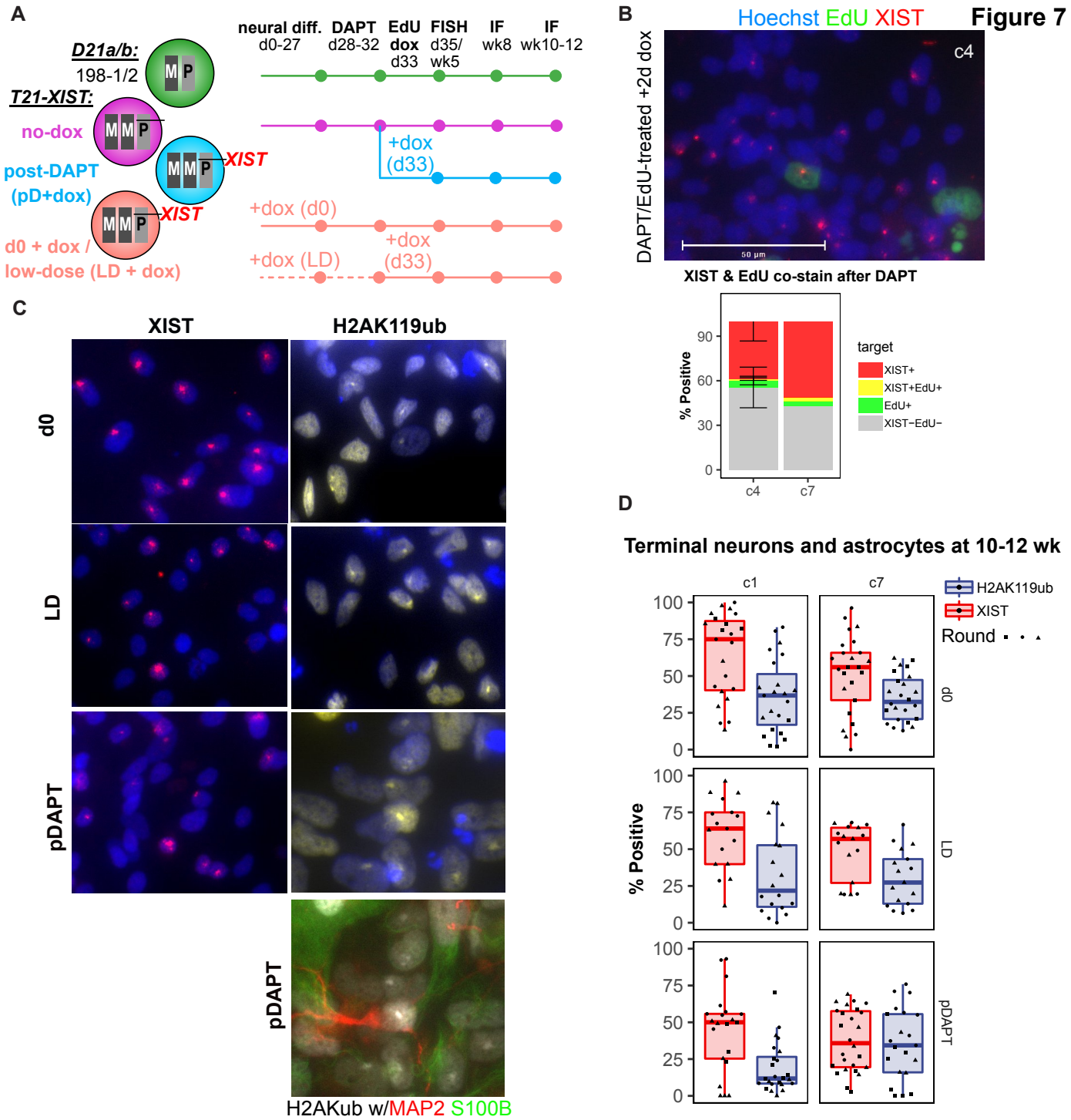


Figure 6
A
c1: d0+dox
c4: d0+dox
c7: hiPSC+dox
H2AK119ub
XIST
Round
NPCs at 4 wk
Channel
H2AK119ub
XIST
c1
c4
c7
% Positive
+dox as hiPSC
+dox on d0
B
Euploid
T21
T21−XIST
−dox
d0
iPSC
pDAPT
% of Cells
MAP2 (neurons)
S100B (astrocyte)
Sum Committed
198−1
198−2
198−5
Type
Committed
MAP2
S100B
Wilcox p-value labels
ns/*** relative euploid
ns/*** relative T21
s100b MAP2 H2A
C
Euploid
Trisomic
Rescue
H2AK119ub
MAP2
s100b
Hoechst
198-1
198-5
c4 iPSC +dox
198-2
c7 -dox
c7 d0 +dox


A
D21a/b:
198-1/2
T21-XIST:
no-dox
post-DAPT
(pD+dox)
d0 + dox /
low-dose (LD + dox)
XIST
XIST
neural diff.
d0-27
DAPT
d28-32
EdU
dox
d33
FISH
d35/
wk5
IF
wk8
IF
wk10-12
+dox
(d33)
+dox (d0)
+dox (LD)
+dox
(d33)
B
Hoechst EdU XIST
Figure 7
DAPT/EdU-treated +2d dox
c4
50 µm
XIST & EdU co-stain after DAPT
% Positive
target
XIST+
XIST+EdU+
EdU+
XIST−EdU−
c4
c7
C
XIST
H2AK119ub
d0
LD
pDAPT
pDAPT
H2AKub w/MAP2 S100B
D
Terminal neurons and astrocytes at 10-12 wk
c1
c7
H2AK119ub
XIST
Round
% Positive
d0
LD
pDAPT

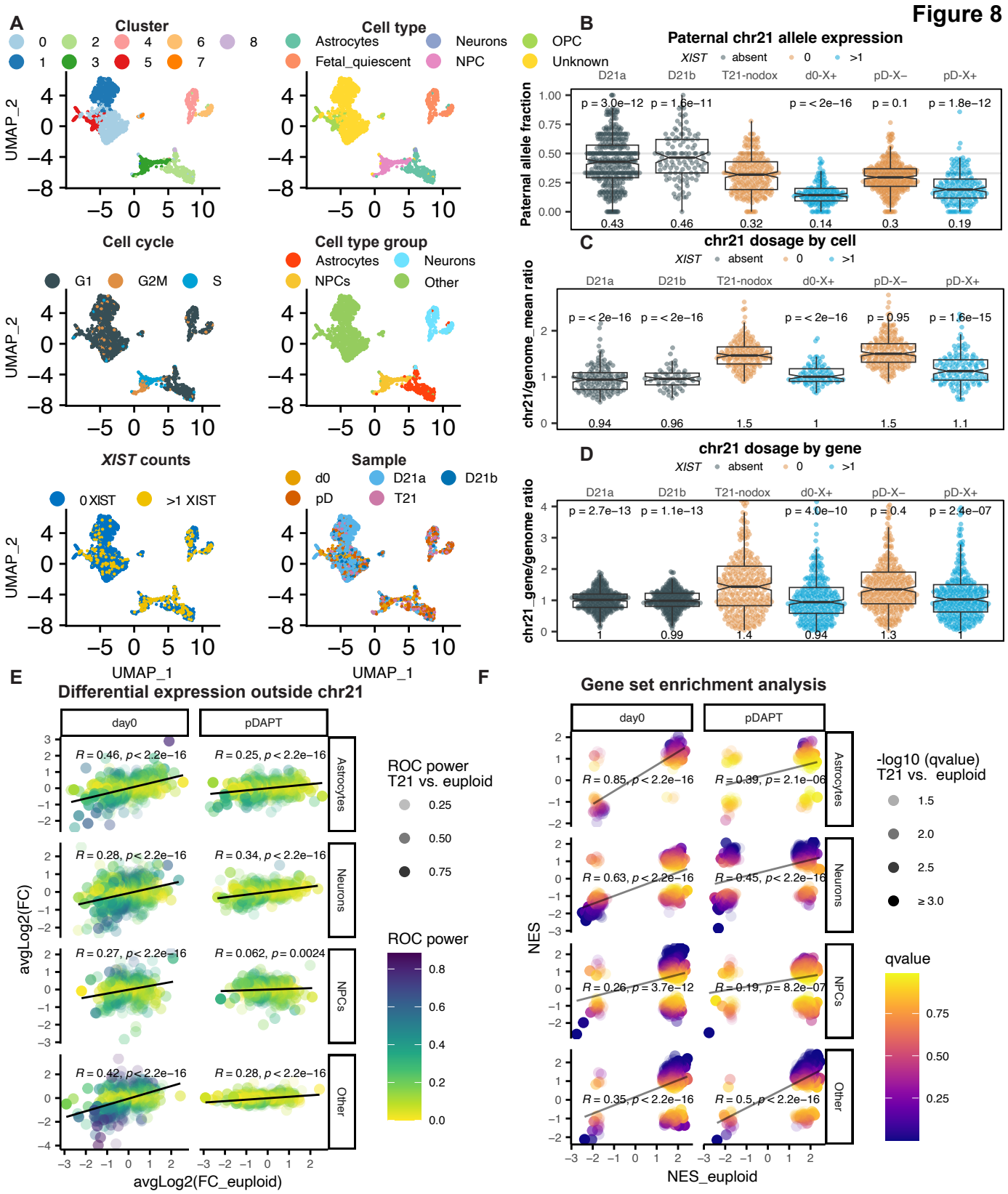


Figure 8
A
Cluster
Cell type
Astrocytes
Fetal_quiescent
Neurons
NPC
OPC
Unknown
Cell cycle
G1
G2M
S
Cell type group
Astrocytes
NPCs
Neurons
Other
XIST counts
0 XIST
>1 XIST
Sample
d0
pD
D21a
T21
D21b
UMAP_1
UMAP_2
B
Paternal chr21 allele expression
XIST absent 0 >1
D21a D21b T21-nodox d0-X+ pD-X− pD-X+
Paternal allele fraction
p = 3.0e−12 p = 1.6e−11 p = < 2e−16 p = 0.1 p = 1.8e−12
0.43 0.46 0.32 0.14 0.3 0.19
C
chr21 dosage by cell
chr21/genome_mean ratio
p = < 2e−16 p = < 2e−16 p = < 2e−16 p = 0.95 p = 1.6e−15
0.94 0.96 1.5 1 1.5 1.1
D
chr21 dosage by gene
chr21_gene/genome ratio
p = 2.7e−13 p = 1.1e−13 p = 4.0e−10 p = 0.4 p = 2.4e−07
1 0.99 1.4 0.94 1.3 1
E
Differential expression outside chr21
day0
pDAPT
Astrocytes
Neurons
NPCs
Other
R = 0.46, p < 2.2e−16
R = 0.25, p < 2.2e−16
R = 0.28, p < 2.2e−16
R = 0.34, p < 2.2e−16
R = 0.27, p < 2.2e−16
R = 0.062, p = 0.0024
R = 0.42, p < 2.2e−16
R = 0.28, p < 2.2e−16
avgLog2(FC)
avgLog2(FC_euploid)
ROC power
T21 vs. euploid
0.25
0.50
0.75
ROC power
0.8
0.6
0.4
0.2
0.0
F
Gene set enrichment analysis
R = 0.85, p < 2.2e−16
R = 0.39, p = 2.1e−06
R = 0.63, p < 2.2e−16
R = 0.45, p < 2.2e−16
R = 0.26, p = 3.7e−12
R = 0.19, p = 8.2e−07
R = 0.35, p < 2.2e−16
R = 0.5, p < 2.2e−16
NES
NES_euploid
-log10 (qvalue)
T21 vs. euploid
1.5
2.0
2.5
≥ 3.0
qvalue
0.75
0.50
0.25