

1 **Loss of MECP2 leads to telomere dysfunction and neuronal stress**

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28 **Abstract**

29 To determine the role for mutations of MECP2 in Rett Syndrome, we generated isogenic
30 lines of human iPSCs (hiPSCs), neural progenitor cells (NPCs), and neurons from
31 patient fibroblasts with and without MECP2 expression in an attempt to recapitulate
32 disease phenotypes *in vitro*. Molecular profiling uncovered a reduction of 5hmC,
33 increased expression of subtelomeric genes including TERRA (a long non-coding RNA),
34 and shortening of telomeres in the absence of MECP2 in hiPSCs, NPCs, and Neurons.
35 Neurons made without MECP2 show signs of stress, including induction of gamma-
36 H2aX, p53, and senescence, which are typical molecular responses to telomere
37 shortening. The induction of p53 appeared to affect dendritic branching in Rett neurons,
38 as p53 inhibition restored dendritic complexity. Examination of Rett patient brains
39 uncovered similar molecular phenotypes suggesting that our disease-in-a-dish model
40 yielded insights into human Rett Syndrome patient phenotypes and point towards a role
41 for MECP2 in regulating telomere function.

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49 Introduction

50 Rett Syndrome is a disease associated with loss of function mutations in the gene
51 MECP2, which was originally identified as encoding a methylated DNA binding protein¹⁻
52 ³. Patient symptoms include microcephaly, intellectual disability, facial dysmorphia, and
53 seizure activity^{4,5}. Studies in murine models recapitulate many of the patient
54 phenotypes and have recently identified a role for MECP2 particularly in inhibitory
55 neurons⁶⁻⁹. These studies demonstrated that loss of MECP2 can lead to defects in
56 transcription¹⁰⁻¹², dendritic branching¹³, nuclear size³, and AKT signaling¹⁴.

57

58 MECP2 is known to bind methylated DNA (both 5mC and 5hmC)^{1,2,15,16}, and the loss of
59 MECP2 was shown to affect 5hmC levels in at least one portion of the murine brain.
60 MECP2 has also been described as a transcription factor with specific targets^{10,11,13},
61 and more broadly as either a transcriptional activator¹⁴ or repressor¹⁷⁻²⁰. However,
62 despite decades of research on MECP2, it is still unclear how mutations in this protein
63 lead to patient symptoms^{3,14,21-23}. To confirm findings made in other models and further
64 study these in a human system, some have turned to modeling Rett Syndrome *in vitro*
65 by taking advantage of Disease in a dish approaches. This involves making hiPSCs
66 from patient somatic cells, or using genome engineering to introduce mutations into WT
67 human pluripotent stem cells. In either case, the pluripotent stem cells created are then
68 differentiated toward the neural lineage, and then comparisons can be made between
69 cells that express MECP2 or lack it.

70

71 Some of these studies have even taken advantage of isogenically controlled lines to
72 identify both transcriptional and electrophysiological effects of loss of MECP2 in human
73 in vitro models^{14,24}. In the current study, we also sought to mitigate the effect of genetic
74 background and variability of differentiation by taking advantage of several isogenic
75 lines of hiPSCs that either express the WT allele or the mutant allele leading to cells
76 that express or lack MECP2²⁵. This allowed for detailed molecular analyses of hiPSCs,
77 NPCs and neurons with and without MECP2 under the same genetic background. In
78 addition, several lines were made and analyzed in each category to avoid variance in
79 differentiation potential amongst isogenic lines. Furthermore, isogenic lines were made
80 from two independent patients with different mutations to highlight only those
81 phenotypes associated with loss of MECP2 expression and not genetic background or
82 variance in hPSC differentiation. Finally, we validated many of these findings using
83 siRNA silencing of MECP2 in WT cells of a distinct genetic background.

84

85 In comparing multiple lines of cells, it is clear from our data that loss of MECP2 leads to
86 profound molecular alterations specifically towards the ends of chromosomes due to a
87 decrease of 5-hydroxymethylation, induction of subtelomeric gene expression, and
88 shortening of telomeres. The telomere defects that arise in neurons appear to be
89 related to defects in dendritic branching that are a hallmark of the patient disease.
90 Together, these results define a heretofore unappreciated role for MECP2 in molecular
91 regulation towards the ends of chromosomes.

92

93 **Results**

94 **An isogenic model of Rett Syndrome *in vitro***

95 To determine how loss of MECP2 expression leads to defects in the nervous system we
96 generated a disease-in-a-dish model using iPSCs. Cognizant of the fact that
97 differentiation from hPSCs is highly variable across individual lines, culture conditions,
98 and time, we developed an isogenic model to study Rett Syndrome *in vitro* to remove
99 the confound of genetic background²⁵. Because female patients with Rett Syndrome
100 are usually heterozygous for mutant alleles of *MECP2*, fibroblasts isolated from these
101 patients display a mosaic pattern where roughly half the cells express either the mutant
102 or WT allele. This is shown in Figure 1A, where fibroblasts isolated from two patients
103 with distinct mutant alleles of MECP2 (R982 and R567) showed that roughly half the
104 cells express MECP2 while the other half lacked detectable amounts of this protein.
105 One of these mutant alleles is predicted to lead to a premature stop codon, while the
106 other leads to failed transcriptional termination. Reprogramming to iPSCs using a small
107 set of transcription factors has been shown to happen at the clonal level, such that
108 individual reprogramming events in single fibroblasts generate isolated hiPSC clones²⁶.
109 Therefore, reprogramming of mosaic fibroblast cultures from two different patients
110 generated single hiPSC clones that either expressed MECP2 protein or lacked it (Fig
111 1B) (Method described in a previous study²⁷). In addition, our work and that of others
112 has shown that under standard conditions, the inactive X chromosome in human
113 fibroblasts does not reactivate upon reprogramming to the pluripotent state^{25,27,28}, which
114 is distinct from murine reprogramming²⁹.

115

116 Thus, we were able to create multiple lines of hiPSCs with and without MECP2 from
117 individual patients and thereby control for differences in genetic background (shown in
118 Fig 1B are clones made from patient 982, clones from 567 look similar). The hiPSCs
119 generated from fibroblasts of both patients appeared to be unaffected by the lack of
120 MECP2, expressed all appropriate markers, and successfully generated teratomas
121 upon injection into the testes of immunocompromised mice, consistent with previous
122 hiPSC models for loss of MECP2 (Fig 1-figure supplement 1)^{14,30-32}. Lack of MECP2 in
123 patient- derived cells and specificity of antibody was also confirmed by western blot (Fig
124 1-figure supplement 2A). Importantly, we never observed reactivation of the silenced X
125 chromosome that would have resulted in re-expression of the WT allele of MECP2 in
126 any cultures regardless of differentiation status or passage. This is consistent with
127 previous data showing that despite evidence for erosion of isolated portions of the
128 silenced X chromosome³³, the portion containing the MECP2 locus was not affected by
129 reprogramming or differentiation.

130

131 As Rett Syndrome primarily afflicts the nervous system and MECP2 is most highly
132 expressed in neurons, we first generated neural progenitor cells from all of the hiPSCs
133 lines following standard protocols³⁴. Across at least two lines per patient with and
134 without MECP2, we measured the rate of neuralization, the morphology of NPCs, and
135 expression of typical marker genes. We were unable to detect consistent differences in
136 these properties between multiple clones of both WT and MECP2- lines derived from

137 both patients (Fig 1C and 1-figure supplement 2B). Furthermore, the growth rate of
138 NPCs with and without MECP2 was not consistently different in NPCs made from either
139 patient (Fig 1-figure supplement 2C). Next, the NPCs were further differentiated by a
140 non-directed differentiation approach that yields both neurons and glia (growth factor
141 withdrawal³⁵) (Fig 1D). Both the neurons and glia made from NPCs adopted typical
142 morphologies regardless of MECP2 expression, and all NPCs from both patients
143 produced neurons and glia at the same rate (Fig 1-figure supplement 2D and figure
144 supplement 2E).

145

146 Previous studies have also shown that loss of MECP2 in neurons can lead to a
147 decrease in AKT signaling¹⁴. A similar pattern was observed here in mutant neurons
148 generated from Rett patient hiPSCs as measured by phosphorylation of AKT and S6,
149 while hiPSCs themselves did not seem to be affected by loss of MECP2 (Fig 1E).
150 Dendritic complexity has been shown extensively to be reliant on MECP2 expression in
151 various models of Rett Syndrome, and we found a statistically significant decrease in
152 complexity in neurons made in the absence of MECP2 by Sholl assay (Fig 1F). In
153 addition, we observed qualitative differences in basic neuronal morphology between WT
154 and mutant neurons, where the neurons lacking MECP2 had shorter, thicker processes,
155 and their soma was not as well defined.

156

157

158 **Loss of MECP2 leads to disruption of hydroxymethylation of DNA**

159 Because MECP2 is a well-established methylated DNA binding protein particularly for 5-
160 hydroxymethylcytosine (5hmC)¹⁶, we analyzed patterns of this mark across the genome
161 in the presence or absence of MECP2 with Methylation-dependent Immunoprecipitation
162 (MEDIP)⁴¹⁻⁴⁴ in hiPSCs. We used a stringent criterion to identify differentially
163 hydroxymethylated regions (DhmRs), whereby the indicated regions had to differ by 0.2
164 per million reads per base pair (Fig 2A). We still observed a large number of
165 differentially hydroxymethylated regions (DhmRs) due to the loss of MECP2 in two
166 clones each from two independent patients (982.15 and 982.17 vs 982.16 and 982.18;
167 567.24 and 567.26 vs 567.25).

168

169 The loss of MECP2 led to many more hypomethylated regions than hypermethylated
170 regions, a strong bias that indicated that MECP2 somehow promotes or stabilizes
171 hydroxymethylation (Fig 2B). This hypomethylation is more clearly identified by plotting
172 the Delta Methylation between the WT and MUT clones from both patients. Both
173 patients showed a dramatic shift towards loss of methylation across both clones (Fig
174 2B). Mapping 5hmC-DhmRs relative to genomic features indicated a de-enrichment
175 away from intergenic regions and enrichment at coding exons (Fig 2C indicated by *).
176 When mapping the 5hmC-DhmRs across chromosomal locations, they were highly
177 enriched towards the ends of chromosomes (Fig 2D). In addition, the effect of loss of
178 MECP2 on 5hmC levels was strong enough to be observed by immunostaining in
179 hiPSCs made from both patients (Fig 2E and F).

180

181 **Loss of MECP2 affects the transcriptome of neurons**

182 It has been suggested that loss of MECP2 only affects gene expression in neurons as
183 opposed to the hPSCs and NPCs from which they were derived¹⁴. Coupled with the fact
184 that 5hmC levels appear to be disturbed in MECP2 null hiPSCs, we sought to determine
185 whether gene expression was affected in hiPSCs, NPCs or neurons in this patient
186 derived *in vitro* model. We therefore proceeded with RNA seq (>120 million reads per
187 sample) of hiPSC, NPC and interneuron cultures. With such sequencing depth, it was
188 possible to analyze the RNA-seq reads for the known mutations present in the patients
189 from which these lines were made (Fig 3-figure supplement 1). This analysis
190 demonstrated that each line studied expressed strictly either the WT or mutant allele of
191 MECP2, and that XCI status was unchanged even after extensive differentiation to
192 neurons.

193

194 To optimize the search for molecular effects of loss of MECP2 in hiPSCs, NPCs or
195 neurons, we generated more defined neuronal cultures by following the newly
196 established 3i (three inhibitor) method to create populations of human interneuron
197 progenitors (Fig 3-figure supplement 1A) and interneurons (Fig 3-figure supplement
198 1B)⁴⁵. Interneurons are particularly relevant in the study of Rett Syndrome as
199 interneuron-specific deletion of *Mecp2* in mice recapitulates many of the disease
200 symptoms^{6,8,46,47}. We validated the purity and quality of differentiation at each step by
201 immunostaining for markers typical of particular cell types (SOX2, SOX1 and NESTIN
202 as well as FOXG1 and NKX2.1 for NPCs; and Tuj1, MAP2 and GABA for interneurons)

203 in both WT and MUT cultures followed by quantification (not shown). We first assessed
204 whether interneurons lacking MECP2 also showed diminished dendritic branching. In
205 fact, in patient-derived interneurons made by 3i, defects in dendritic branching as
206 measured by the number of endpoints were clearly observed (Fig 3A).

207

208 First, we quantified the expression level of MECP2 in WT cells across these three
209 stages of development and found that the average RPKM was 3.1 for hiPSCs, 4.3 for
210 NPCs, and 7.75 for interneuron cultures. This is consistent with consensus that MECP2
211 is enriched in neuronal cells, but also demonstrates that it could potentially be relevant
212 to hiPSC and NPC physiology as well. However, high stringency analyses (FDR <0.05)
213 of the RNA-seq data yielded very few gene expression changes due to loss of MECP2
214 in hiPSCs or NPCs derived from Rett patients (Fig 3B), consistent with Li et al¹⁴. On the
215 other hand, interneuron cultures made from patient 982 showed many gene expression
216 changes when comparing two individual WT and MUT clones (Fig 3B). Gene ontology
217 analysis uncovered many neuronal physiology- related pathways were downregulated
218 due to loss of MECP2 in neurons, while genes associated with extracellular remodeling
219 and cell migration appeared to be induced (Fig 3C).

220

221 We then mapped the interneuron DEGs according to chromosomal location and found
222 an interesting pattern whereby genes that many of the upregulated genes in the
223 absence of MECP2 were enriched towards the ends of chromosomes (Fig 3D).
224 Moreover, the same pattern emerged in analysis of low stringency DEGs (p value

225 <0.05) from hiPSCs and NPCs lacking MECP2 (Fig S4C and S4D) for the upregulated
226 genes. We did not observe this pattern for the downregulated genes in hiPSCs, NPCs
227 and neurons. These data suggested that MECP2 could play a role in gene regulation
228 particularly at the ends of chromosomes, and was consistent with the pattern observed
229 for hypomethylation of 5hmC in the absence of MECP2. Finally, there was also a small,
230 but statistically significant overlap of 5hmC-DhMRs with DEGs in hiPSCs suggesting a
231 link between the two (Fig 3E).

232

233 We validated a number of the subtelomeric gene expression changes induced by loss of
234 MECP2 by RT-PCR in independent preparations of hiPSCs, NPCs and Neurons (Fig
235 4A). Many of the subtelomeric genes upregulated are typically not expressed at all in
236 hiPSCs, NPCs or neurons, thus the loss of MECP2 led to an aberrant expression
237 pattern as opposed to a reinforcement or suppression of a typical pattern in these cell
238 types. Furthermore, the subtelomeric genes that were induced in the absence of
239 MECP2 were still typically present at less than 1 RPKM, clouding the potential
240 physiological consequence of the induction of these genes.

241

242 To determine whether these effects were specific to the genetic background of the cells
243 used or whether defects in reprogramming to the pluripotent state in the absence of
244 MECP2 affected the downstream gene expression pattern, we silenced MECP2 in WT
245 NPCs and assessed gene expression patterns in this context. Several different siRNA
246 targeting oligos were assayed for their ability to silence MECP2 by RT-PCR, western

247 blot, and immunostaining (Fig 4-figure supplement 1). Silencing of MECP2 in either
248 WT-NPCs derived from other pluripotent stem cells or WT-NPCs derived from 15 week
249 old fetal Medial Ganglionic Eminence (MGE) brain tissue, a source of cortical
250 interneuron progenitors³⁵, led to strong induction of expression of subtelomeric genes
251 (Fig 4B and C).

252
253 Recently, it was discovered that a long non-coding RNA (lncRNA) is also transcribed
254 from the subtelomeric domain into the telomeric sequence itself⁴⁸⁻⁵². This lncRNA is
255 both known to be induced by telomere shortening and to potentially negatively regulate
256 telomere length by competing for telomere priming within telomerase⁵¹⁻⁵³. TERRA
257 transcripts are difficult to detect by RNA-seq because they contain mostly telomeric
258 repeat sequences. This also makes it difficult to design PCR primers that are specific to
259 a single TERRA transcript. However, we used established RT-PCR primers^{51,52} to show
260 that, similar to many subtelomeric genes, several TERRAs were strongly induced in the
261 absence of MECP2 in isogenic derivatives (hiPSCs, NPCs, interneurons) (Fig 4D). In
262 addition TERRA was induced in WT-NPCs derived from pluripotent stem cells or from
263 tissue (Fig 4D).

264
265 **Loss of TET activity phenocopies loss of MECP2**

266 To assess the possibility that MECP2 regulation of 5hmC levels is linked to the
267 regulation of subtelomeric gene expression, we targeted TET enzymes by siRNA. TET
268 enzymes convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and their

269 deletion or downregulation is known to severely diminish levels of 5hmC^{44,54}. According
270 to RNA-seq, the three TET enzymes are expressed similarly in both WT and MUT
271 patient neurons (Average RPKM: TET1, 4.1; TET2, 1.6; TET3, 3.6). RT-PCR
272 demonstrated the ability to silence TET 1, 2, and 3 isoforms using a combination of
273 siRNA oligos in neurons (Fig 4E). Assaying for subtelomeric genes, including TERRA
274 transcripts demonstrated that TET inhibition led to strong increases in not only
275 subtelomeric coding genes, but also TERRA transcripts (Fig 4F), in a similar manner as
276 in loss of MECP2, suggesting a link between 5hmC and subtelomeric gene expression.
277 Together, these data confirm that loss of MECP2 can lead to induction of subtelomeric
278 genes, including TERRA, and that this dysregulation could be due to an effect on 5-
279 hydroxymethylation. Left unclear is how the loss of MECP2 leads to changes in 5hmC
280 levels.

281

282 **Loss of MECP2 leads to telomeric abnormalities**

283 One of the most established functions for TERRAs is their ability to interfere with
284 telomerase function by acting as a competitive inhibitor for telomere priming⁵⁵, leading
285 to shortened telomeres. In addition, others have shown that decreased 5hmC in murine
286 embryonic stem cells can also lead to telomere shortening⁵⁶, and, as shown here, loss
287 of MECP2 led to decreased 5hmC in hiPSCs (Fig 2). Therefore, we attempted to
288 determine the physiological consequence of TERRA induction in the absence of
289 MECP2. qPCR for telomere length in fact showed that cells without MECP2 showed
290 shorter telomeres, regardless of their stage of differentiation (Fig 5A) (a complete list of

291 cell lines used for these analyses is provided in Supplemental Table 1). NPCs with
292 transient siRNA knockdown of MECP2 also showed shorter telomeres, indicating that
293 this effect was not simply due to defects during reprogramming or differential expansion
294 of cell lines (Fig 5A). Quantitative fluorescence in situ hybridization (qFISH) was used
295 to determine telomere length at the single cell level. qFISH demonstrated that NPCs
296 without MECP2 showed significantly shorter telomeres relative to centromeric regions
297 (Fig 5B). To further validate these findings we performed southern blot with a telomere
298 probe on NPCs with and without MECP2 and found telomere shortening in cells lacking
299 MECP2 (Fig 5C). These data point towards telomere erosion in the absence of
300 MECP2.

301

302 Telomere dysfunction is known to be present in some cancers, and also in cells driven
303 to senescence due to telomere shortening, but this process has yet to be implicated in
304 Rett Syndrome etiology⁵⁷⁻⁶⁰. Telomere dysfunction is characterized by short telomeres,
305 induction of PML, gammaH2aX, and p53⁶¹. We assayed for evidence of telomere
306 dysfunction by immunostaining for gamma-H2aX (Fig 6A) and PML (Fig 6B) in NPCs.
307 WT NPCs with silencing of MECP2 by siRNA and neurons lacking MECP2 also showed
308 clear induction of these marks (data not shown), consistent with telomere dysfunction
309 induced by the absence of MECP2.

310

311 **Induction of P53 and senescence pathways in the absence of MECP2**

312 It is well established that shortening of telomeres puts significant stress on cells, which
313 can lead to senescence or even apoptosis^{55,62-64}. As Rett Syndrome is caused by
314 neuronal defects specifically, we determined how neurons lacking MECP2 respond to
315 telomere shortening at the molecular and physiological level. Cells under stress due to
316 telomere shortening are known to induce p53, which can then activate various response
317 pathways downstream such as DNA repair, senescence, and apoptosis⁶⁵. Interestingly,
318 p53 induction due to telomere shortening was previously shown to cause defects in
319 dendritic branching^{64,66}, which is also the dominant phenotype in Rett Syndrome.
320 Immunostaining for p53 in neurons with and without MECP2 showed a strong increase
321 in p53 protein in the absence of MECP2 (Fig 6C). p21, a transcriptional target gene of
322 p53, was also induced in MECP2 null neurons at the protein level (Fig 6C). In addition,
323 telomere shortening in NPCs due to overexpression of the Progerin allele, which is
324 associated with accelerated aging also induced p53 expression (Fig 6-figure
325 supplement 1E).

326

327 Because telomere shortening is known to also drive cellular senescence, we looked for
328 signs of defective proliferation *in vitro*. While attempting to make clones of fibroblasts
329 from patients with Rett syndrome, we repeatedly found that clones lacking MECP2 did
330 not expand well after a passage (14 MECP2 null clones were created, none expanded),
331 while clones expressing the WT allele expanded without problem (42 MECP2+ clones
332 were created, and 4 out of 4 all expanded). To determine whether MECP2 null
333 fibroblasts encounter senescence, we performed assays to detect endogenous beta-

334 galactosidase, which is known to be a hallmark of this process⁶⁷. Indeed, MECP2 null
335 fibroblasts showed strong activity in this senescence assay (Fig 6D).

336

337 We did not encounter such difficulties with clonal expansion once hiPSCs or hiPSC-
338 derived NPCs were made from patients, presumably because during reprogramming,
339 telomerase is strongly induced to restore telomere length at least beyond the critical
340 threshold⁶⁸⁻⁷³. In fact, our RNA-seq data showed that hiPSCs made from patients had
341 very high expression of TERT, and NPCs still expressed moderate levels, while neurons
342 did not express appreciable levels (average RPKM for TERT: hiPSC, 8.8; NPC, 1.6;
343 neuron, 0.006). Importantly, the same endogenous galactosidase activity assay on
344 interneurons showed a dramatic increase in senescence activity in neurons lacking
345 MECP2 (Fig 6E). On the other hand, similar assays on NPCs lacking MECP2 did not
346 show any induction of senescence (data not shown). Together, these data indicate that
347 loss of MECP2 leads to the generation of neurons that show evidence of telomere
348 dysfunction.

349

350 Probing RNA-seq data, we also found that MECP2 null interneuron cultures showed a
351 strong increase in a group of genes that are known to be induced by senescent cells,
352 known as the Senescence Associate Secretory Program (SASP). Fig 6-figure
353 supplement 1A shows that SASP genes were strongly induced in MECP2 mutant
354 neurons, providing further evidence of a senescence phenotype. These senescence
355 phenotypes are also intriguing in light of the transcriptional data suggesting an increase

356 in aging- related genes by gene ontology analysis (Fig 3C). The only previous report
357 linking MECP2 loss to senescence was performed by partial silencing of this protein in
358 mesenchymal stem cells, but the results were consistent with those shown here for
359 patient derived MECP2 null fibroblasts⁷⁴.

360

361 To demonstrate whether the induction of senescence and p53 observed here was due
362 to telomere shortening as opposed to other molecular phenotypes due to loss of
363 MECP2, we deliberately shortened telomeres in otherwise wildtype NPCs. We took
364 advantage of the progerin allele of the Lamin-A gene. This truncated allele is similar to
365 what is found in patients suffering from Progeria, a premature aging disorder typified by
366 telomere shortening^{75,76}. Induction of the progerin allele by lentiviral infection of cDNA
367 in WT NPCs showed a significant telomere shortening as expected (Fig 6-figure
368 supplement 1B). In addition, induction of the progerin allele caused an increase in
369 expression of the same subtelomeric genes and TERRA transcripts that were induced
370 by the loss of MECP2 (Fig 6-figure supplement 1C and D). This was presumably due to
371 the Telomere Position Effect, whereby telomere shortening is known to lead to induction
372 of subtelomeric gene expression^{77,78}. Importantly, progerin expression also led to a
373 strong induction of p53 expression (Fig 6-figure 1E), consistent with what was observed
374 in MECP2 null neurons.

375

376 **Blocking induction of P53 can rescue dendritic branching defects due to loss of**
377 **MECP2**

378 Previous evidence from a murine model of telomere shortening as a result of loss of
379 telomerase complex (TERT) led to defects in dendritic branching, and this effect was
380 strictly dependent on induction of p53⁶⁴. A more recent study also showed that
381 experimentally aging the neural lineage with telomerase inhibition led to neurons with
382 signs of aging, including reduced dendritic branching⁷⁹. Therefore, we posited that
383 inhibition of P53 in MECP2 null neurons with shortened telomeres could potentially
384 restore appropriate dendritic branching.

385

386 To determine whether blocking the action of P53 could improve dendritic branching in
387 MECP2 null interneurons, we took advantage of Pifithrin- α , a potent inhibitor of P53
388 target gene activation⁸⁰. Treatment of MECP2 null interneurons with Pifithrin- α showed
389 evidence of p53 inhibition as measured by RT-PCR for GADD45⁶⁵, a target gene
390 important for DNA repair (Fig 6F). After 24-48 hours of p53 inhibition by Pifithrin- α ,
391 MECP2 null interneurons appeared to adopt an improved neuronal morphology typified
392 by increased physical distinction between the soma and neurites, longer, thinner
393 neurites, as well as increased dendritic branching as shown and quantified in Fig 6F.
394 These data provide evidence that neurons with shortened telomeres due to loss of
395 MECP2 respond by inducing P53 activity, which then inhibits the formation of complex
396 neuronal processes. In summary, our in vitro model in human neurons suggests that
397 loss of MECP2 leads to aberrant molecular regulation at the ends of chromosomes,
398 leading to telomere shortening and a resulting induction of cell stress pathways such as
399 p53 and senescence (Fig 6G).

400

401 **Rett patient brains show evidence of telomeric dysfunction**

402 To determine whether any of the phenotypes discovered in this *in vitro* model of Rett
403 Syndrome have relevance to patients afflicted with the disease, we acquired tissue
404 specimens from Rett patients and aged matched controls. We first quantified the
405 degree of chimerism of female Rett patient neurons due to skewing of X chromosome
406 inactivation to determine the relative ratio of neurons that express MECP2 versus those
407 that did not. One of the Rett patient brains showed roughly 75% of its neurons lacking
408 MECP2, while others appeared to have less than 25% MECP2 null neurons (Fig 7A).
409 Southern blotting of the patient brain with 75% mutant neurons compared to an aged-
410 matched control demonstrated that this Rett brain had shorter telomeres (Fig 7B). We
411 then measured telomere length by PCR from genomic DNA isolated from small
412 specimens of brain tissue from a group of Rett patients, and found that some aged
413 matched Rett patient brains showed small decreases in average telomere length (Fig
414 7C), though not in every case (data not shown). Because of both the limited availability
415 of Rett patient brains, and the variable chimerism of WT and MUT neurons within these
416 Rett brains, perhaps it is not surprising that we were unable to detect trends across all
417 brains analyzed using a method that cannot distinguish between WT and mutant cells.
418 This chimerism, coupled with the known variability of telomere length across even
419 normal individuals and even across brain regions and cell types precludes an accurate
420 assessment of general telomere length differences in Rett brain until more samples
421 become available for study.

422

423

424 On the other hand, we did find that the TERRA transcript was induced in nearly all Rett
425 patient brains as measured by RT-PCR (Fig 7D), suggesting that all the Rett patients
426 display dysregulation at the ends of their chromosomes. We did identify two Rett patient
427 brains with a high proportion of MECP2 null neurons and subjected these to further
428 investigation for signs of telomere dysfunction *in situ*. In patients 1815 and 5784,
429 MECP2 null neurons showed a strong increase in both P53 and PML levels compared
430 to adjacent neurons that expressed MECP2 (Fig 7E and F). This is consistent with the
431 response to telomere shortening due to loss of MECP2 observed *in vitro*. These data
432 are particularly intriguing in light of data showing that telomere shortening diminishes
433 dendritic branching in various types of neurons and that this process can be dependent
434 on p53 activity^{64,66}.

435

436 Discussion

437 Taken together, these data demonstrate that loss of MECP2 leads to telomere
438 shortening, which in neurons results in clear signs of stress such as H2aX induction,
439 p53/p21 induction, and initiation of a senescence program, all of which suggest that
440 neurons in Rett Syndrome could be in suboptimal health, leading to neurophysiological
441 defects such as dendritic arborization^{13,22}. Many of these phenotypes first observed in
442 the *in vitro* model also appeared to be consistent with what could be observed in Rett
443 patient brains, suggesting disease relevance for these findings.

444

445 It is curious that telomere defects have not been reported in previous models of Rett
446 Syndrome. While one paper suggested that RNAi-mediated silencing of MECP2 could
447 affect the telomeres of mesenchymal cells⁷⁴, decades of work on Rett Syndrome have
448 not uncovered a role for MECP2 in relation to telomeres in a wide variety of models
449 such as various transgenic mouse line, human patient post-mortem analyses, *in vitro*
450 human models. Our study certainly benefited from analyses of multiple isogenically
451 controlled cells from two patients and from the single cell analyses of patients with both
452 WT and MUT neurons in the same area of the brain. This allowed for high confidence
453 comparisons without having to correct for genetic background, or differences in tissue
454 preparation. In addition, the study of telomeres in MECP2 mutant mice could be
455 hampered by the simple fact that telomeres from inbred mouse strains typically used for
456 these studies are on average much longer than human telomeres. Therefore, it is
457 possible that telomere shortening in murine models does not proceed to such an extent
458 by which one would expect induction of p53 in murine models.

459

460 Patients with Rett Syndrome are typically characterized by a normal development at
461 birth and subsequent failure to thrive leading to microcephaly and intellectual disability
462 that develops with age. As a result, Rett Syndrome is thought to be caused by
463 experience-dependent loss of neuronal function, which would correlate with data
464 suggesting that MECP2 regulates activity dependent gene expression^{10,13,37}. The
465 microcephaly has been proposed to be a function of decreased nuclear size and

466 dendritic arborization of affected neurons^{13,22}. Could the telomere shortening induced
467 senescence described here underlie patient phenotypes? Several studies have looked
468 at the effects of telomere shortening specifically in the neural lineage and found
469 consistently that shortened telomeres leads to upregulation of p53 and decreased
470 dendritic arborization^{64,66,81}, a phenotype widely described to afflict MECP2 null neurons
471 *in vitro* and *in vivo* (Fig 1).

472

473 These results presented here raise the question of whether telomere defects could be
474 common to the etiologies of other ID syndromes. The phenotypes described here show
475 a striking similarity to those observed in hiPSCs and neural derivatives made from
476 patients with Immunodeficiency, centromeric region instability, facial anomalies
477 syndrome (ICF) Syndrome⁸²⁻⁸⁴. Two independent studies showed that ICF patient-
478 derived hiPSCs displayed subtelomeric hypomethylation, induction of subtelomeric
479 gene expression, TERRA induction and telomere shortening that was coupled to
480 senescence of somatic derivatives such as fibroblasts. ICF Syndrome only partially
481 overlaps with Rett Syndrome in terms of patient phenotypes, but is caused by mutations
482 in DNMT3B, a *de novo* DNA methyltransferase⁸⁵. These findings together are highly
483 relevant as DNMT3B is a key *de novo* methyl transferase to create methylated DNA
484 (5mC), which is the substrate for Tet oxigenases to create 5-hydroxymethylated DNA
485 (5hmC). Recently, another study showed that deletion of Tet enzymes, which are
486 critical to generate the 5hmC mark, led to shortened telomeres^{56,86}. Together, these
487 studies demonstrate that DNA hydroxymethylation is important in the regulation of

488 telomere length, and our data suggest that MECP2 is potentially an important mediator
489 of this effect.

490

491 ATRX-related syndrome shares many phenotypic features with Rett Syndrome (ID,
492 seizures, and microcephaly), and the causative gene, ATRX, is known associate with
493 MECP2 both genetically as well as biochemically⁸⁷⁻⁹². In murine models of loss of
494 ATRX, telomere shortening and reduced lifespan are observed⁹³. The fact that the
495 causative mutations of ICF, Rett and ATRX syndromes are in genes whose products
496 are thought to interact in the regulation of DNA methylation and all possess telomeric
497 defects suggests that the similarities of patient phenotypes could be the result of
498 neuronal response to telomere shortening. This molecular overlap could even form the
499 basis of novel therapeutic strategies that either reverse telomere shortening or block the
500 response of the cell to telomere defects with agents such as Pifithrin, as shown in Fig
501 6F.

502

503 Considering the phenotypes of ICF derived cells, and those of Tet-deleted cells, it
504 seems reasonable to suggest that telomere deficiency could be related to intellectual
505 disability. In addition, subtelomeric and telomeric dysfunction has been implicated in up
506 to 10% of all intellectual disability syndromes⁹⁴⁻⁹⁶. Another ID syndrome, Hoyeraal-
507 Hreidarsson, is caused by mutations in RTEL1 (regulator of telomere elongation
508 helicase 1), a factor that interacts with shelterin complex and is critical for telomere
509 elongation⁹⁴. These patients are characterized by low birth weight, microcephaly and

510 immunological dysfunction. Therefore, mutations that specifically result in telomere
511 shortening lead to disease phenotypes similar to those found in patients with loss of
512 MECP2. As a result, we cannot exclude the possibility that telomere shortening during
513 *in utero* development generates neurons that are less well equipped to deal with post-
514 natal stimulation. Furthermore, our analysis of the single male Rett brain specimen
515 available to us suggested that brains completely lacking MECP2 also had shorter
516 telomeres.

517

518 Another possible interpretation of these data is that instead of a failure to mature, Rett
519 Syndrome neurons instead show aspects of premature aging. The fact that MECP2 null
520 neurons have shorter telomeres (Fig 5), show induction of aging related genes including
521 p53 (Fig 3 and 6), and show senescence (Fig 6) are all consistent with this idea⁹⁷. In
522 addition, the fact that WT-NPCs and neurons transduced with the Progerin allele⁹⁸,
523 which is known to cause premature aging, show similar phenotypes as neurons lacking
524 MECP2 is also consistent with this idea. On the other hand, while Rett patients suffer
525 from a post-natal cognitive decline, and long term survivors show phenotypes
526 associated with Parkinson's disease⁹⁹, the typical phenotypes presented in young
527 female patients are not consistent with premature aging. Whether the physiological
528 response to loss of MECP2 is truly akin to premature aging or whether patients suffer
529 from the effects telomere dysfunction that is unrelated to aging is worthy of continued
530 investigation.

531

532 Regardless, it is tempting to speculate that treatments that could relieve telomere
533 dysfunction or abrogate the p53 mediated stress response could potentially ameliorate
534 patient outcomes. Pifithrin- α has already been shown to be an effective treatment to
535 restore neuronal function in murine models of injury or stroke¹⁰⁰⁻¹⁰². Significant future
536 effort will be devoted to determining both whether telomere dysfunction is a common
537 trigger for ID Syndromes, and whether telomere restoration could potentially help
538 patients.

539

540 **Acknowledgements**

541 We are grateful to Lorenz Studer for sharing the Progerin-GFP and Nuclear-GFP
542 constructs. Human tissue was obtained from the NIH Neurobiobank at the University of
543 Maryland (Baltimore, MD). AZX and TPW are partially supported by Connecticut
544 Regenerative Medicine Foundation (13-SCB-YALE-12). This work was funded by
545 training grants to MO (NIH-Virology and Gene Therapy, UCLA), PL (CIRM, UCLA), CS
546 (CIRM-Bridges, Cal-State-Northridge), DA (HHURP, UCLA). PJ was supported by
547 grants from NIH (KP was supported by NS051630, NS079625 and MH102690). AZX
548 and TPW were supported by the Connecticut Regenerative Medicine Foundation (13-
549 SCB-YALE-12). WEL was supported by a Rose Hills Scholar award through the Eli and
550 Edythe Broad Center for Regenerative Medicine. WEL and KP were supported by NIH
551 (P015P01GM099134). This research was also supported by the Allen Distinguished
552 Investigator Program, through The Paul G. Allen Frontiers Group.

553

554 **Materials and Methods**

555 *Generation of isogenic Rett Syndrome iPSCs*

556 Two primary fibroblast lines GM17567 (1461A>G in the gene encoding methyl-CpG
557 binding protein 2 (MECP2)), and GM07982 (frameshift mutation, 705delG, in the gene
558 encoding methyl-CpG binding protein 2 (MECP2)), from patients with Rett Syndrome
559 were obtained from Coriell Cell Repositories. 1×10^5 fibroblasts were plated in a gelatin
560 coated well of a 6 well plate in MEF media (DMEM/F12 + 10% FBS). After 8-12 hours,
561 the cells were infected with reprogramming lentivirus that harbors polycistronic human
562 Yamanaka factors (Oct4, Klf4, Sox2, cMyc) in DMEM medium containing 10ug/ml of
563 polybrene and incubated overnight at 37°C in 5% CO₂ incubator. The following day, the
564 viral media was aspirated, replaced with MEF media and cultured for 3 additional days.
565 Cells were re-plated on the 5th day onto irradiated MEFs in MEF media. On day 6, the
566 culturing media was changed to human ES media containing DMEM/F12 supplemented
567 with L-glutamine, nonessential amino acids (NEAA), penicillin-streptomycin, knockout
568 serum replacement (Invitrogen), and 10 ng/ml basic FGF. Cells were cultured in hiPSC
569 media until iPSC-like colonies were formed. Reprogrammed colonies were further
570 identified by live immunofluorescence staining with TRA-1-81 (Chemicon) then
571 mechanically isolated. Individual colonies were isolated and maintained for at least 2
572 passages before genotyping analysis. For early passages, the iPSCs were propagated
573 mechanically, whereas collagenase was used for subsequent passaging. hiPSCs were
574 cultured as described previously in accordance with the UCLA ESCRO.

575

576 *Generation of teratomas*

577 Generation of teratoma was previously described¹⁰³. Briefly, a single incision was made
578 in the peritoneal cavity of adult SCID mice and the testis was explanted through the
579 incision site. Approximately 60,000 iPSC in a volume of 50 µl 0.5X Matrigel (BD) were
580 transplanted into the testis using a 27-gauge needle. Four to six weeks after surgery,
581 mice were euthanized and the tumors removed for histology. Surgery was performed
582 following Institutional Approval for Appropriate Care and use of Laboratory animals by
583 the UCLA Institutional Animal Care and Use Committee (Chancellor's Animal Research
584 Committee (ARC)).

585

586 *Differentiation in vitro and analysis*

587 Neural specification with neural rosette derivation, neuroprogenitor (NPC) purification,
588 and further differentiation to neurons and glia were performed as described previously
589 ^{34,104,105}. Relative neuralization efficiency was analyzed by counting the number of
590 neural rosette containing colonies over total number of iPSC colonies. 6-12 35 mm
591 wells were analyzed over four separate experiments. The proliferation efficiency of
592 NPCs was determined by at days 1, 3, and 5 by the total number of cells present in
593 35mm wells seeded at 200,000 cells on day 0. The cells were detached from the plates
594 using accutase (Millipore) then total number of cells per well analyzed using Z1 Coulter
595 particle counter (Beckman Coulter).

596

597 For spontaneous terminal neuronal differentiation by growth factor withdrawal, NPC
598 cultures were subjected to growth factor withdrawal (removal of EGF and FGF) and
599 cultured in basic medium (DMEMF12 + N2 + B27) with three quarter exchange of media
600 every three days. Cells were cultured up to 20 weeks. Neural differentiation efficiency
601 was analyzed four weeks after growth factor withdrawal by counting the number of cells
602 positive for neuronal markers (MAP2 and Tuj1) over the total number of cells visualized
603 by DAPI. NPCs were transfected with DCX-GFP reporter one day prior to differentiation
604 using Lipofectamine 2000 (Invitrogen). Sholl analysis of DCX-GFP positive neuronal
605 neuritis were also measured using ImageJ. All data values were presented as mean +/-
606 SEM. Student's t-tests were applied to data with two groups. ANOVA analyses were
607 used for comparisons of data with greater than two groups.

608

609 For directed differentiation of interneurons, iPSCs were grown on plates coated with
610 matrigel (Corning) until 80% confluency with mTeSR (Stem Cell Technologies). Cells
611 were then treated with DMEM/F12 (GIBCO) containing NEAA (GIBCO), GlutaMAX
612 (GIBCO), bovine serum albumin (Sigma-Aldrich), β -mercaptoethanol (Sigma-Aldrich),
613 N2 (GIBCO), B27 (GIBCO), SB431542 (10 μ M; Cayman Chemical), LDN-193189
614 (100nM; Cayman Chemical) and XAV939 (2 μ M; Cayman Chemical) later transitioning to
615 the media containing sonic hedgehog (20ng/mL; R&D) and purmorphamine (1 μ M;
616 Cayman Chemical) as previously described (Maroof et al., 2013). Cells were further
617 differentiated into interneurons with neurobasal medium (GIBCO) containing N2
618 (GIBCO), B27 (GIBCO), ascorbic acid (Sigma-Aldrich), GlutaMAX (GIBCO), bovine
619 serum albumin (Sigma-Aldrich), β -mercaptoethanol (Sigma-Aldrich), neurotrophin-3

620 (10ng/mL; R&D), brain-derived neurotrophic factor (10ng/mL; R&D), and glial cell-
621 derived neurotrophic factor (10ng/mL; R&D).

622

623 *Neuronal activation*

624 8 weeks *in vitro* differentiated neuronal culture were subjected depolarizing stimulation
625 with 55mM of KCl in basic media for 0hr, 1hr, 5hr and 7hr then isolated for RNA
626 analysis and coverslips fixed with 4% paraformaldehyde for immunostaining.

627

628 *Western blot*

629 Cells were lysed on ice with RIPA buffer (Pierce) that contains Halt Protease Inhibitor
630 Cocktail (Thermo Fisher Scientific) and Halt Phosphatase Inhibitor Cocktail (Thermo
631 Fisher Scientific). The total protein concentration was measured using BCA Protein
632 Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. The lysates
633 containing the equal amount of total protein were mixed with NuPAGE sample buffer
634 (Invitrogen) with 5% mercaptoethanol and denatured at 95 °C for 10 min. Supernatant
635 was electrophoresed onto NuPAGE 4-12% Bis-Tris Protein Gels (Invitrogen) using
636 MOPS running buffer (Invitrogen). Gels were then electroblotted using semi-dry
637 transfer apparatus onto a membrane. The membrane was blocked with 5% non-fat milk
638 for 1 hr and incubated overnight with primary antibodies at 4°C. The next day the
639 membrane was washed and incubated with HRP-conjugated secondary antibodies for 1
640 hr at room temperature. The membrane was then incubated with ECL Western Blotting

641 Substrate and developed.

642

643 *Immunofluorescence and image quantification*

644 Cells on coverslips were washed with PBS, fixed in 4% paraformaldehyde for 15 min at
645 room temperature, blocked for 1 hr at room temperature with 10% serum and 0.1%
646 Triton-X-100, then incubated overnight at 4 °C with primary antibodies. Frozen tissue
647 sections were thawed to room temperature, fixed in 4% paraformaldehyde for 15 min at
648 room temperature, permeabilized with 0.2% Triton-X-100 for 15 min at room
649 temperature and blocked with 10% serum at 4 °C overnight, followed by incubation with
650 primary antibodies at 4 °C for 16-24 hr. Following primary antibody incubation, the
651 coverslips were incubated with Alexa Fluor (Invitrogen) or Jackson immunoresearch
652 secondary antibodies at room temperature for 1 hr. Cells were counterstained with
653 DAPI and mounted in Prolong Gold (ThermoFisher). Antibodies used include the
654 following: mouse anti-OCT3/4 (1:100, Santa Cruz Biotechnology Inc.), rabbit anti-SOX2
655 (1:300, Cell Signaling Technology), rabbit anti-Nanog (1:100, Cell Signaling
656 Technology), mouse anti-Tra-1-81 (1:250, Chemicon), mouse anti-NESTIN (1:1000,
657 Neuromics), chicken anti-MAP2 (1:2000, Biolegend), chicken anti-GFAP (1:2000,
658 Abcam), rabbit anti-Tubulin β 3 (1:500, Covance), mouse anti-p53 (1:500, Cell
659 Signaling), rabbit anti-p21 (1:250, Santa Cruz), mouse anti-PML (1:100, Santa Cruz),
660 mouse anti-phospho-Histone H2A.X (1:2000, EMD Millipore), rabbit anti-5hmc (1:100,
661 Active Motif), rabbit anti MECP2 (1:1000, Diagenode), rabbit anti Foxg1 (1:1000,
662 Abcam), and mouse anti NKX2.1 (1:300, Novocastra). Secondary antibodies

663 conjugated with Alexa 488, 568, 594, 647 (1:500, Life Technologies) were used.
664 Imaging was performed on Zeiss Axio Imager A1 or Zeiss LSM780 confocal microscope
665 using a 40X or 63X objective on randomly selected cells. Mean intensity or a number of
666 foci were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>). At least 100 cells per
667 condition were used for each independent experiment.

668

669 *RT-qPCR*

670 RNA from cultured cells was collected using the RNeasy Mini Kit from Qiagen according
671 to the manufacturer's instructions. The concentration and purity of RNA were measured
672 using nanodrop spectrophotometers (Thermo Scientific). RNA with an A260/A280 ratio
673 in between 1.8 and 2.0 as well as an A260/A230 ratio in between 2.0 and 2.2 was used.
674 RNA was then reverse transcribed using the Super Script III First-Strand cDNA
675 Synthesis kit with Random Hexamers (Invitrogen) according to the manufacturer's
676 instructions. Quantitative PCR was performed using SYBR Green master mix (Roche).
677 Primers were used at a final concentration of 1 μ M. Reactions were performed in
678 duplicate and duplicate CT values were averaged and then used for standard $\Delta\Delta$ CT
679 analysis. Expression levels were normalized to beta actin. See Supplementary Table 2
680 for qPCR primer sequences.

681

682 *Data collection and statistical analysis*

683 All the experimental data (RT-qPCR, qPCR assay for telomere length, immunostaining,
684 β -Galactosidase Senescence Assay) were presented as mean +/- SEM based on at
685 least three biological replicates from independent experiments using the cell lines
686 indicated in Supplementary Table 1. Student's t tests were applied to data with two
687 groups. ANOVA analyses were used for comparisons of data with greater than two
688 groups. A p value < 0.05 was considered as statistically significant.

689

690 *siRNA gene silencing*

691 All knockdown experiments were performed using trilencer siRNAs (from OriGene
692 Technologies) and RNAimax (ThermoFisher) in Opti-MEM media (ThermoFisher).
693 Trilencers were used at a concentration of 20 nM. Transfection media was prepared
694 and then 500,000 cells were plated on top of the transfection media in 6-well plates. The
695 medium was changed to normal NPC media the next day and cells were collected for
696 analysis at the time points indicated.

697

698 *β -Galactosidase Senescence Assay*

699 β -Galactosidase Senescence Assay was performed using the Senescence β -
700 Galactosidase Staining Kit from Cell Signaling according to manufacturer's instructions.
701 Briefly, the cells were fixed on coverslips, incubated with X-gal overnight at 37°C, then
702 mounted on glass slides and imaged using a brightfield microscope. The number of
703 blue cells and number of total cells were quantified using the Cell Counter plugin in

704 ImageJ.

705

706 *Quantitative fluorescence in situ hybridization*

707 Cells were fixed with 4% paraformaldehyde for 15 min at RT and permeabilized with
708 0.5% Triton X for 10 min at RT. After dehydration series of 80, 95, and 100% cold
709 ethanol, cells were then treated with RNase (100ug/mL in 2xSSC) for 30 min at 37°C.
710 After washing and another dehydration series, cells were denatured at 85°C for 15 min
711 in the hybridization mix (70% DI formamide, 10 mM Tris-HCl, pH 7.5, 2xSSC, 0.1ug/mL
712 salmon sperm DNA) containing PNA probes (TelC-FITC and Cent-Cy3, Panagene) at
713 1ug/mL and then incubated for 2 hr at RT in dark. After hybridization, cells were washed
714 3X for 5 min in 2xSSC/50% DI formamide, in 2xSSX with 0.1 Tween20, and in 1xSSC.
715 Cells were then counterstained with DAPI and mounted with ProLong Gold
716 (ThermoFisher).

717

718 *Southern blot analysis of terminal restriction fragments (TRF)*

719 Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen)
720 following the manufacturer's instruction. Fresh genomic DNA with high purity (an
721 A260/A280 ratio of 1.8) was used for experiments. The integrity of genomic DNA was
722 determined by gel electrophoresis. Southern blotting was carried using a TeloTTAGGG
723 assay kit (Roche Applied Sciences) following the manufacturer's protocol with some
724 modifications (Kimura et al., 2010).

725

726 *Quantitative PCR assay for average telomere length measurement*

727 Total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen)
728 following the manufacturer's instruction. The concentration and purity of genomic DNA
729 were measured using nanodrop spectrophotometers (Thermo Scientific). Fresh
730 genomic DNA with high purity (an A260/A280 ratio of 1.8) was used for experiments.
731 The integrity of genomic DNA was determined by gel electrophoresis. QPCR was
732 performed as previously described (Cawthon, 2002) with further modifications (Jodczyk
733 et al., 2015). Briefly, two qPCR reactions were carried using either primers for single
734 copy reference gene (albumin) or telomeres at a final concentration of 900nM (See
735 Supplementary Table 2 for primer sequences). In each reaction, a standard curve was
736 made by serially diluting one reference DNA by 2 fold ranging from 1.56 ng to 50 ng in
737 order to ensure the PCR efficiency of 90-100% with the linear correlation coefficient
738 greater than 0.98 for each independent experiment. 5ng of sample genomic DNA was
739 added in a 384 well plate with a total reaction volume of 15uL. Each reaction was
740 performed in duplicate and duplicate CT values were averaged and then used to
741 calculate for relative telomere copy number to single gene copy number (T/S) ratio.

742

743 *Quantification of Dendritic Arborization*

744 Neuronal cultures were immunostained for Tuj1 in order to identify mature neurons and
745 visualize entire cells. The stained cells were then imaged at 20x and dendritic arbors of
746 individual cells were traced using the Simple Neurite Tracer plugin for ImageJ. The

747 number of process ends per cell were counted using the Cell Counter plugin for ImageJ.
748 Only mature neurons—identifiable by their thin processes and condensed somas—were
749 used for analysis. The number of process ends per cell are presented as mean ends
750 per cell +/- SEM. Means were compared using the Student's T-Test for data with two
751 groups. A p value <0.05 was used as the cutoff for significance.

752

753 *RNA expression profiling*

754 RNA purification was performed with Qiagen RNAeasy kit following the manufacturer's
755 instruction. Libraries were prepared according to the manufacturers guidelines using
756 The TruSeq V2 kit (Illumina). Microarray profiling was performed with Affymetrix Human
757 HG-U133 2.0 Plus arrays as described¹⁰⁶. For RNA sequencing, the datasets were
758 mapped with RASER and HISAT2. The reads were counted per gene. Genes were
759 defined by the exon union from the hg19 ensembl annotations. The function of DESeq
760 in DESeq2 package was used to first normalize the gene read counts data and then
761 identified the differentially expressed genes. The MA plot was generated with the
762 function of plotMA in DESeq2 package. Q-value of 0.05 is regarded as the stringent
763 cutoff of calling DEGs while p-value less than 0.05 is regarded as the low stringency
764 cutoff. For the meta-chromosome plot of DEGs, all the chromosomes (except
765 chromosome Y) were first divided equally into 20bins with different length, and then the
766 number of DEGs in each bin was counted. GO analysis was performed using DAVID.

767

768 *Analysis of 5hydroxymethyl-cytosine*

769 *5hmC capture (hmC-Seal)*

770 Chemical labeling-based 5hmC enrichment was described previously¹⁰⁷. In brief, five
771 microgram of genomic DNA was sonicated to 100-500 bp, and then mixed with 100 μ l
772 reaction buffer (50 mM HEPES at pH 8.0, 25 mM MgCl₂, 250 μ M UDP-6-N₃-Glu and
773 2.25 μ M wild-type β -glucosyltransferase (β -GT)). Reactions were incubated at 37°C for
774 1 hour, and DNA substrates were purified by Qiagen DNA purification kit. 150 μ M
775 dibenzocyclooctyne modified biotin were mixed with β -GT-modified DNA. The labeling
776 reaction was performed at 37°C for 2 hours. The biotin-labeled DNA was then enriched
777 by Streptavidin-coupled Dynabeads (Dynabeads MyOne™ Streptavidin T1, Life
778 Technologies) and purified by Qiagen DNA purification kit. The quantity and quality of
779 purified DNA were analyzed by Qubit 3.0 Fluorometer (Invitrogen) and Agilent 2100
780 BioAnalyzer using DNA high sensitivity analysis kits (Agilent Technologies).

781

782 *MeDIP-seq*

783 Methylated DNA Immunoprecipitation (MeDIP) experiments were performed according
784 to the manufacturer's protocol (Active Motif) and described previously¹⁰⁸. In brief, five
785 microgram of genomic DNA were sonicated to 100-500 bp, and incubated with 5mC-
786 specific antibody (Active Motif) at 4°C overnight. Enriched methylated DNA will be
787 extensively washed and purified by Qiagen DNA purification kit. The quantity and quality
788 of purified DNA were analyzed by Qubit 3.0 Fluorometer (Invitrogen) and Agilent
789 Bioanalyzer using DNA high sensitivity analysis kits (Agilent Technologies).

790

791 *Library Preparation and High-throughput Sequencing*

792 Enriched DNA from MeDIP and hME-Seal was subjected to library construction using
793 the NEBNext CHIP-Seq Library Prep Reagent Set for Illumina according the
794 manufacturer's protocol. In brief, 25 ng of input genomic DNA or experimental enriched
795 DNA were utilized for each library construction. 150-300 bp DNA fragments were
796 selected by AMPure XP Beads (Beckman Coulter) after the adapter ligation. An Agilent
797 2100 BioAnalyzer were used to quantify the amplified DNA, qPCR were applied to
798 accurately quantify the library concentration. 20 pM diluted libraries were used for
799 sequencing. 50-cycle single-end sequencing reactions were performed. Image
800 processing and sequence extraction were done using the standard Illumina Pipeline.

801

802 *Analysis*

803 Bowtie software was used to map the sequenced reads back to the human genome
804 (hg19) with the parameter of allowing up to two mismatches. Only the uniquely mapped
805 reads were then used to generate the piled-up genome coverage of methylation signals.
806 The methylation signals were further normalized by per million mapped reads for
807 following analysis. The human genome was then segmented into bins of 1kilo-base
808 pairs, which allows for the identification of bins which shows most dramatic methylation
809 signals differences between wild-type and mutant samples. The delta methylation signal
810 of 0.2 per million reads was chosen as the cutoff of calling the Differential Methylation
811 Regions. For the meta-chromosome plot, all the chromosomes (except chromosome Y)
812 were divided equally into 20 bins with different length. We then summarized the total
813 methylation signal within each bin and subtracted the signal between wild-type samples

814 and mutant samples. The subtracted signal was then plotted as the meta-chromosomal
815 plot of methylation differences.

816

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1090 **Figure Legends**

1091 **Figure 1. Generation of isogenic model of Rett Syndrome *in vitro***

1092 **A**, Fibroblasts isolated from Rett Syndrome patients (R982 and R567) heterozygous for
1093 MECP2 mutations exhibit a mosaic pattern of MECP2 expression due to random XCI.
1094 Note that roughly 50% of fibroblasts from each patient express MECP2. **B**, Multiple
1095 isogenic hiPSC lines were produced from patient 982 with a typical Yamanaka protocol
1096 yielding individual isogenic clones with and without MECP2 expression from the same
1097 patient, as judged by NANOG and OCT4 staining. **C**, Specification of 982 patient
1098 derived hiPSCs towards neural progenitor cells yielded homogenous cultures of NPCs
1099 with and without MECP2. **D**, terminal differentiation of 982 patient derived NPCs
1100 towards neurons and glial by growth factor withdrawal yielded normal neural derivatives
1101 as measured by immunostaining for MAP2 and GFAP. **E**, MECP2⁺ and MECP2⁻
1102 hiPSCs and neurons were generated from patient 982 (R982.16 and R982.15) and
1103 assayed for activity of the AKT pathway by western blot with antibodies that recognize
1104 the active forms of Akt and its downstream target S6. **F**, Sholl assay of dendritic
1105 complexity was performed on WT vs MUT neurons derived from patient 982. Increased
1106 # of branch points indicates increased dendritic complexity, measured as a function of
1107 distance from the cell body. *p value < 0.05 according to student's t test. Bar graphs
1108 represent mean +/- SEM.

1109 **Figure 2. Hypomethylation of 5-hydroxymethylcytosine in MECP2 null cells**

1110 **A**, The overall delta methylation signal distribution is shown. The cutoff was made
1111 based on the difference of 0.2 per million reads per base pair. **B**, Number of differential

1112 5hmC regions (DhmRs) are presented as either gain of 5hmC (hypermethylated) or loss
1113 of 5hmC (hypomethylated) in each patient line, comparing MECP+ clones to MECP2-
1114 clones. Differential hydroxymethylation pattern between clones from 982 and 567 shows
1115 the overall delta-methylation as hypomethylation. **C**, Localization of DhmRs within
1116 various genomic features relative to the portion of those features in the genome. The
1117 highest concentration of DhmRs was found in coding exons (light green). **D**, Mapping
1118 DhmRs across metachromosomes representing the relative location across all
1119 chromosomes shows an increase in DhmRs towards the ends of chromosomes. The y-
1120 axis represents the differences of normalized methylation signal (piled-up signal per
1121 million mapped reads) between wild-type and mutant. **E**, Immunostaining for 5hmC in
1122 hiPSC clones from patients 982 and 567 indicated that levels of this DNA methylation
1123 mark are considerably lower in MECP2 null hiPSC clones. **F**, 5hmC staining was
1124 quantified in hiPSCs derived from both patient 567 and 982. *p value < 0.05 according
1125 to student's t test. Bar graphs represent mean +/- SEM.

1126

1127 **Figure 3. Loss of MECP2 is associated with differential gene expression**
1128 **particularly in neurons.**

1129 **A**, Immunostaining neurons generated from patient 982 for TuJ1, a neuronal-specific
1130 marker. **Right**, quantification of dendritic complexity by counting endpoints shows a
1131 significant difference between neurons with and without MECP2 made from patient 982.
1132 **B**, Volcano plots of differentially expressed genes (DEGs) in hiPSCs, NPCs and
1133 Neurons shows that loss of MECP2 has a profound effect on gene expression in

1134 neurons. **C**, Gene ontological analysis of DEGs increased versus decreased in MECP2
1135 null neurons. **D**, DEGs were mapped to a metachromosome to determine their relative
1136 location across chromosomes. In general, upregulated DEGs were enriched towards
1137 the ends of chromosomes, while downregulated DEGs showed no clear pattern of
1138 location. **E**, DEG and DhmrRs are statistically significantly overlapped in hiPSCs. This
1139 analysis was performed by randomly select the same number of genes with iPSC FDR
1140 DEG from the hg19 genes, then calculating the overlapping with hmC DMRs in a
1141 permutation test performed 5,000 times. The permuted number of overlapping genes
1142 is shown in the parentheses.

1143

1144 **Figure 4. Loss of MECP2 leads to induction of subtelomeric genes including**
1145 **TERRA, a long non-coding RNA**

1146 **A**, RT-PCR for subtelomeric genes in hiPSCs, NPCs and neurons derived from
1147 patients. **B**, RT-PCR for subtelomeric genes in WT NPCs with silencing of MECP2 by
1148 siRNA. **C**, RT-PCR for subtelomeric genes in WT brain tissue derived NPCs with
1149 silencing of MECP2 by siRNA. **D**, RT-PCR with the same samples described in **A**, **B**
1150 and **C** for TERRA transcripts. **E**, Silencing of TET expression by siRNA was assessed
1151 by RT-PCR. **F**, Knockdown of TET followed by RT-PCR for TERRA transcripts and
1152 subtelomeric genes also showed that loss of 5hmC is associated with induction of
1153 subtelomeric gene expression. In this figure, all data presented are the resulting relative
1154 fold change differences found in at least three biologically independent experiments. In
1155 addition, student's t-test was performed across all three or more experiments, and those

1156 with a p-value < 0.05 are indicated with an asterisk. Bar graphs represent mean +/-
1157 SEM. The identity of cells used in each replicate experiment are described in
1158 Supplemental Table 1.

1159

1160 **Figure 5. Loss of MECP2 is associated with telomere shortening**

1161 **A**, Quantitative PCR for telomere length based on a ratio of telomere product versus an
1162 autosomal locus (T/S ratio) showed that loss of MECP2 in patients or by siRNA for
1163 MECP2 is associated with shorter telomeres in hiPSCs, NPCs and neurons. The data
1164 presented are the result of at least three biologically independent experiments, and
1165 asterisks indicated p-value < 0.05 according to student's t test. Bar graphs represent
1166 mean +/- SEM. A complete sample list across all experiments used is provided in
1167 Supplemental Table 1. **B**, As an independent method, quantitative FISH was performed
1168 for telomere length as a function of centromere size. **Bottom**, quantification of telomere
1169 length in NPCs in two separate experiments from patient 982. **C**, Southern blotting with
1170 genomic DNA and a telomere specific probe showed telomere shortening in the
1171 absence of MECP2 in hiPSCs and NPCs derived from patient 982.

1172

1173 **Figure 6. Physiological consequences to telomere shortening in the absence of** 1174 **MECP2**

1175 Immunostaining for H2aX and PML can identify cells with telomere dysfunction. **A**,
1176 Immunostaining NPCs in the absence of MECP2 showed a strong increase in H2aX,

1177 which was quantified as a function of SOX2 immunostaining in 567 derived NPCs. **B**,
1178 Immunostaining NPCs in the absence of MECP2 showed a strong increase in PML,
1179 which was quantified as a function of SOX2 immunostaining in 982 derived NPCs. **C**,
1180 Immunostaining for p53 and p21, a target of p53, showed an increase of these stress
1181 markers in MECP2 null neurons derived from patient 567. **D**, Cells undergoing
1182 senescence show upregulation of endogenous b-galactosidase activity. Clones of
1183 fibroblasts lacking MECP2 showed strong b-gal activity, while those of WT fibroblasts
1184 did not. **E**, The senescence assay applied to neuronal cultures showed a strong
1185 increase in the absence of MECP2. **F**, Treatment of MECP2-null neurons with DMSO
1186 or Pifithrin, followed by immunostaining with antibody for TuJ1 shows a change in
1187 dendritic branching and morphology following treatment with Pifithrin. Bottom left, RT-
1188 PCR for GADD45, a p53 target gene, showed that Pifithrin reduced p53 activity. Bottom
1189 right, Quantification of branching phenotype across three independent experiments
1190 showed a strong increase in branching as measured by the number of endpoints. **G**,
1191 Schematic to depict molecular events known to regulate the ends of chromosomes.
1192 Shown on the right is the result of loss of MECP2, which according to immunostaining
1193 and senescence activity assays, led to neuronal stress and TIF. In this figure, all data
1194 resulted from at least three independent experiments. *p value<0.05 according to
1195 student's t test. Bar graphs represent mean +/- SEM.

1196

1197 **Figure 7. Rett patient brains show telomere shortening and induction of p53**

1198 **A**, Female Rett patient brains show variable XCI skewing in neurons as judged by
1199 immunostaining for MECP2, and quantified as a function of DAPI and MAP2 staining.
1200 **B**, Southern blotting of genomic DNA with a telomere specific probe shows the average
1201 telomere length in both control (5559) and Rett (1815) brain compared to ladder (right).
1202 **C**, qPCR from genomic DNA of aged matched Rett patient brains and control brains. **D**,
1203 As measured by RT-PCR, Rett patient brains show an increase in TERRA transcripts
1204 compared to aged-match controls. $N \geq 3$ independent experiments. Bar graphs
1205 represent mean +/- SEM. **E**, Extended characterization of patient 1815 and 5784
1206 showed increased expression of PML specifically in MECP2 null neurons in each of
1207 these two patient brains. Yellow inset is a magnification of box showing high
1208 magnification of PML staining specifically in MECP2 null neurons. Right panel shows
1209 quantification of PML signal in Rett patient brain, comparing the signal in MECP2+
1210 versus MECP2- neurons. **F**, Immunostaining Rett brain for MECP2 and p53 shows
1211 higher levels of p53 specifically in MECP2- neurons (quantified on the right).

1212

1213 **Figure 1-figure supplement 1. hiPSCs lacking MECP2 are pluripotent**

1214 Teratoma assay was performed to establish pluripotency of hiPSCs made from Rett
1215 patient fibroblasts. The resulting tumors each showed evidence of differentiation
1216 towards all three embryonic germ layers.

1217

1218 **Figure 1-figure supplement 2. Similarity of NPCs generated with and without** 1219 **MECP2**

1220 **A**, NPCs were produced from isogenic hiPSCs of Rett patient, and assessed by western
1221 blot to validate loss of MECP2 and specificity of antibody. Top panel shows that the
1222 antibody only recognizes MECP2. Bottom panel shows that in NPCs from both
1223 patients, individual clones either express or lack MECP2. **B**, The ability of hiPSCs to
1224 generate NPCs was assayed in Rosette formation assay. Lack of MECP2 did not affect
1225 rosette formation across multiple lines from both patients. N=4 independent
1226 experiments. *p value < 0.05 according to student's t test (for patient R567) or ANOVA
1227 (for patient R982). Bar graphs represent mean +/- SEM. **C**, Growth curves show that
1228 loss of MECP2 does not affect proliferation of NPCs made from either patient. **D**, 3
1229 weeks of growth factor withdrawal drives NPCs to differentiate into neurons and glia as
1230 measured here by immunostaining for MAP2/Tuj1 or S100/GFAP in patient 567 derived
1231 cultures. There was no consistent difference in differentiation potential across lines
1232 from either patient. N=2 independent experiments. Bar graphs represent mean +/-
1233 SEM. **E**, Patient 982 derived cultures also do not show dramatic differences in the
1234 presence of neurons or astrocytes as measured by MAP2 and S100. N=3 independent
1235 experiments. Bar graphs represent mean +/- SEM.

1236

1237 **Figure 3-figure supplement 1. RNA-seq analysis to determine the relative ration**
1238 **of WT versus MUT transcripts of MECP2 in Rett patient derived lines.** Detection of
1239 WT and MUT transcripts from each of the lines indicated demonstrated a clear bias
1240 towards individual alleles in each patient derived line. This analysis indicates XCI status
1241 for each allele, and demonstrates that XCI status is unchanged, even after
1242 differentiation to neurons.

1243

1244 **Figure 3-figure supplement 2. A**, Immunofluorescence of interneuron progenitors
1245 from WT (**top**) or MECP2null (**bottom**) clones. **B**, Immunofluorescence of interneurons
1246 generated from a MECP2null hiPSC clone generated by 3i protocol. **C**, Volcano plots of
1247 lower stringency DEGs in hiPSCs and NPCs between MECP2+ versus MECP2- clones
1248 (p-value < 0.05). **D**, Mapping of low stringency DEGs in hiPSCs and NPCs across
1249 metachromosome to measure enrichment of DEG location.

1250

1251 **Figure 4-figure supplement 1. Silencing MECP2 by siRNA**

1252 MECP2 was downregulated by RNA interference, quantified by RT-PCR (left), for
1253 protein by western blot (middle), and as demonstrated by immunostaining for MECP2
1254 (right). N=3 independent experiments. *p value < 0.05 according to student's t test. Bar
1255 graphs represent mean +/- SEM.

1256

1257 **Figure 6-figure supplement 1. Transduction of Progerin leads to phenotypes**

1258 **similar to loss of MECP2. A**, Cells undergoing senescence are known to induce and
1259 secrete a group of genes called SASP. RNA-seq data from neurons were mined for
1260 SASP genes, and shown are those SASP genes that were differentially expressed
1261 between patient derived neurons with and without MECP2. **B**, qPCR for telomere length
1262 in WT NPCs showed that Progerin infected cells have on average shorter telomeres.
1263 N=3 independent experiments. *p value < 0.05 according to student's t test. Bar graphs
1264 represent mean +/- SEM. **C**, RT-PCR from progerin infected NPCs showed an

1265 induction of various subtelomeric genes. N≥3 independent experiments. *p value<0.05
1266 according to student's t test. Bar graphs represent mean +/- SEM. **D**, RT-PCR for
1267 TERRA transcripts following progerin transduction. N≥3 independent experiments. *p
1268 value < 0.05 according to student's t test. Bar graphs represent mean +/- SEM. **E**,
1269 Immunostaining for p53 following progerin expression. Quantification of p53 in infected
1270 cells (either Nuclear-GFP or Progerin-GFP) is shown on the right. *p value < 0.05
1271 according to student's t test. Bar graphs represent mean +/- SEM.

1272

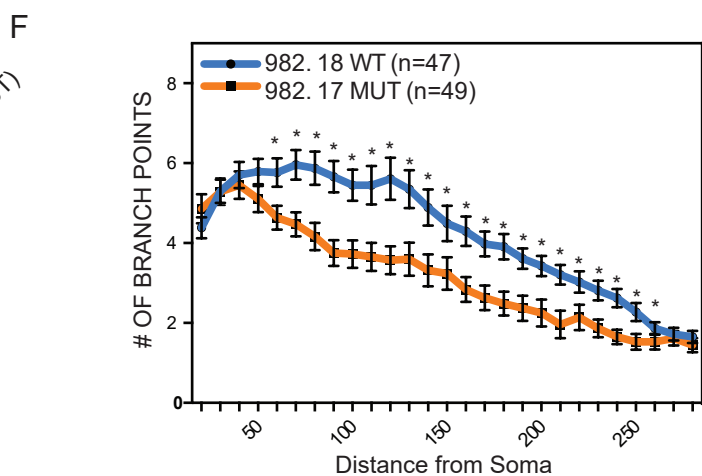
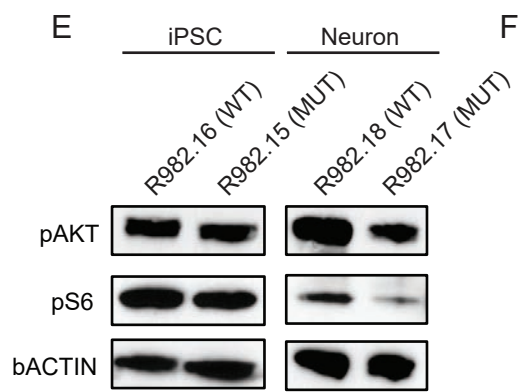
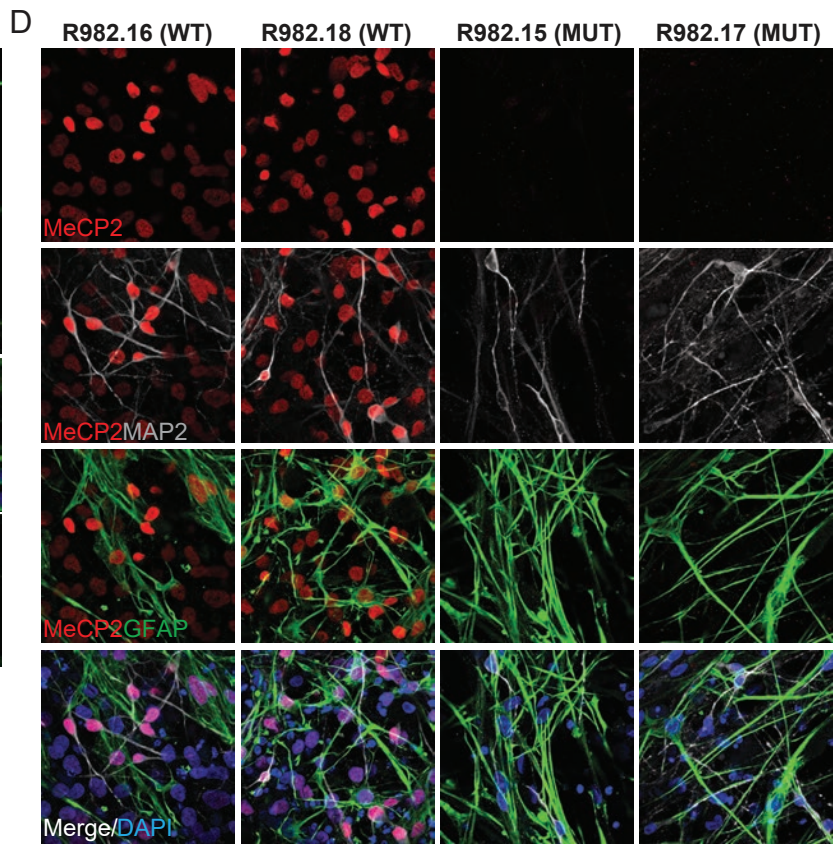
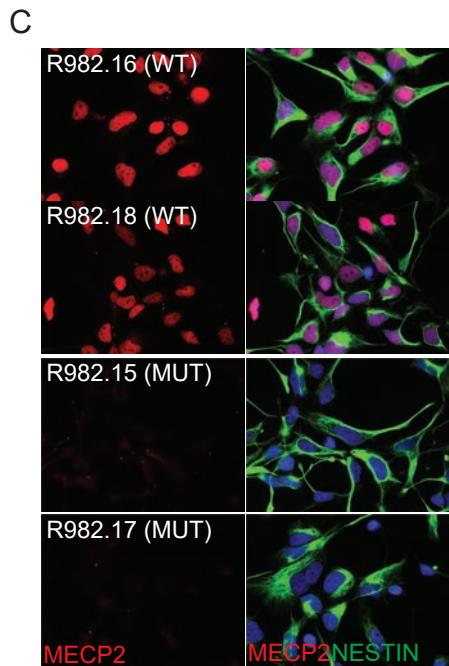
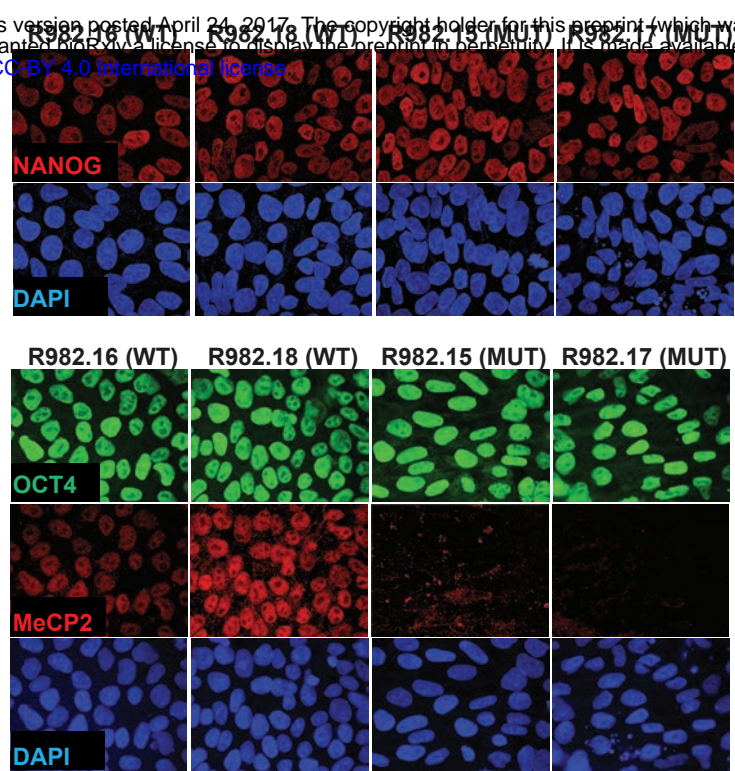
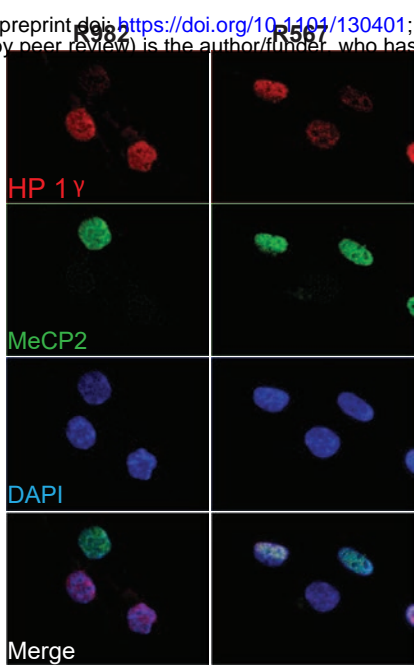
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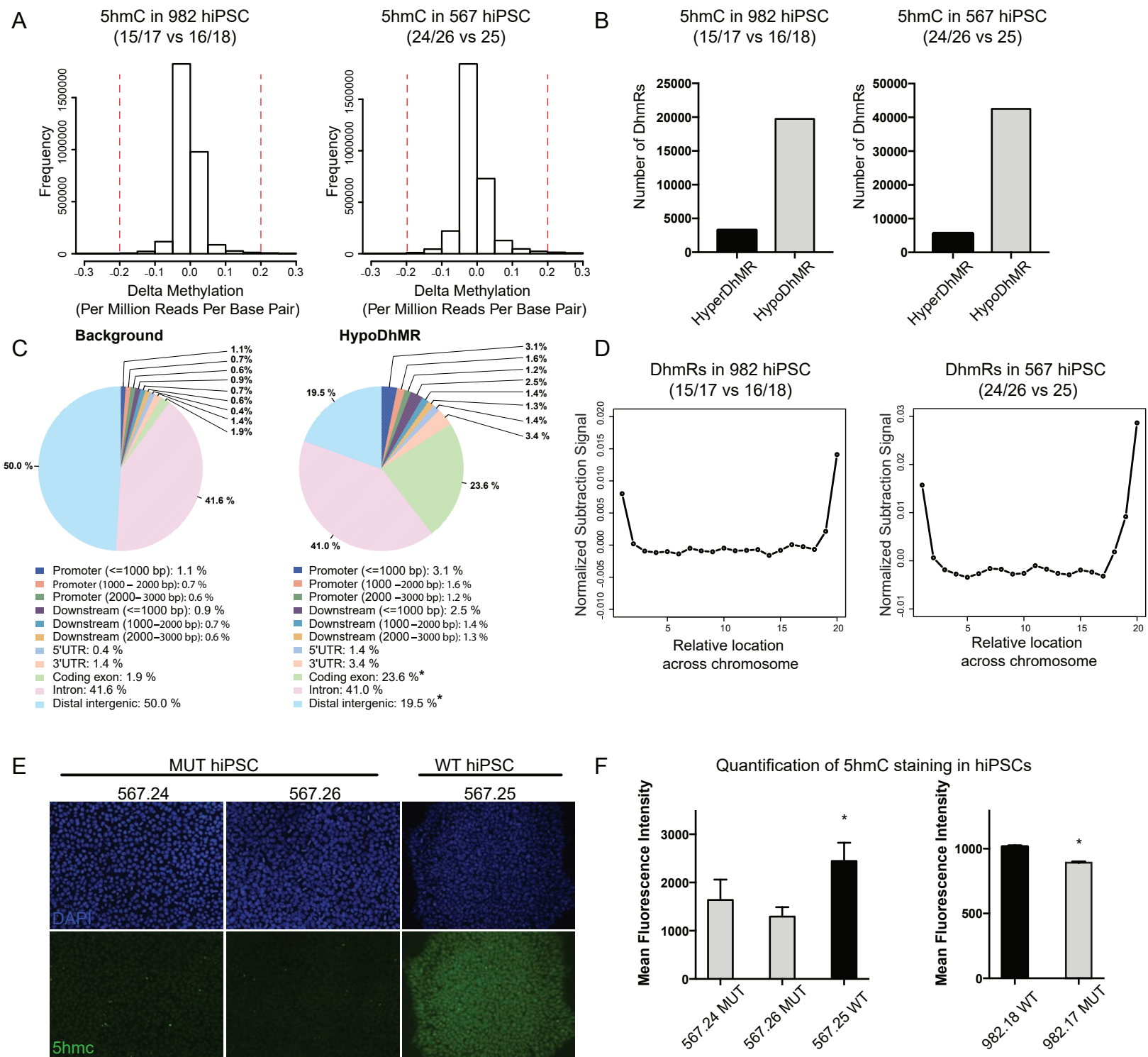
1274 Provided is a list of all cell types analyzed across the RT-PCR and Telomere qPCR
1275 experiments performed in this manuscript.

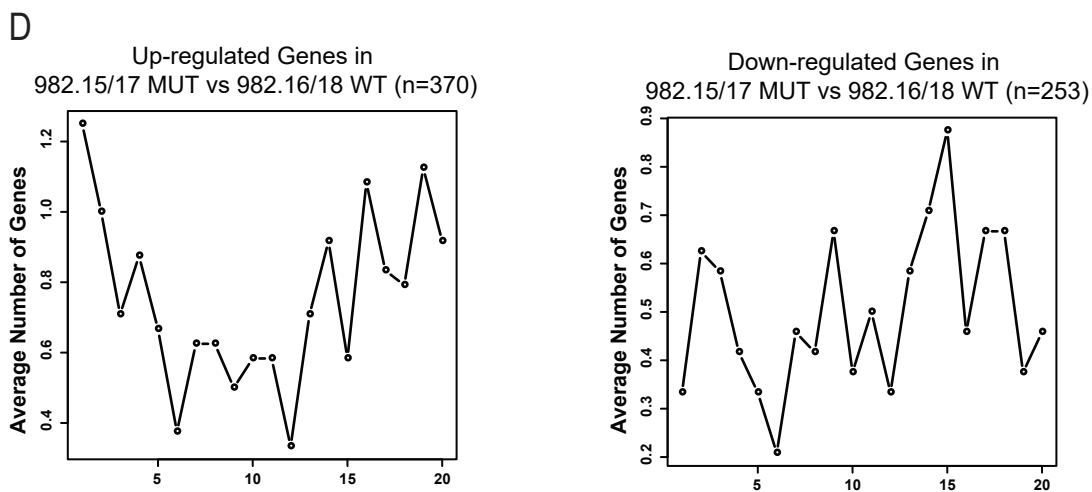
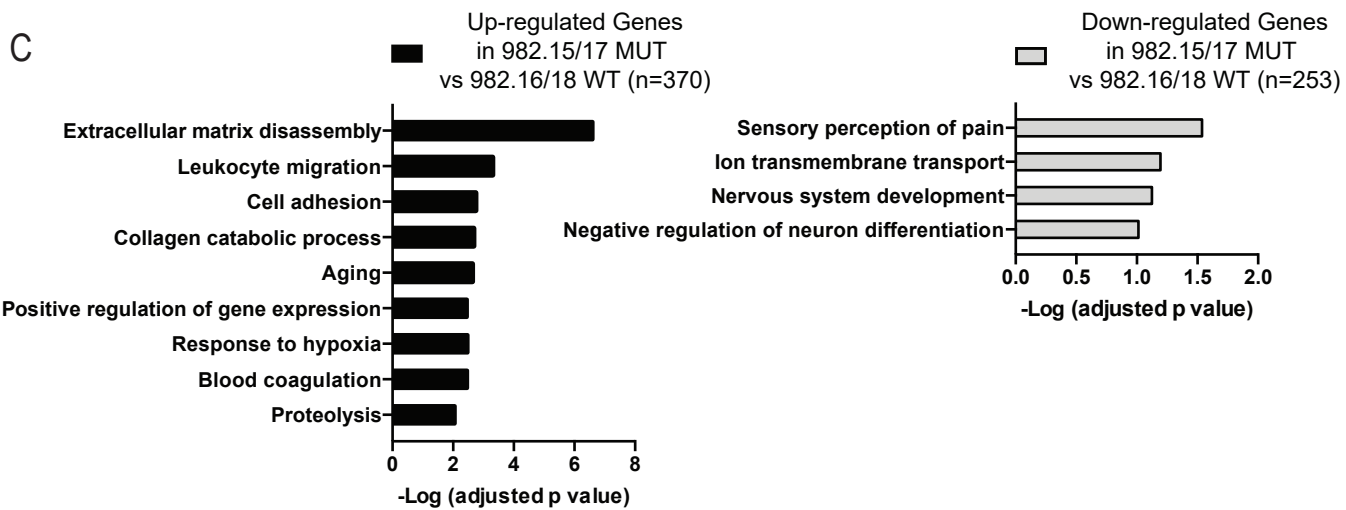
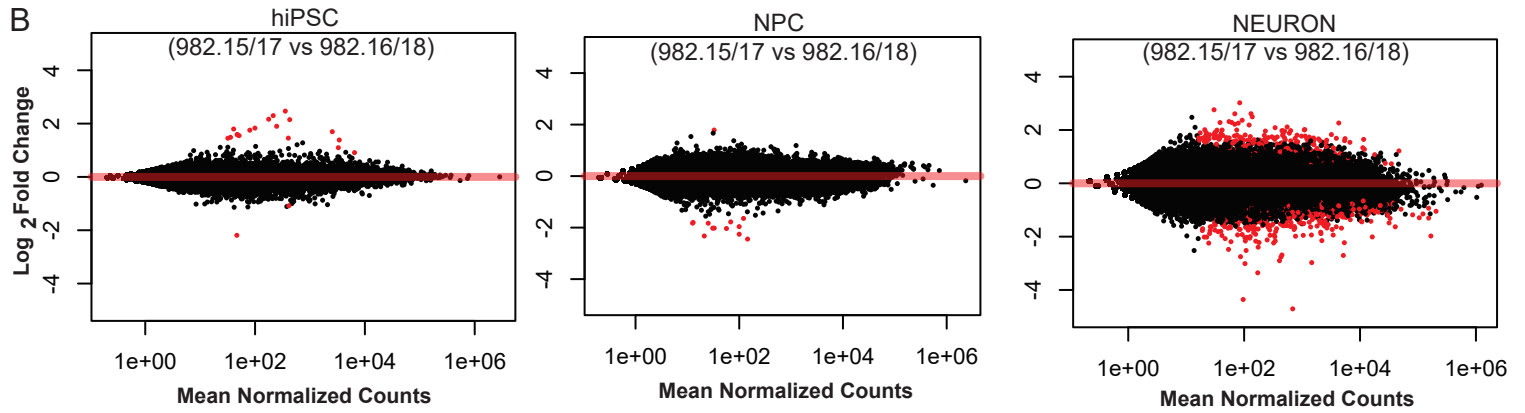
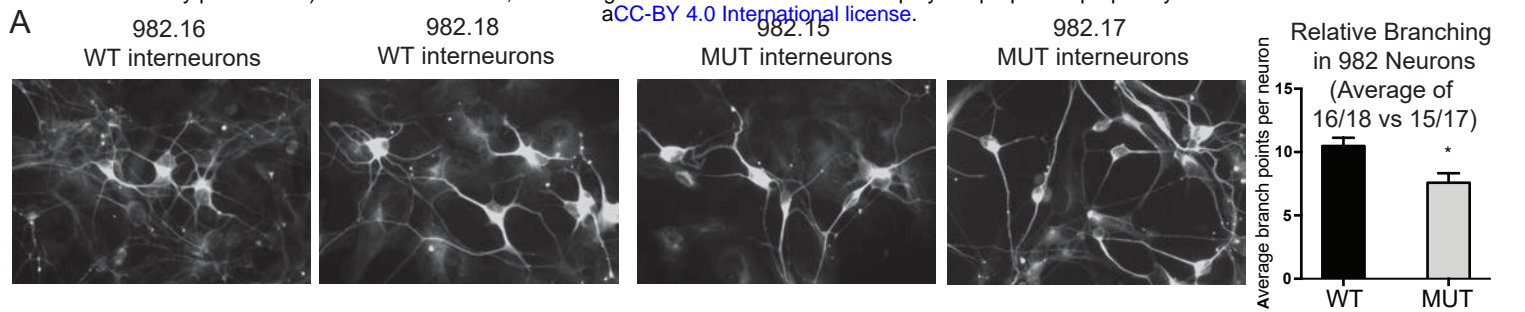
1276 **Supplemental Table 2.**

1277 Provided is a list of all the primers used in this study.

1278

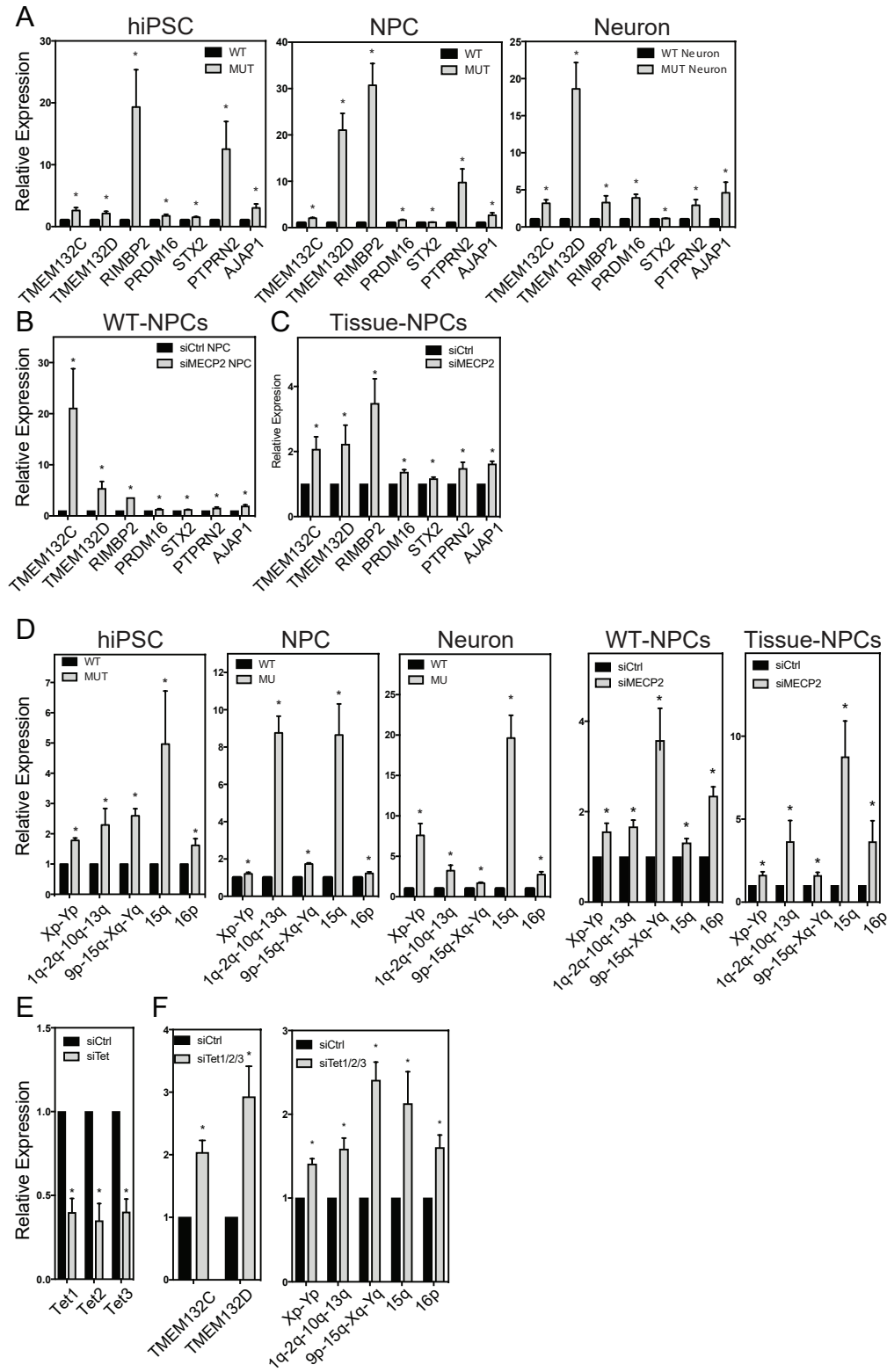


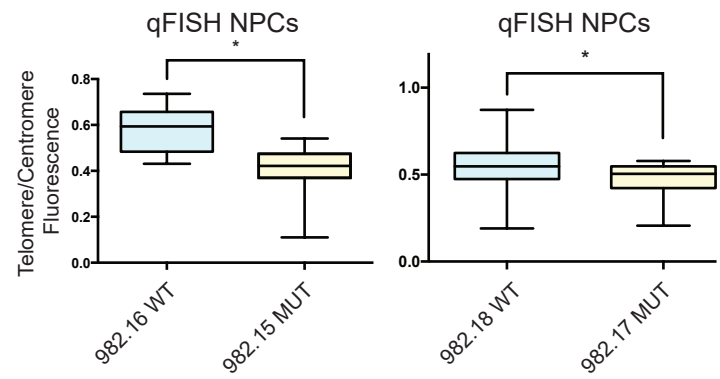
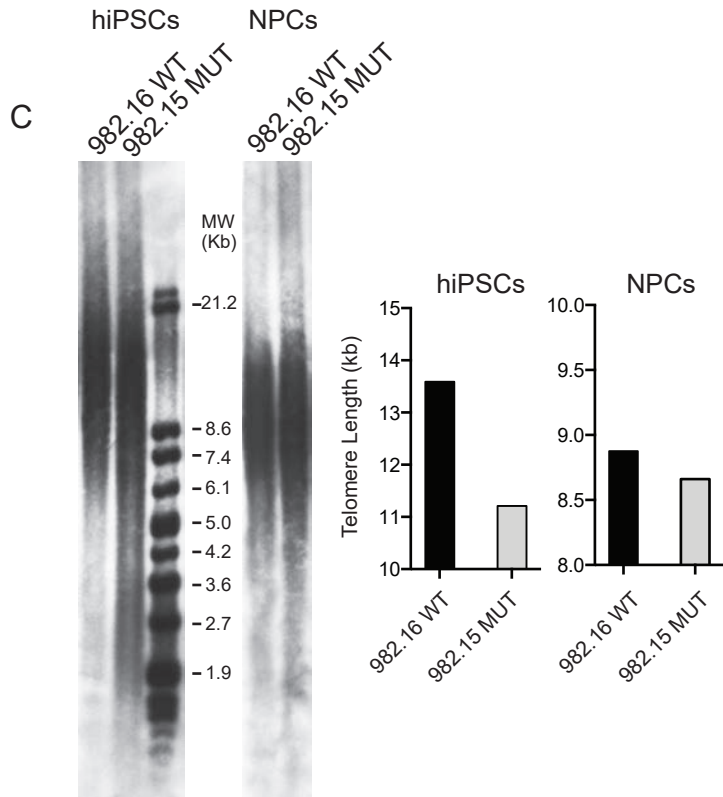
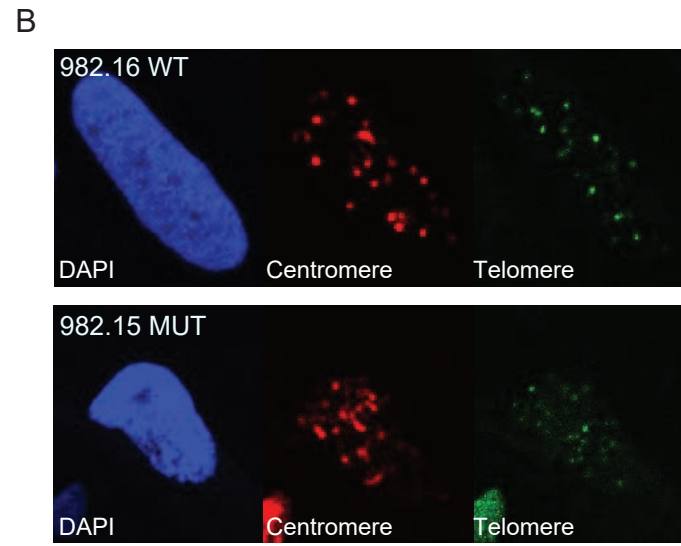
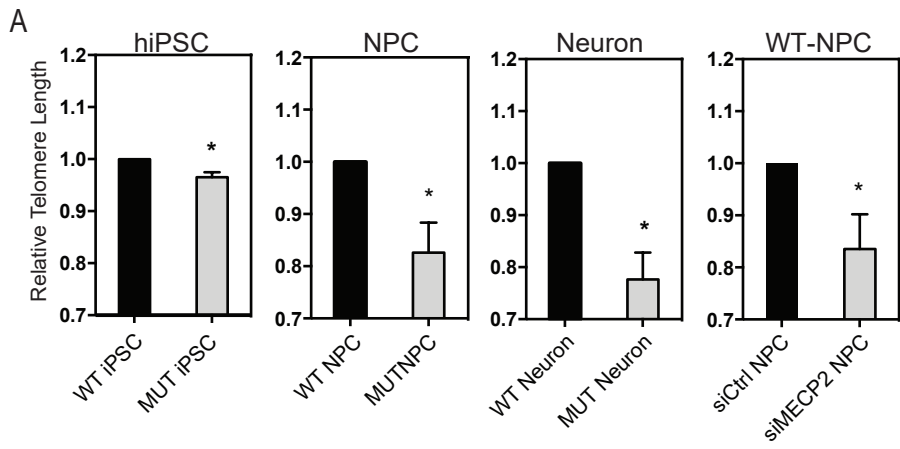


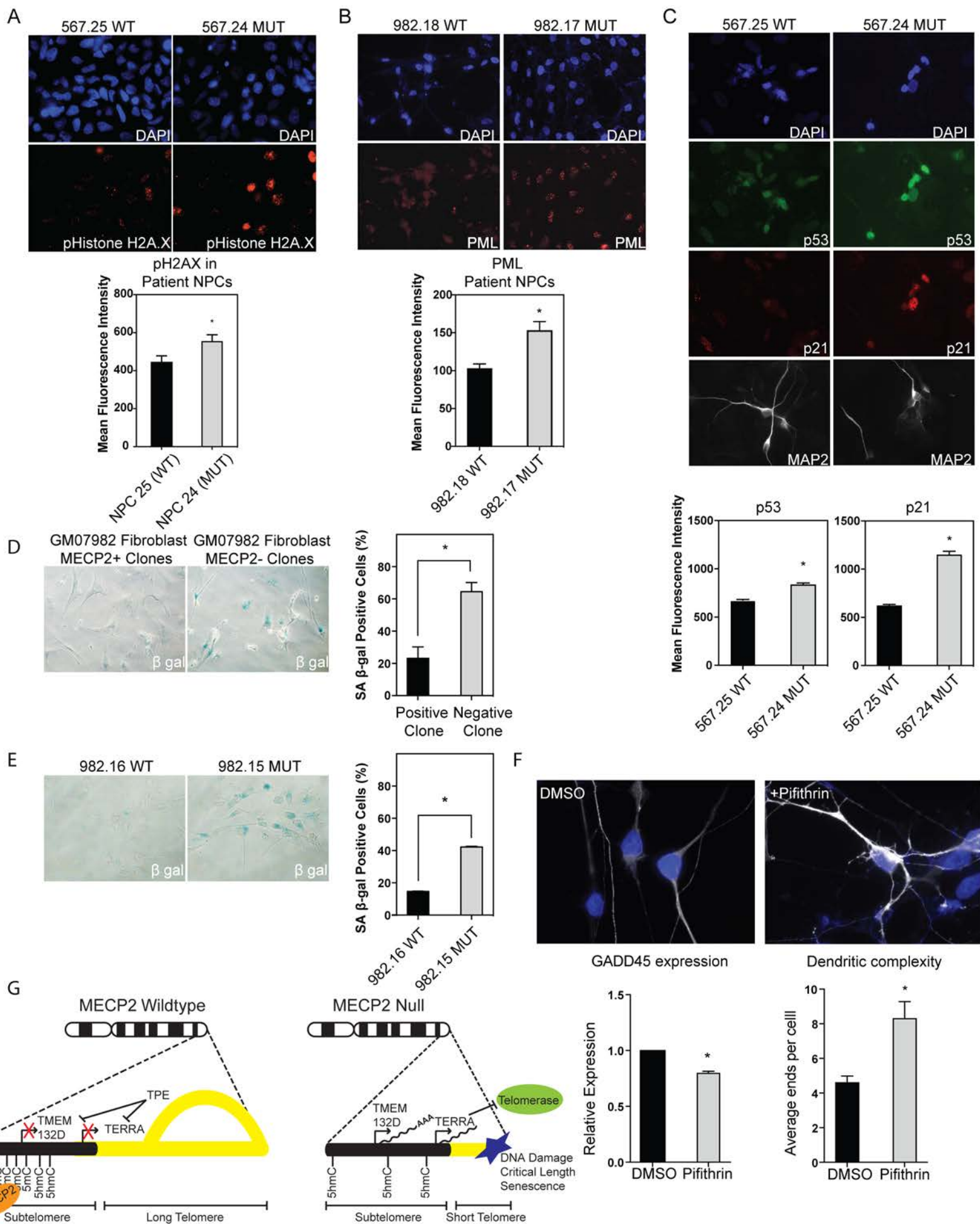


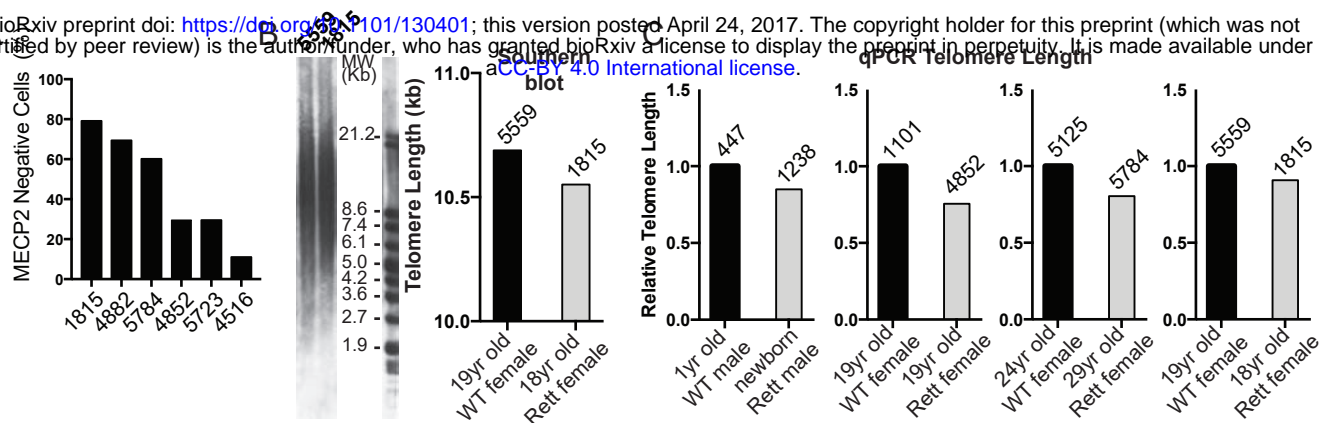
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Number of DEG (hiPSCs)	Overlap with Hyper DhMR	Overlap with Hypo DhMR
20	5 (1)	19 (6)

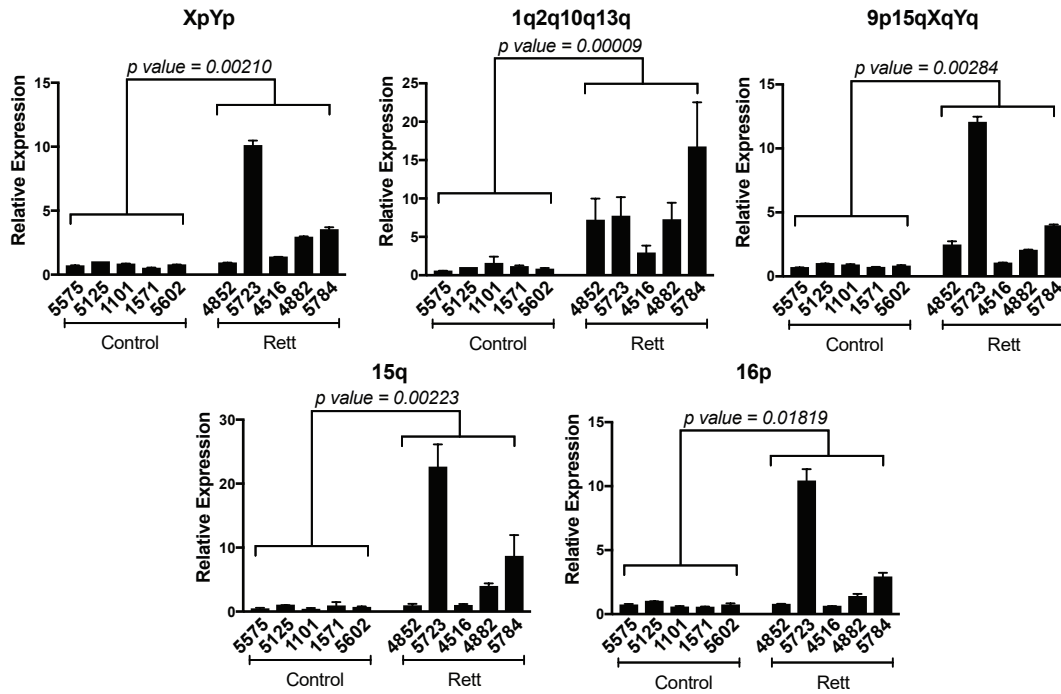




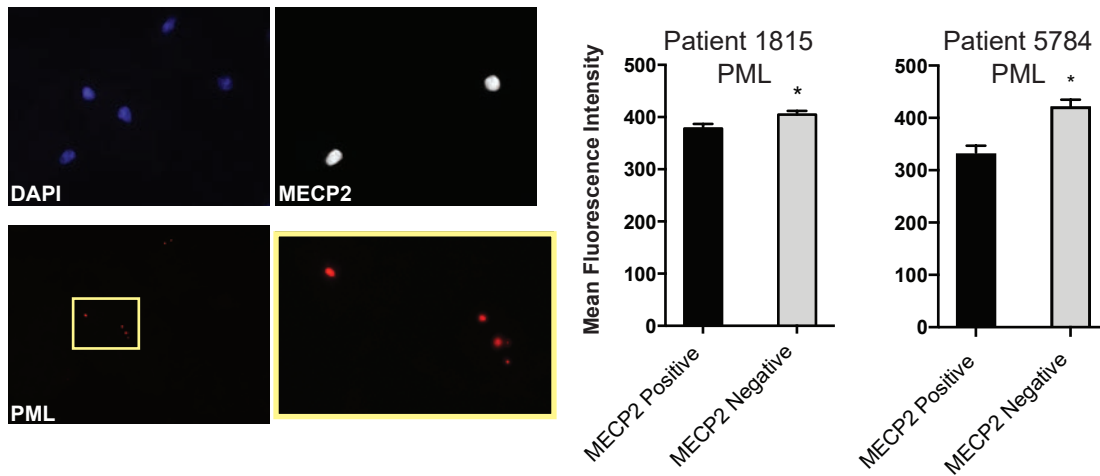




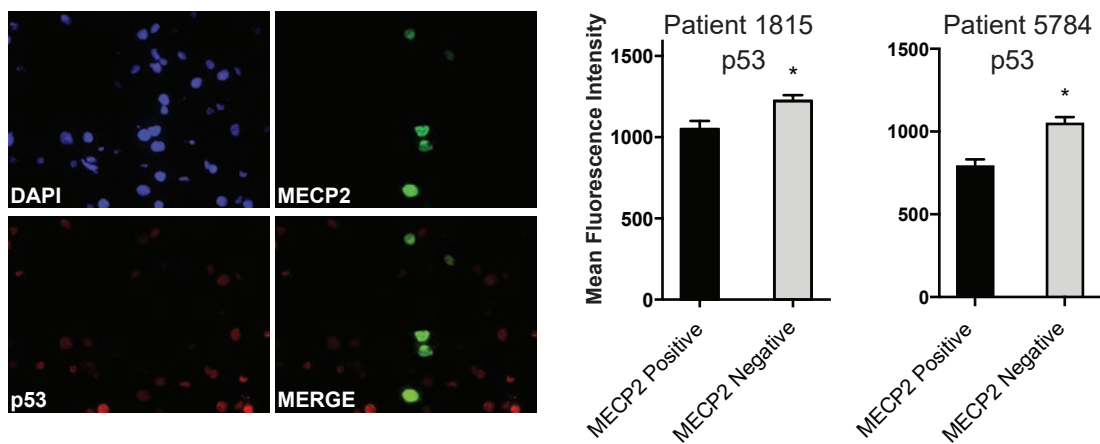
D



E



F



	N (biological gene	Sample	Correspondir	Date of Experiments
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	5 1q_2q_10q_1	25/26	Fig4D	20151028
		15/16	Fig4D	20160602
		15/16	Fig4D	20160222
		24/25	Fig4D	20151028
		25/26	Fig4D	20151028
		15/16	Fig4D	20151102
	3 9p_15q_Xq_1	15/16	Fig4D	20160602
		15/16	Fig4D	20160222
		24/25	Fig4D	20151028
	6 15q	15/16	Fig4D	20160420
		15/16	Fig4D	20160512
		15/16	Fig4D	20151102
		17/18	Fig4D	20160420
		24/25	Fig4D	20151102
		15/16	Fig4D	20160602
		3 16p	17/18	Fig4D
	15/16		Fig4D	20160222
	24/25		Fig4D	20151102
24/25	Fig4D		20151102	
NPC Terra	4 Xp-Yp	17/18	Fig4D	20151113
		17/18	Fig4D	20151113
		17/18	Fig4D	20151113
		17/18	Fig4D	20151027
	3 1q_2q_10q_1	17/18	Fig4D	20151027
		17/18	Fig4D	20151027
		17/18	Fig4D	20151109
	3 9p_15q_Xq_1	17/18	Fig4D	20151027
		17/18	Fig4D	20151027
		17/18	Fig4D	20151109
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		15/16	Fig4D	20151113
		17/18	Fig4D	20151113
		17/18	Fig4D	20150508
	Neuron Terra	3 Xp-Yp	17/18	Fig4D
25/26			Fig4D	20160825
25/26			Fig4D	20160825
3 1q_2q_10q_1		24/25	Fig4D	20160527
		26/25	Fig4D	20160527
3 1q_2q_10q_1	26/25	Fig4D	20160323	
	15/16	Fig4D	20160323	
	26/25	Fig4D	20160323	
		17/18	Fig4D	20160527

HIPS Subtel	3 9p_15q_Xq_	17/18	Fig4D	20160527
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		L15/L16	Fig4D	20160624
	3 15q	26/25	Fig4D	20160323
		24/25	Fig4D	20160527
		26/25	Fig4D	20160527
	3 16p	L17/18	Fig4D	20160323
		17/18	Fig4D	20160527
		24/25	Fig4D	20160527
	3 TMEM132D E	24/25	Fig4A	20151028
		25/26	Fig4A	20151028
		25/26	Fig4A	20151028
	TMEM132C E	25/26	Fig4A	20161104
		25/26	Fig4A	20161104
		25/26	Fig4A	20161104
	4 RIMBP2	15/16	Fig4A	20160411
		17/18	Fig4A	20160411
		24/25	Fig4A	20160411
		25/26	Fig4A	20160411
	4 PTPRN2	17/18	Fig4A	20160411
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3 STX2	15/16	Fig4A	20160224	
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NPC Subtel	4 TMEM132D €	17/18	Fig4A	20151109
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		17/18	Fig4A	20160429

Neuron Subt	3	PTPRN2	17/18	Fig4A	20160429
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	4	TMEM132D	E P15/16	Fig4A	20160323
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			L15/16	Fig4A	20160323
			L17/18	Fig4A	20160323
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			17/18	Fig4A	20160527
			25/26	Fig4A	20160825
	4	RIMBP2	L17/L18	Fig4A	20160218
			P15/P16	Fig4A	20160218
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			25/26	Fig4A	20160218
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			24/25	Fig4A	20160527
			24/26	Fig4A	20160527
			17/18	Fig4A	20160527
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		3mt 24/25	Fig4A	20160810	
4	STX2	L15/16	Fig4A	20160218	
		L17/L18	Fig4A	20160218	
		P15/P16	Fig4A	20160218	
		P17/P18	Fig4A	20160218	
3	PRDM16	L17/L18	Fig4A	20160218	
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	N (biological replicates)	Telomere qPCR Sample	Corresponding	Date of Experiments	
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NPC	4	Telomere	15/16	Fig5A	20160516
			17/18	Fig5A	20160516
			24/25	Fig5A	20160516
			17/18	Fig5A	20150421
Neuron	3	Telomere	15/16	Fig5A	20160405
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