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1 **Mutations in *ACTL6B* cause neurodevelopmental deficits and epilepsy and lead to**
2 **loss of dendrites in human neurons**

3

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89

90

91

92

93 **Abstract**

94 We identified individuals with mutations in *ACTL6B*, a component of the chromatin
95 remodelling machinery including the BAF complex. Ten individuals harbored bi-allelic
96 mutations and presented with global developmental delay, epileptic encephalopathy and
97 spasticity, and ten individuals with de novo heterozygous mutations displayed intellectual
98 disability, ambulation deficits, severe language impairment, hypotonia, Rett-like
99 stereotypies and minor facial dysmorphisms (wide mouth, diastema, bulbous nose). Nine
100 of these ten unrelated individuals had the identical de novo c.1027G>A mutation. Human
101 derived neurons were generated that recaptured *ACTL6B* expression patterns in
102 development from progenitor cell to post-mitotic neuron, validating the use of this cell
103 model. Engineered knock-out of *ACTL6B* in wildtype human neurons resulted in
104 profound deficits in dendrite development, a result recapitulated in two individuals with
105 different bi-allelic mutations, and reversed on clonal genetic repair or exogenous
106 expression of *ACTL6B*. Whole transcriptome analyses and whole genomic profiling of
107 the BAF complex in wildtype and biallelic mutant *ACTL6B* NPCs and neurons revealed
108 increased genomic binding of the BAF complex in *ACTL6B* mutant cells, with
109 corresponding transcriptional changes in several genes including *TPPP* and *FSCN1*,
110 suggesting that altered regulation of some cytoskeletal genes contribute to altered
111 dendrite development. Assessment of biallelic and heterozygous *ACTL6B* mutations on
112 an *ACTL6B* KO human background demonstrated that biallelic mutations mimic
113 engineered deletion deficits while heterozygous mutations do not, suggesting that the
114 former are loss-of-function and the latter are gain-of function. These results reveal a role
115 for *ACTL6B* in neurodevelopment, and implicate another component of chromatin
116 remodelling machinery in brain disease.

117

118 **Introduction**

119 *ACTL6B* (MIM: 612458) encodes an actin-related protein (ARP), which are a
120 class of proteins that resemble actin and have roles in chromatin remodelling and
121 histone acetylation¹. Though *ACTL6B*, known as BAF53B, may interact with multiple
122 complexes in a particular spatiotemporal order, most investigations have focused on its
123 role in the BAF (BRG1/BRM-Associated Factor), or SWI/SNF complex², which serves as
124 an important regulator of gene expression by remodeling nucleosomes in an ATP-
125 dependant fashion³⁻⁵. In order to regulate different sets of genes during development,
126 BAF subunits can be exchanged with homologous alternatives³. One such switch in BAF
127 subunit composition occurs in developing neural cells as they exit the cell cycle. During
128 this time, the neural progenitor specific BAF (npBAF) complex transitions to the neural
129 specific BAF (nBAF) complex through the exchange of several subunits, including
130 BAF53A for its paralog BAF53B⁶. This is partly achieved through increased expression
131 of miR-9* and miR-124 in post mitotic neurons, which repress the expression of the gene
132 that encodes BAF53A, *ACTL6A* (MIM: 604958)⁷. nBAF complexes can bind the
133 transactivator CREST and be recruited to genes crucial for dendritogenesis through
134 interactions mediated by BAF53B⁸. As a result, loss of BAF53B protein levels during
135 neuronal development results in impaired dendritic outgrowth. An *Actl6b* KO mouse has
136 previously been generated, and found to have deficits in dendritic spine and synapse
137 function, leading to impaired long-term memory and poor survival⁹.

138 While different genes that contribute to the BAF complex have been found to be
139 associated with human disease (e.g., Nicolaides-Baraitser syndrome MIM: 601358,
140 *SMARCA2* MIM:600014; Coffin-Siris syndrome MIM: 135900, *ARID1B*; MIM: 614556)¹⁰;
141 ¹¹, *ACTL6B* has not been conclusively reported to have a deleterious role in human
142 neurological diseases. In this study, we identified individuals with neurodevelopmental

143 disorders with either inherited recessive mutations or dominantly acting *de novo*
144 mutations in *ACTL6B*, and sought to understand how mutations in *ACTL6B* might affect
145 the development of human neurons.

146

147 **Materials and methods**

148 **Description of studied individuals.** Individuals had whole exome sequencing as part of
149 local neurodevelopmental studies on developmental delay and intellectual disability,
150 autism or epilepsy (R1, R2a/b, R3a/b, R4, R5, R7, R9, R10, D2, D3, D7, D8). Informed
151 consent for participating in the genetic studies was obtained on protocols approved by
152 institutional review boards of local hospitals. Individuals D1 and D4 were enrolled in the
153 DDD study and provided informed consent for this study. Other individuals had exome
154 sequencing at GeneDx as part of clinical care (individuals R6, R8a/b, D5, D6, D9), and
155 after *ACTL6B* was identified as a candidate gene, provided informed consent for the
156 sharing of photographs or samples as applicable.

157

158 **Experimental procedures for sequencing.** DNA was extracted from peripheral blood
159 from affected individuals and parents using standard protocols. For individuals who had
160 Whole Genome Sequencing (R1, R2a/b, R10), the DNA libraries were prepared by using
161 the Illumina TruSeq DNA PCR-Free kits using the manufacturer's protocol. For
162 individuals who had Whole Exome Sequencing, the exome libraries were prepared using
163 Agilent SureSelect kits (R3ab, R4, R6, R8ab, R9, D1, D2, D4-D9), Roche-NimbleGen EZ
164 exome kits (R5, D3) and Illumina Nextera kits (R7). More details included in Tables 1
165 and 2. All libraries were then sequencing on Illumina HiSeq systems.

166

167 **Analysis of sequencing data.** Sequences were aligned using BWA, GATK, Novoalign,
168 Isaac, or LifeScope software. The variants were called using GATK, SAMtools, Annovar,
169 CarpeNovo, Isaac, LifeScope and in-house pipelines. More details can be found in
170 Tables S1 and S2. After identification of candidate variants in *ACTL6B*, their segregation
171 was confirmed by Sanger sequencing using standard protocols.

172 **Fibroblast reprogramming to induced pluripotent stem cells (iPSCs)** Fibroblasts
173 were obtained from biopsies or from Coriell (Table S3), and cultured in DMEM
174 (Invitrogen) supplemented with 10% bovine serum albumin (Invitrogen). Fibroblasts
175 were reprogrammed using episomal reprogramming vectors containing Oct4, Sox2,
176 Myc3/4, Klf4, ShRNA P53 (ALSTEM) and a puromycin resistance gene using a Neon
177 Transfection System (Invitrogen). Following transfection, cells were plated on tissue
178 culture plates coated with Matrigel (Corning) in TesR-E7 media (Stem Cell
179 Technologies) supplemented with 2ug/ml puromycin (Sigma). Following 48hrs of
180 puromycin selection, fresh TesR-E7 media was exchanged, until distinct and robust
181 iPSC colonies formed, at which point mTESR1 media (Stem Cell Technologies) was
182 used to maintain and proliferate the colonies. Quality control experiments for iPSCs
183 include mycoplasma testing, short tandem repeat profiling to ensure no sample mix-ups,
184 assessment of endogenous pluripotency factor, immunocytochemistry for pluripotency
185 markers, and molecular karyotyping.

186

187 **Molecular Karyotyping** To ensure no chromosomal abnormalities occurred as a result
188 of iPSC induction or gene editing, DNA from all generated iPSC lines was sent to Prince
189 of Wales Hospital (Shatin, Hong Kong) for sequencing on an Ion Torrent Hi-
190 Q Sequencer (ThermoFisher). Samples were sequenced with an average of 4 million
191 150bp reads per sample, for an average coverage of 0.0014X. Analysis was performed
192 using CNV-Seq¹². Positive controls included cells of origin and cells from families with
193 first-degree relationships where we could detect Mendelian inheritance of CNVs >1Mb.

194

195 **Differentiation of iPSCs to forebrain neural progenitor cells (NPCs)** iPSCs were
196 differentiated to forebrain NPCs according to our previously described methods^{13; 14}.
197 iPSC colonies were dissociated and resuspended in DMEM/F12 media supplemented
198 with N2 (Invitrogen) B27 (Invitrogen), and BSA [1 mg/ml], Y27632 [10 μ M] (AduoQ
199 Bioscience), SB431542 [10 mM](Selleckchem), and Noggin [200 ng/ml](GenScript),
200 onto non-adherent plates to form organoids. After one week of maintenance as
201 organoids, cells were dissociated and plated on Matrigel coated plates in DMEM/F12
202 supplemented with B27, bFGF (20ng/ml), EGF (20ng/ml), and laminin[1ug/ml] for a
203 further seven days of differentiation, with media exchanged every three days. Cells were
204 assessed for NPC morphology, and stained for markers of forebrain NPCs (PAX6,
205 SOX2, TUJ1) and OCT4.

206

207 **Differentiation of NPCs to Post-mitotic Neurons** For short term (5 days)
208 differentiation, NPCs were plated in DMEM/F12 media supplemented solely with B27. If
209 longer term (>5 days) differentiation was required, NPCs, were plated in BrainPhys SM1
210 (Stem Cell Technologies) and N2-A supplemented media (Stem Cell Technologies).
211 50% of this media was exchanged every three days. Previous work has shown that
212 neurons generated using this methodology express both GABAergic (~30%) and
213 Glutamatergic (60%) markers¹⁴ and are negative for midbrain markers, such as tyrosine
214 hydroxylase. Approximately 5-10% of cells stain for GFAP, an astrocyte marker.

215

216 **Whole Cell Recordings** For whole-cell patch-clamp recordings, individual coverslips
217 containing differentiated hPSC-Derived Neurons were transferred into a heated
218 recording chamber and continuously perfused (1 ml/min) with BrainPhys™ Neuronal

219 Medium (Catalog # 05791; StemCell Technologies) bubbled with a mixture of CO₂ (5%)
220 and O₂ (95%) and maintained at 25 °C. Whole-cell patch clamp recordings were
221 obtained using borosilicate pipettes (3–6 MΩ), filled with intracellular solution that
222 contained the following (in mM): 5 HEPES, 2 KCl, 136 potassium gluconate, 5 EGTA, 5
223 Mg-ATP, 8 creatine phosphate, and 0.35 GTP. The pH was adjusted to 7.27 with KOH,
224 and the osmolarity adjusted with distilled water or concentrated potassium gluconate if
225 needed to between 295 and 298 mOsm with an osmometer (3320; Advanced
226 Instruments). After a recording was completed, the nominal membrane potential in
227 voltage- and current-clamp recordings was corrected for the calculated 10 mV liquid
228 junction potential. All potential values reported reflect this correction. Once whole-cell
229 recording had been established, neurons were routinely held in voltage clamp at -70 mV
230 except when examining changes in the resting membrane potential and rheobase, which
231 was performed in current clamp. Cells were only studied if they exhibited a stable
232 holding current and access resistance for at least 10 min before experimental
233 manipulations. Data were acquired using a Digidata 1550A/ Multiclamp 700B (Axon
234 Instruments) and Clampex 10.5 (Molecular devices). Currents were filtered at 2 kHz and
235 digitized at 20 kHz.

236

237 **CRISPR/Cas9 gene editing** A double nickase CRISPR/Cas9 gene editing system with a
238 Paprika RFP (pRFP) reporter and gRNA targeting the first exon of the *ACTL6B* was
239 used for KO experiments. For *ACTL6B*ext33 repair experiments, a wild-type
240 CRISPR/CAS9-pRFP gene editing system was used to target the mutation in the stop
241 codon of exon 14 of *ACTL6B*. One μg of construct was added per transfection reaction,
242 and transfection was carried out simultaneously with iPSC induction to ensure clonality,
243 as previously described¹⁴. Following transfection, cells were selected for puromycin

244 resistance and RFP visualization as described⁸ allowing for cell expansion from a single
245 edited fibroblast. Potentially edited colonies were expanded and stored as cell lines after
246 which DNA was extracted and Sanger sequenced at Genome Quebec.

247

248 **RNA Sequencing** RNA samples with RIN values >9.0 were submitted to Genome
249 Quebec for RNA sequencing. Eight libraries were run per lane of an Illumina HiSeqV4
250 2500 flow cell (125 bp paired-end reads), which achieved an average of ~40 million
251 reads per library. For bioinformatic processing, we used FASTX-Toolkit, TopHat¹⁵
252 Bowtie¹⁶, and Cufflinks²¹⁷ with default parameters to preprocess, align, and assemble
253 reads into transcripts, estimate abundance, and test differential expression. More
254 detailed methods can be found here¹⁸.

255

256 **Western Blot** Cells were lysed with RIPA buffer (Sigma) supplemented with
257 SIGMAFAST™ Protease Inhibitor Tablets (Millipore-Sigma). Protein concentrations were
258 determined using a Pierce BCA Protein Assay Kit (ThermoFisher). Approximately 15 µg
259 of protein was loaded per well in Mini-PROTEAN® TGX Stain-Free™ Precast Gels
260 (Biorad). Gels were run at 150V for approximately 75 minutes, and then transferred to a
261 nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System (Biorad).
262 Membranes were blocked in 4% non-fat milk dissolved in TBS-T buffer (tris-buffered
263 saline-tritonX; Sigma-Aldrich) for twenty minutes, and then incubated with primary
264 antibodies overnight at 4°C with shaking. Blots were washed three times in TBS-T for
265 five minutes, and then incubated with appropriate mouse or rabbit secondary antibodies
266 for one hour at room temperature. Blots were washed a further three times in TBST for
267 five minutes, then imaged using a ChemiDoc™ XRS+ System (Biorad). Blots were
268 imaged and analysed using ImageLab (Biorad) software, and statistical analysis was

269 preformed using student T-tests when two samples conditions were present and a one-
270 way ANOVA when more than two sample conditions were present. Blots were
271 normalized to β -actin. Further details on the antibodies used for WB can be found in
272 Table S5.

273

274 **Quantitative PCR** Reverse transcriptions were done on the total RNA fraction in order to
275 obtain cDNA in 40 μ l volume containing 1 μ g of total RNA, 0,5 μ g random primers, 0.5
276 mM dNTPs, 0,01 M DTT and 400 U M-MLV RT (Invitrogen). The reactions were
277 performed in a total volume of 20 μ l volume on a 384 well plate either using an Applied
278 Biosystems 7900 HT (Applied Biosystems) or a QuantStudio 6 (Thermofisher) PCR
279 Machines. For each well, PCR mix included 10 μ l of Power SybrGreen PCR Mastermix
280 (Life Technologies), 1 μ l of primers/probe mix, 2 μ l of cDNA, H₂O up to 20 μ l. Serial
281 dilutions of a mix of cDNA ranging between 0.003052 ng and 50 ng were used to
282 generate a calibration curve for an absolute quantification. Protein levels were given as a
283 ratio between the relative quantities of the gene of interest and the endogenous control.
284 GAPDH was used as internal control for normalization. The normalized expression levels
285 were then compared between cell lines using either a student's t-test or an ANOVA with
286 post-hoc t-test. Further details on the primers used for qPCR can be found in Table S6.

287

288 **Immunofluorescence** Adherent Cells were washed with PBS, then fixed with 3%
289 paraformaldehyde (Sigma-Aldrich) on slides for fifteen minutes. Samples were
290 permeabilized with 0.5% TX-100 (Sigma-Aldrich) in 0.5% PBS-BSA for fifteen minutes,
291 and then blocked in 0.5% PBS-BSA for an additional fifteen minutes. Primary antibodies
292 were added in appropriate dilutions (Table S5) in 0.5% PBS-BSA and added to samples

293 for 30 minutes. Samples were washed in 0.5% PBS-BSA containing an appropriate
294 dilution of secondary antibody (Table S5) was added to the samples and incubated for
295 thirty minutes in the dark. Samples were washed with 0.5% PBS-BSA. Samples were
296 then visualized on an Apotome Florescent microscope (Zeiss). Neurolucida Tracing
297 Software (MBF Bioscience) was used to measure nuclei surface area, soma surface
298 area, and projection length. Images were processed and scale bars added in Image J.

299

300 **Chromatin immunoprecipitation (ChIP) sequencing** Samples were prepared for
301 ChIP-Seq and ChIP-qPCR using a Magna ChIP-Seq™ kit (Millipore-Sigma). Cells were
302 cross linked at day 0 and day 5 of differentiation by immersion in 37% formaldehyde.
303 Glycine was added at a final concentration of 125uM to the samples to inactive cross-
304 linking. DNA was sheared using a S220 Sonicator (Covaris), and precleared using a
305 protein A or G agarose beads. All samples were then probed using both a mouse
306 monoclonal (Santa Cruz, sc-17796) and rabbit polyclonal (Santa Cruz, sc-10768)
307 antibody directed against BRG1 overnight at 4°C. A IGG control was ran for both rabbit
308 polyclonal and mouse monoclonal antibodies using a pooled sample composed of equal
309 parts of all samples used for ChIP. A 0.2M glycine solution pH 2.6 was used for elution
310 of cross-linked proteins and DNA from the beads. DNA was purified using Agencourt®
311 AMPure® XP Beads (Beckman Coulter). Libraries were constructed using an NGS
312 Library Preparation Kit (Millipore-Sigma) and sent to Genome Quebec, where they were
313 sequenced using a Illumina HiSeqV4 2500 flow cell (125 bp paired-end reads) with
314 between 11-12 samples sequenced per lane.

315 **ChIPSeq Analysis**

316 **Quality trimming and pre-processing** Sequencing adaptors were clipped using Trim

317 Galore. Quality trimming was done with same tool. A phred score cut-off value of 20 was
318 used. Reads shorter than 20bp were filtered out. Reads were aligned to the Human
319 Reference Genome (hg19) using BWA software version 0.7.10. Resulting bam files
320 were filtered for minimal mapping quality (MAPQ \geq 20) and all alignments with samflag 4
321 (read unmapped) were excluded using SAMtools (version 0.1.19). Duplicates reads were
322 removed using the MarkDuplicates module of Picard (version 1.141) with the option
323 REMOVE_DUPLICATES=true.

324

325 **Peak calling** The identification of CHIP-seq enriched regions (peaks) was performed
326 using MACS2 (version 2.1.1); (macs2 callpeak --format BAM --broad --nomodel -q 0.05
327 --broad-cutoff 0.1 --extsize 500). Differential binding-sites analysis were done using the
328 DiffBind Bioconductor R package (version 2.6.6)[4]. Diffbind calls some of the DESeq2
329 (version 1.18.1) functions to perform the contrast analysis between pairwise
330 p.*427Aspext*33 or Control D5 vs D0 groups (dba.analyze (method = DBA_DESEQ2)).
331 For each comparison, DiffBind generated a set of consensus peaks with the requirement
332 that peaks must be in at least two of the samples (minOverlap = 2). Standardized
333 differential analysis was then performed using the following default settings for
334 dba.analyze: method=DBA_DESEQ2, FDR \leq 0.05, bSubControl parameter set to
335 TRUE, bFullLibrarySize set to TRUE. Thus, raw number of reads in the control sample
336 was subtracted and the library size was computed for each sample and used for the
337 normalization. sizeFactors is called with the number of reads in the BAM files for each
338 CHIP sample, divided by the minimum of these. The final normalized counts returned are
339 the raw reads (adjusted for control reads) divided by the normalization factors (result of
340 calling sizeFactors()). Significantly different peaks were then annotated with HOMER
341 (version 4.7)[6] using RefSeq annotations (distal_distance = -10000, distance5d_lower
342 =-10000, distance5d_upper = -100000, gene_desert_size=100000, proximal_distance=-

343 2000).

344

345

346 **Results**

347 **Identification of French Canadian families with homozygous mutations in *ACTL6B***

348 By exome sequencing of families with neurodevelopmental disorders in Quebec, we
349 identified a family with two children with a homozygous mutation in *ACTL6B* that
350 eliminates the stop codon (c.1279del, NM_016188.4) and extends the reading frame by
351 an additional 33 amino acids (p.*427Aspext*33; NP_057272.1; Individual R3, Figure 1,
352 Table 1, Table S1).

353

354 Sequencing both affected children, their unaffected older brother, and both parents from
355 family R3 revealed that c.1279del was the only mutation identified in the family that was
356 protein altering, followed an autosomal recessive inheritance model and was absent
357 from all genomic databases. Both parents and the unaffected brother were carriers, and
358 all are healthy. The phenotype of the disorder is severe: both brothers died early (4 and
359 6 years) of aspiration asphyxiation, were non-verbal, non-ambulatory, and required 24-
360 hour care for all needs. Parents reported incessant crying (10+ hours per day), seizures
361 beginning at 3 months, and sleep difficulties, (Table 1). An MRI of the brain was provided
362 and had no indications from the reviewing radiologist. Careful tracing of the lineage of
363 family R3 using Catholic Church records⁷ revealed a common ancestor, which we
364 determined to be the most likely origin of the mutation in Family R3 (Figure S1A and
365 Supplemental Methods). We genotyped five other members from family R3 across four
366 generations and could identify appropriate inheritance of the mutation from the predicted
367 founder (Figure S1B). The R3 parents of the proband can be traced to a brother and
368 sister going back six generations in the mother and five generations in the father, an
369 event unknown to the R3 parents prior to birth of the probands (Figure S1B).

370

371 Another French Canadian family (defined as both great grandparents being born in the
372 province of Quebec, Canada) with child R10 (Table 1) also had this same mutation with
373 almost identical phenotype, suggesting that this mutation is not private to the R3 family
374 but rather may be a specific but rare variant in the French Canadian population. We
375 assume that these families are distantly related but could not identify the branch point at
376 which the pedigrees may overlap between families R3 and R10.

377

378 **Biallelic mutations in *ACTL6B* cause a severe neurodevelopmental disorder**

379 We were able to identify eight additional families with a similar phenotype (Table
380 1, Table S1) with biallelic mutations. A majority of these identified mutations resulted in
381 premature termination codons, and were located in highly conserved sequences (Figure
382 1B-C). We considered it very likely that most of these mutation sites resulted in
383 nonsense mediated decay (NMD) of the transcript, as they occur well in advance of the
384 penultimate exon¹⁹, and strongly suggests that the disease is due to loss of function of
385 the *ACTL6B* gene. However, some mutations, including the c.1279del mutation were
386 located in the final exon of *ACTL6B* (Figure 1B), and were therefore not predicted to lead
387 to NMD¹⁹. Heterozygous stop mutations are present in healthy parents, suggesting a
388 recessive disorder. Selected case vignettes can be found in the Supplemental section.

389

390 **De novo missense mutations at specific loci in *ATCL6B* cause a different, severe** 391 **neurodevelopmental disorder**

392

393 Over the course of identifying subjects with mutations in *ACTL6B*, we found ten
394 individuals with heterozygous *de novo* missense mutations in *ACTL6B* with hypotonia,
395 intellectual disability, developmental delay, autism, and Rett-like stereotypies such as
396 handwringing (Figure 1, Table 2, Table S2). Detailed case vignettes of some subjects

397 are presented in the Supplemental section. This was surprising, given that we observed
398 heterozygous stop/Frameshift mutations in healthy individuals arguing for a recessive
399 inheritance model. Nine of ten of these individuals possess the same well conserved
400 c.1027G>A mutation (Figure 1C and Table 2). p.Gly343Arg (NM_016188.4,
401 HG19:chr7:100244258; Exon 12), is not seen in the 60,706 ExAC subjects. The same
402 holds true for the other observed variant, p.Asp77Gly (NM_016188.4; HG19: chr7:
403 100253082; exon 3), both of which are likely gain-of-function mutations since
404 heterozygous stop mutation carriers have no disease. 3D modeling of the *de novo*
405 dominant and the recessive biallelic mutations (Figure 1D) shows no spatial clustering of
406 mutation sites. BAF53B has an actin-related domain, which is subdivided into four
407 subdomains²⁰. Subdomain I and III are structural, and also contain residues that interact
408 with ATP²¹. Subdomain II is the smallest domain, and enables the protein to have polar
409 and non-polar orientations. Previous work has shown that mutations in this subdomain
410 impair dendritic outgrowth⁴. Subdomain IV interacts with subdomain 1²⁰ and is
411 necessary for the interaction of the protein with actin²¹. We mapped these subdomains
412 onto a model of BAF53B derived from the *S.cervisiae*; ARP4 structure, and found that
413 the variants occurred in all subdomains. Specifically, p.Phe147del, p.Cys425,
414 p.Arg130Gln and p.Gln411* variants occur in Subdomain I, the p.Asp77Gly variant
415 occurred in Subdomain II, p.Gly343Arg, p.Gly349Ser and p.Arg130Gln occur in
416 Subdomain III, and p. Gly425*, occur in Subdomain IV (Table 1-2). We did not find any
417 concentration of mutations in a particular domain.

418

419 Two other BAFopathies, Nicolaides-Baraitser syndrome (MIM:600014) and
420 Coffin-Siris syndrome (MIM: 614556), so called because they affect genes that code for
421 proteins that can be incorporated into the BAF complex, have sparse scalp hair and

422 coarse facial features, though this is a wide spectrum in affected individuals. We
423 obtained images of several probands in this study and did not observe coarse features in
424 the majority of subjects (Figure 1A). However, in individuals with the dominant mutations,
425 we did find common features such as a wide mouth, diastema and bulbous tip of the
426 nose. In the case of MRI brain structure this was grossly normal, with subtle but not
427 specific features (common across many MRI scans of children with neurodevelopmental
428 diseases) detected for some individuals (Table S1, S2)).

429

430 **Modeling the p.*427Aspext*33 variant in human neurons**

431 Human stem cells are a powerful model for functional genetic studies as mutations can
432 be assayed on a relevant genetic background and are amenable to genetic engineering.
433 All iPSC lines generated in this study had normal chromosomal integrity, presented
434 typical hallmarks of pluripotency (Figure 2A), including expression of endogenous
435 pluripotency genes (Figure S2A-B), and had the capacity to differentiate into all three
436 germ layers (Figure S2C). iPSCs were utilized to generate forebrain neural progenitor
437 cells (NPCs), which expressed neural and forebrain specific markers (Figure 2B; Figure
438 S3). Mature neurons generated from wild type cells expressed markers of cortical
439 neurons and displayed electrophysiological characteristics typical of high quality iPSC-
440 derived neurons, including spontaneous action potentials and excitatory post-synaptic
441 currents (Figure 2D-F).

442

443 To assess the validity of iPSC-derived neurons to model *ACTL6B* mutation
444 syndrome, we sought to recapitulate the developmental expression increase² in *ACTL6B*
445 in wildtype neurons, where *ACTL6B* expression is absent from dividing cells but is
446 present in post-mitotic cells². We found that *ACTL6B* increased in expression from day 1

447 to day 5. To minimize time in culture which can increase experimental variation, we
448 selected five days differentiation as our timepoint for post-mitotic transcriptomic analysis,
449 where we could be confident *ACTL6B* would be well expressed (Figure 3A and 3B). To
450 characterize the basic expression pattern of key genes involved in the BAF complex in
451 both p.*427Aspext*33 and control cells, we assessed the expression of *ACTL6B*,
452 *ACTL6A* and *SMARCA4* (MIM: 603254) a core DNA binding component of the BAF
453 complex. Genes were assessed at Day 0 (D0) and Day 5 (D5) of differentiation. *ACTL6B*
454 expression increased significantly in both p.*427Aspext*33 and control cells as cells
455 differentiated. We also detected a significant decrease of *ACTL6B* in p.*427Aspext*33
456 compared to control cells at D5 (Figure 3C). *ACTL6A* had high expression in proliferating
457 cells with a significant decrease after 5 days, but yet was still clearly expressed at day 5
458 in both control and p.*427Aspext*33 cells (Figure 3C). We detected no significant
459 difference in the expression level of *SMARCA4* between any cell line or timepoint (Figure
460 3C). To confirm and validate these mRNA based data, we performed western blot on
461 protein extracted from p.*427Aspext*33 and control cells at proliferative and post-mitotic
462 timepoints (Figure 3D). These data suggest that there is no difference in protein level of
463 any of BAF53A, BAF53B, and BRG1 between control and p.*427Aspext*33 cells. By
464 developmental period (proliferating and post-mitotic) we observe consistent protein
465 levels of BAF53A and BRG1, and absent BAF53B in proliferating cells.

466

467

468 **Engineered homozygous deletion of *ACTL6B* in human neurons causes severe**
469 **loss of dendrites**

470 What is the function of *ACTL6B* in developing human cells and what is its role in
471 human disease? Our previous experiments suggest that BAF53B is specific to post-
472 mitotic cells, as reported in rodents²², so we opted to inactivate *ACTL6B* to determine
473 cellular phenotypes resulting from complete gene loss.

474 We knocked out *ACTL6B* from control human cells using a clonal genetic
475 engineering technique²³ (Supplemental Methods). We generated two independent
476 *ACTL6B* knockout cell lines that had different homozygous frameshift mutations in exon
477 1 (KO1 and KO2; referred to collectively as KO), and compared them to the isogenic cell
478 line that had undergone no editing event (Control) (Figure 4A-B). The use of two
479 independently edited cell lines with the same outcome (homozygous loss of *ACTL6B*) is
480 one way to ensure against cell line artefacts, where we do not expect the same artefact
481 to be present in both cell lines. To further ensure this, we Sanger Sequenced the five
482 genomic regions most likely to be edited by the gRNAs used, all of which were unedited,
483 suggesting no off-target effects, as has been reported and systematically assessed
484 previously²⁴. We also performed long-range (1.6Kb) sequencing to ensure that these
485 mutations were in fact homozygous and not due to a large deletion in one allele, in
486 addition to DNA based qPCR to confirm equal gene dosage between edited and
487 unedited lines (Supplemental Methods). Following clonal gene editing and careful
488 genomic integrity assessment, we planned to investigate dendritic length anomalies in a
489 more mature neuronal state, since mouse KO *Actl6b* neurons show deficits in dendrite
490 development⁴. To do this, we differentiated human *ACTL6B* KO cell lines and their
491 matched isogenic controls for 15 days (D15 – a timepoint where we routinely see
492 extensive neuronal arborisation in culture²³) and confirmed the loss of *ACTL6B*
493 expression at the mRNA and protein level (Figure 4C-D). We used MAP2 and TUJ1 as
494 dendritic and neuronal markers, respectively, since these are routinely used in

495 neuroscience research for this purpose^{25; 26}. We observed virtually no MAP2 staining in
496 *ACTL6B* KO cells, while MAP2 was clearly present in most cells in the isogenic controls
497 (Figure 4E). We also observed a larger nuclear size in the deleted cells, as assayed by
498 DAPI, an effect that is obvious on cell examination (Figure 4F).

499 **The p.*427Aspext*33 mutation phenocopies *ACTL6B* KO dendritic deficits**
500 **and is reversible upon biallelic genetic repair**

501 From our genotype-phenotype data from affected individuals and their first
502 degree relatives, we reasoned that recessive mutations cause a loss-of-function of
503 *ACTL6B*, and thus may mimic the cellular phenotype of the engineered *ACTL6B* KO
504 cells. To demonstrate this, we reasoned that using these cell lines and comparing them
505 to a clonally repaired version should provide interpretable data.

506 We biallelically repaired the *ACTL6B*ext33 line to a wildtype genotype and
507 simultaneously reprogrammed these edited cell lines, where we had several
508 unsuccessful repairs that could be used as isogenic controls. Homology directed repair
509 was performed using a wildtype template in p.*427Aspext*33 fibroblasts plated at low
510 density and iPSCs colonies derived from a single fibroblast were isolated, ensuring
511 clonality and purity of repair²³. After iPSC expansion of many colonies, we extracted
512 DNA and Sanger sequenced around the mutation site (Method S2-S4). A colony with
513 c.1279del mutation repaired to a wildtype genotype was identified and labelled as
514 Successful Repair (SR) and differentiated to NPCs in tandem with an Unsuccessful
515 Repair (UR) line, which was derived from a colony that received the CRISPR complex
516 and repair template but where no editing event occurred (Figure 5 A-B). We
517 differentiated these cells from NPCs to D15 neurons and then stained for MAP2 and
518 TUJ1, identical in design to the KO study. As shown in Figure 5D-E, affected individual

519 cells recapitulate the loss of MAP2 and increased nuclei size observed in the KO, a
520 result that is reversed on repair of the homozygous base change in *ACTL6B*. The
521 similar cellular phenotype between affected individual and engineered KO neurons
522 suggest that the *ACTL6B*^{ext33} recessive mutation causes similar deficits to the
523 complete KO, and thus could be interpreted as causing a loss-of-function.

524

525 **Loss of dendrites due to loss of function of *ACTL6B* is likely due to delayed**
526 **maturation of young neurons**

527 Is the observed decrease in MAP2 staining due to immature differentiation,
528 differential differentiation, or a specific deficit in dendrite development? To try to address
529 these questions, we first asked whether the cell types in each condition were equivalent.
530 To investigate this question we stained D15 cultures from repaired and KO cells and
531 their control with an astrocyte (GFAP) and a neuronal marker (TUJ1), with the
532 hypothesis that perhaps deficits in *ACTL6B* bias NPCs towards becoming astrocytes.
533 Figure S4A shows the results of this experiment; we could not detect different numbers
534 of cells that stained for GFAP. We include in this experiment a positive control where we
535 add 0.1% BSA which can glialize cell cultures. To support the idea that deficits of
536 *ACTL6B* do not lead cell cultures to become more glial and to provide more specificity
537 than just GFAP, we assessed the transcriptomic data of p.^{427Aspext}³³ and control
538 cells we had generated in an RNA-Seq experiment. We found no consistent pattern in
539 mRNA expression levels of glial markers *ALDH1L1*, *GFAP*, and *GJA1* and neuronal
540 markers *RBFOX3* and *TUBB3* to suggest an increase in expression of glial related
541 genes in *ACTL6B*^{ext33} cells compared to control cells (Figure S4B). These data
542 indicate that deficits in *ACTL6B* do not lead NPCs to become more astrocytic. We

543 therefore ruled out the loss of MAP2 staining being due to cells being pushed towards an
544 astrocytic fate.

545 As a simple measurement of differentiation, we opted to photograph control,
546 ACTL6Bext.33, ACTL6B KO and Repair cells across several developmental timepoints.
547 Figure S5 shows that in contrast to repaired and control cell lines, unrepaired and KO
548 *ACTL6B* cells are not branched prior to day 20, whereas at day 25 through day 50, all
549 lines show branching.

550 These data suggest that deficits in *ACTL6B* lead to a delay in differentiation in
551 early post-mitotic states. This delay in differentiation, if true *in vivo*, may lead to cell
552 connectivity deficits.

553

554 **The p.*427Aspext*33 mutation alters BRG1 genomic binding and affects gene** 555 **expression**

556 This project began with the index case R3 (p.*427Aspext*33) which had fibroblasts
557 collected prior to mutation identification, thus our study is heavily biased towards this
558 case. To this end, we opted to continue experiments with these cells, with the idea that
559 we might recruit cells from other subjects or design exogenous templates for validation
560 studies.

561

562 We wanted to understand the molecular consequences of the p.*427Aspext*33
563 variant and how this might lead to neurodevelopmental deficits. Due to a lack of ChIP
564 grade antibodies directed specifically to BAF53B, we chose to perform a ChIP-Seq
565 experiment targeting BRG1, a key subunit of the BAF complex with ATPase activity that

566 is found both BAF53B and BAF53A containing BAF complexes ¹¹. We chose the D0 and
567 D5 time points for proliferating and post-mitotic cells respectively, and performed ChIP in
568 control and *ACTL6B*ext33 cells using eight replicates per subject per time period (32
569 ChIP experiments). We performed several QC experiments with different anti-BRG1
570 antibodies prior to sequencing to ensure appropriate parameters (not shown), and chose
571 two antibodies to provide overlapping datasets of Brg1-containing BAF complex binding
572 (Figure 6A). After sequencing and QC, we analyzed differential binding in D5 cells to
573 understand how the genomic targeting of Brg1-containing BAF complexes may be
574 altered by a mutant BAF53B subunit. ChIP peaks were called in at least 2/8 lines, and
575 differential analysis used reads from all replicates within the peak. 10,222 peaks were
576 common across all data points (Figure 6B), suggesting that BRG1 remains mostly at the
577 same location in the genome, even in mutant *ACTL6B* cells and irrespective of
578 developmental state.

579 We focused our primary analysis on D5, since this is when *ACTL6B* is
580 expressed, and tried to determine whether there was differential binding of BRG1 at
581 peaks called in both p.*427Aspext*33 and control cells. Using FDR <0.05, we found no
582 significant differences; however, using an uncorrected p-value of 0.05 revealed 382
583 common genomic regions that were significantly differentially bound and every one of
584 these showed increased binding in affected individual cells (Figure 6C). Loss-of-function
585 of BAF53B may lead to increased affinity or stabilization of the BAF complex to its
586 genomic targets, possibly through retention of BAF53A. More than half of the 382 sites
587 that BRG1-containing BAF complexes were found to bind to were associated with genes
588 (Figure 6C). PANTHER GO terms associated with the differentially bound regions were
589 related to cell adhesion and neurodevelopment (Figure 6D). This list included autism
590 associated genes including *AUTS2*, *PTEN*, *FOXP2*, and *SMARCA2*.

591 To further assess whether mutant *ACTL6B* leads to increased binding of BAF to
592 genomic regions, we performed a within-subjects analysis in proliferating (D0) and
593 differentiating (D5) cells, looking for peaks present at both developmental stages using
594 FDR<0.05 for peak calling. While we did not find the same peaks that were called
595 between cases and controls (suggesting the experiment was underpowered since we
596 used 8 replicates in each block), we found evidence for a general decrease in BRG1
597 binding in differentiated cells compared to proliferating cells in those genomic regions
598 present at both D0 and D5 in control conditions, in contrast to the p.*427Aspext*33 cells
599 where there was a consistent increase in BAF binding at D5 compared to D0 (Figure 6E)
600 at all sites. These data support the notion that a recessive *ACTL6B* mutation leads to
601 increased association of BRG1 to certain areas of the genome.

602

603 How does genomic BAF binding affect gene expression and how might this differ when
604 *ACTL6B* is mutated? We performed RNAseq in affected individual and control cells (n=4
605 independent replicates per subject) at D0 and D5 and looked only at those genes that
606 were detected in the ChIPSeq analysis. We were interested in those genes that showed
607 significant changes between D0 and D5 in the ChIPSeq data and which also showed
608 significant change in the RNAseq data between D0 and D5 (within-subjects); Also, we
609 selected those genes that showed RNAseq differences between mutant cells and control
610 cells at D5 (Table S2). We highlight *TPPP* (Table S4), a microtubule binding protein
611 involved in cell process extensions^{27; 28}, and *FSCN1*, which has been shown be involved
612 in neurite shape and trajectory in prior studies in mice²⁹. Due to their biological function
613 and significance levels in our experiments, we chose to use *TPPP* and *FCSN1*
614 expression levels to assess the external validity of our findings. We note the prevalence
615 (Table S4) of genes that might be implicated in sphingolipid biology or myelin processing

616 (*SOX8*³⁰, *CERK*³¹, and *A4GALT*³²), consistent with Wu et al, (2007) who observed a
617 severe myelination defect in *Actl6b* KO mice.

618 We used *TPPP* and *FSCN1* expression as output markers to assess direct
619 versus correlational effects of mutant *ACTL6B*. We posed two initial questions to test
620 direct versus correlational effects. First; does the *ACTL6B* KO show a similar pattern of
621 expression to *ACTL6B*ext33 compared to its isogenic control? Second; do we see the
622 opposite effect in the UR cells compared to the SR cells? We began by validating the
623 RNA-Seq data, using the same RNA that was used to make RNA-Seq libraries (Figure
624 7A). Next, we examined the expression of these genes between D0 and D5 timepoints in
625 *ACTL6B* KO cells and their isogenic controls, as well as in UR and SR cells. SR cells,
626 when compared to UR cells showed a significant increase in *TPPP* and decreased
627 expression of *FSCN1* as NPCs mature from D0 to D5. In *ACTL6B* knock-out cells
628 compared to their isogenic controls, we observed significant and opposite effects to that
629 observed with repaired cells: *FSCN1* expression did not decrease, while *TPPP*
630 expression did not increase as the cells differentiated (Figure 7A). These data provide
631 isogenic evidence that complete loss of *ACTL6B* and a repair of p.*427Aspext*33
632 recapitulate and reverse, respectfully, expression alterations in *TPPP* and *FSCN1* and
633 suggest that expression changes in these genes are directly caused by disruption of
634 *ACTL6B*.

635

636 **External validity of *TPPP* and *FSCN1* expression levels as markers of an *ACTL6B***
637 **recessive, loss-of-function disease in human neurons using constructs derived**
638 **from different *ACTL6B* variants.**

639 External validity can be provided by KO rescue and by recapitulating expression
640 effects using different mutations in *ACTL6B* identified in our cohorts (Figure 7B-D). If
641 *TPPP* and *FSCN1* expression levels are markers of loss-of-function of *ACTL6B*, the
642 exogenous re-introduction of *ACTL6B* on a KO background should help restore their
643 expression towards levels observed in lines with wild-type *ACTL6B*. Further, expressing
644 mutant *ACTL6B* to match other variants found in the recessive cohort should re-establish
645 expression changes on an *ACTL6B* KO background. We therefore made *ACTL6B*
646 constructs of two recessive mutations c.441_443 del and c.1275C>A, and the most
647 prevalent dominant mutation c.1027 G>A, as well as the WT construct itself. Expressing
648 these variants from transiently delivered vector on an *ACTL6B* KO background, may
649 give us an indication if the dominant and recessive variants mediate their effects through
650 the same molecular pathways and cause similar effects on the expression of *FSCN1* and
651 *TPPP*. At a D5 timepoint, cells transfected with WT *ACTL6B* showed decreased
652 expression of *FSCN1* and increased expression of *TPPP*, consistent with what we
653 observed in the initial KO experiment (Figure 7D), meaning that the WT construct can
654 rescue the expression changes observed in *ACTL6B* KO cells. We observed that the
655 two recessive variants (c.441_443 del and c.1275C>A) mimicked the effects observed in
656 the recessive p.*427Aspext*33 variant, while the dominant mutation mimicked wildtype
657 cells (Figure 7D).

658

659 **Confirmation of dendritic deficits and gene expression markers using neurons**
660 **derived from *ACTL6B* mutant c.617T>C/ c.724C>T**

661 We obtained fibroblasts from individual R9 with compound heterozygous
662 mutations in *ACTL6B* (c.617T>C, p.Leu206Pro and c.724C>T, p.Gln242*) (Table 1). We

663 induced the fibroblasts to become iPSCs, differentiated the iPSCs to D15 neurons and
664 confirmed the mutant genotype of this line (Figure 8A-B). We compared this “*ACTL6B*
665 compound heterozygous mutant” line to healthy control cells differentiated to a day 15
666 timepoint, and found a similar decrease of *MAP2* staining and increased nuclei size as
667 compared to the *ACTL6B* KO and *ACTL6Bext33* lines (Figure 8C-D). Assessing the
668 expression of *TPPP* and *FSCN1* at D5 and D0 timepoints in the *ACTL6B* compound
669 mutant and control lines also produced results similar to those seen with the
670 *ACTL6Bext33* line, with the *ACTL6B* compound mutant showing a lack of increased
671 expression of *TPPP*, as well as a lack of decreased expression of *FSCN1* during
672 differentiation compared to control cells (Figure 8E).

673

674 **Discussion**

675 These data describe two distinct neurodevelopmental diseases caused by
676 dominant or recessive mutations in *ACTL6B*. This work positions *ACTL6B* mutations as
677 causing both a recessive neurological disease characterized by severe epileptic
678 encephalopathy, and a dominant intellectual disability syndrome with severe speech and
679 ambulation deficits.

680 Previous studies have identified mutations in genes that code for other subunits
681 of the nBAF and npBAF complexes that are capable of causing disease through
682 dysregulated BAF function, collectively called “Bafopathies”³³. The two foremost
683 diseases among the Bafopathies, Coffin-Siris (CSS) (MIM: 135900) and Nicolaides–
684 Baraitser (NCBRS) (MIM: 601358) syndrome show interesting parallels and differences
685 to the diseases described here^{34; 35}. While NCBRS is a monogenic disease, caused
686 exclusively by mutations in *SMARCA2* (MIM: 600014) that are autosomal dominant, and
687 CSS is a genetically diverse disease, and can be caused by mutations in *ARID1B* (MIM:
688 614556) and a variety of other genes that play a role in the BAF complex, that vary in
689 their inheritance pattern, common symptoms appear to exist in both these conditions and
690 the diseases described here. Common symptoms reported across conditions include
691 intellectual disability, developmental delay, hypotonia and some form of dysmorphic
692 facial features ³⁴. Like individuals with recessive mutations in *ACTL6B*, individuals with
693 NCBRS also show early-onset seizures³⁶, and seizures are also reported in individuals
694 with CSS, although they are not necessarily early-onset ^{34; 37; 38}. Individuals with NCBRS
695 also show short phalanges³⁶, as observed in some individuals with dominant mutations
696 in *ACTL6B*. However, some of the specific developmental symptoms observed in these
697 diseases, such as sparse scalp hair³⁹ or an absent fifth digit³⁶ do not appear in
698 individuals with either recessive or dominant mutations in *ACTL6B*. This could suggest

699 that while a general disruption of the BAF complex in a variety of protein subunits
700 neurodevelopment will inevitably lead to intellectual disability and developmental delay,
701 the specific protein subunit that is affected will determine the presence and nature of
702 dysmorphisms and epilepsy.

703 To understand why mutations in *ACTL6B* cause disease, we modeled the
704 disease in human NPCs and neurons. We first confirmed that *ACTL6B* expression was
705 induced upon neural cells becoming post-mitotic. We then went on to make several
706 different cell models in the hope of reducing variation across experimental variables. We
707 made forebrain progenitor cells from a healthy individual with experimentally induced
708 knock-out of *ACTL6B* to understand the effects of complete loss of the gene, in addition
709 to cells derived from individuals R9 and R3 with an isogenic engineered repaired cell
710 line.

711 While we cannot precisely determine the mechanism of the disease that appears
712 to be caused by bi-allelic mutations in *ACTL6B*, our results do illuminate several key
713 features of the etiology of the disease. First, the presence of BAF53B in the
714 *ACTL6Bext33* cell line at D5 eliminates the possibility that the symptoms are caused by
715 an absence of BAF53B, as is likely the case in other variants of BAF53B where NMD is
716 predicted to occur¹⁹. Instead, it seems plausible that the symptoms are the result of a
717 loss of function of BAF53B stemming from changes in the structure of the protein. This
718 hypothesis is supported by our observations in the *ACTL6B* KO model, which shows
719 similar deficits in both MAP2 staining and the expression of key genes identified in the
720 *ACTL6Bext33* that are regulated by BAF. *ACTL6B* KO cells expressing the recessive
721 mutations in *ACTL6B* observed in our recessive cohort fails to rescue aberrant
722 expression of genes, whereas reintroduction of wildtype *ACTL6B* does, strongly
723 suggesting that the phenotype is the result of a loss of function.

724 However, how the recessive mutations render BAF53B non-functional could be
725 due to one of several possibilities. Perhaps the most intuitive answer is that mutations
726 disrupt the ability of BAF53B to bind to the BAF complex. If this fails to occur, this might
727 allow BAF53A to remain bound in the complex. Given that there are a great many BAF
728 complexes that dynamically exchange parts to affect cell differentiation at any one time,
729 a high proportion of BAF53A in BAF might cause increased affinity of BAF to the
730 genome in a differentiating cell state compared to both a proliferating cell state when
731 BAF53B is absent or a differentiated cell state where BAF53B is present but not
732 functional and/or when the interaction with the complex is impaired. The increased
733 presence of BAF53A in the BAF complex, associated with a more proliferative neuronal
734 cell state, might also explain the delayed differentiation we observed in disease cells.
735 Another explanation could be that recessive mutations do not prevent BAF53B from
736 binding to the BAF complex, but instead prevent the various components of the complex
737 from interacting properly, and thus prevent the BAF complex from interacting with the
738 genome and other proteins in yet unknown ways. Finally, there is the possibility that
739 recessive mutations do not disrupt BAF complex function significantly at all, but instead
740 prevent BAF53B from properly interacting with the other complexes such as SRCAP⁴⁰,
741 TIP60/NuA4¹⁹ and INO80⁴¹. Future work will need to look at how mutant BAF interacts
742 with different proteins.

743

744 With respect to the dominant mutation identified in eight unrelated individuals, we failed
745 to observe the transcription effects we found in the KO, *ACTL6B* compound
746 heterozygous mutant or the *ACTL6B*^{ext33} cells, suggesting dominant and recessive
747 mutations in *ACTL6B* cause disease through distinct molecular pathways. This is also
748 consistent with the observation of different symptoms in individuals with recessive/

749 dominant mutations in *ACTL6B*. Based on the limited molecular information we have for
750 dominant mutations, there exists many potential explanations for how a point mutation in
751 *ACTL6B* might cause the observed symptoms. Given that these subjects all have a
752 functioning copy of BAF53B which presumably incorporates normally into BAF or other
753 complexes, it is reasonable to suggest that the *ACTL6B* dominant mutations identified
754 here may be gain-of-function. One of the few clues that we have at this stage of
755 investigation is that the highly specific nature of the p.Gly343Arg variant suggests a very
756 precise interaction is being disrupted or created. Previous work in mice⁴² has shown that
757 deletion of the hydrophobic domain of BAF53b results in a dominant negative form of
758 the protein, causing deficits in memory, LTP, and gene expression. These deficits were
759 likely caused by altering the ability BAF53B to interact with other proteins⁴². It is possible
760 that the dominant mutations observed in this study cause disease by altering the
761 hydrophobic domain of BAF53B through a similar mechanism.

762

763 Several genes of note may be important targets of the BAF complex at different
764 developmental stages. *FSCN1* is one of these and is strongly associated with the
765 formation of actin, particularly in early neurodevelopment^{29; 43}. The lack of increase of
766 *FSCN1* observed in our models of *ACTL6B* dysfunction as cells differentiate may
767 therefore be the result of neuronal cells remaining in a more proliferative or immature
768 state due to an impairment in the ability of *ACTL6B* to transition the BAF complex from
769 promoting genes associated with proliferation to those associated with neuronal
770 outgrowth⁴⁴.

771 The stable expression levels of *TPPP* observed in models of *ACTL6B*
772 dysfunction as NPCs differentiate may reflect the cytoskeletal changes observed both
773 within our own models and in the deficits in dendritic spine and synapse function

774 observed in mouse models of *Actl6b* KO. It should also be noted that our CHIP-SEQ
775 data that initially highlighted these genes as being dysregulated is based upon only
776 BRG1-containing BAF complexes. As BRG1 and BRM are mutually exclusive
777 components of the BAF complex that are both found in dividing and post-mitotic neural
778 cells¹¹, it is possible that the dataset we generated is only a partial picture of the regions
779 of the genome where BAF may bind in these cells.

780 This work brings together extensive clinical samples and human stem cell
781 modeling to demonstrate that mutations in *ACTL6B* in human cause severe neurological
782 disorders. Substantially more work will need to be done to understand the precise
783 mechanisms of how recessive or dominant mutations in *ACTL6B* affect incorporation into
784 the BAF complex, and how this incorporation can alter differential genomic binding and
785 gene expression patterns.

786

787 **Supplemental Data**

788 Supplemental Data can be found with this article online and includes five supplementary
789 figures, four supplementary tables and supplementary methods which provide additional
790 clinical details of individuals and further details of the generation, characterization, and
791 quality control of the cell lines generated in this study.

792 **Declaration of Interests**

793 Amber Begtrup, Ingrid Wentzensen, and Amy Crunk are employees of GeneDx. Carl
794 Ernst is president of ManuStem.com and has commercial interests with Stem Cell
795 Technologies. The other authors declare no conflict of interest.

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802 a custom antibody to BAF53B. CE is grateful to Family R3 who collaborated with his lab
803 from mutation detection to human neuron modeling.

804

805

806 **Figure Legends**

807 **Figure 1. Location of mutations in *ACTL6B* found in individuals with potential**
808 **recessive or dominant disease causing mutations**

809 A) Photos of individuals with *ACTL6B* mutations. Note broad mouth of individuals D1,
810 D2, D3 and D7, diastema in D1, D3, D7, bulbous tip of the nose in all D individuals, and
811 hypertelorism with telecanthi in individual D8. Lower right: MRI images of individuals with
812 recessive *ACTL6B* mutations. For individual R4, note white matter T2 hyperintensity
813 (arrows). For individual R8, note enlarged lateral ventricles and asymmetric gyral pattern
814 (left, arrows). On the right, note thin corpus callosum (arrows). B) Linear graph of
815 mutations in *ACTL6B* (introns not drawn to scale). C) Conservation of the residues
816 affected by amino acid substitutions. D) 3D model generated with SWISSMODEL based
817 on *S. cerevisiae* Arp4 (yeast homolog of *ACTL6B*), visualized with Swiss-PdbViewer
818 showing that recessive mutations are not focused in one region. Note however that the
819 dominant mutations seem to lie at the periphery of the protein thus they might affect
820 protein-protein interactions.

821

822 **Figure 2. Generation of iPSC-derived neurons for BAF53 studies**

823 A) Representative images of quality control staining done on iPSCs. B) Representative
824 quality control staining on NPC cultures. C) Representative staining of control cells for
825 TUJ1 and MAP2 at D15 of differentiation. D) Representative trace of miniature EPSCs
826 from D25 neurons held at -40 mv. E) Representative recordings showing spontaneous
827 activity of D25 neurons in current-clamp mode. F) Trace of a hyperpolarizing pulse
828 showing a depolarizing sag followed by multiple rebound action potentials. The first
829 action potential is shown at a higher temporal resolution. All scale bars represent 40 μ m

830

831 **Figure 3. Comparison of control and *ACTL6Bext*33* before and after expression of**
832 ***ACTL6B***

833 A) Diagram illustrating the production of control and *ACTL6Bext*33* iPSC-derived NPCs
834 from fibroblasts B) *ACTL6B* expression normalized to *GAPDH* expression plotted against
835 number of days of differentiation of NPCs. N=4, error bars represent standard error
836 around the mean. C) Expression of key genes in the SWI/SWF complex in 706
837 *ACTL6Bext*33* and control NPCs in proliferating and post-mitotic states. Genes are
838 normalized to *GAPDH* expression. (n \geq 3) Student's t-test, *P<0.05, **P<0.01. H) Western
839 blots assessing the level of proteins encoded by the genes displayed in G.

840

841 **Figure 4. Generation and characterization of *ACTL6B* KO neurons reveals a loss of**
842 **dendrites.**

843 A) Diagram of the experimental approach taken to generate *ACTL6B* KO NPCs. B)
844 Sanger sequencing traces of two *ACTL6B* KO lines. C) *ACTL6B* expression in control
845 and *ACTL6B* KO NPCs at a D0 and D5 timepoint (n \geq 3) D) Western Blots assessing the

846 protein levels of *BAF53A/B* in *ACTL6B* KO lines. E) Representative TUJ1 and MAP2
847 staining of control and *ACTL6B* KO D15 immature forebrain neurons. F) Quantification of
848 the surface area of the nucleus in the cell lines shown in E, (n>50). Student's t-test,
849 *P<0.05, **P<0.01.

850

851 **Figure 5. Repair of the *ACTL6B* c.1279del mutation restores morphological and**
852 **dendritic deficits.**

853 A) Schematic detailing *ACTL6B* CRISPR repair. B) Sanger sequencing traces of a
854 Successful Repair (SR) and Unrepaired (UR) cell line generated from *ACTL6Bext*33* cell
855 line. C) *ACTL6B* expression in SR and UR NPCs at a D5 timepoint (n=6) D)
856 Representative TUJ1 and MAP2 staining taken from SR and UR forebrain immature
857 neurons at D15. Scale bars represent 40µm. E) Quantification of the surface area of the
858 nucleus and soma, and the length of projections in the cell lines shown in D, (n>50).
859 Student's t-test *P<0.05, **P<0.01, ***P<0.001

860 **Figure 6. *ACTL6Bext*33* variant leads to increased binding of BRG1-BAF complex**
861 **to the genome.**

862 A) Diagram illustrating the ChiP-Seq experiment. B) Venn diagram showing overlap of
863 genes that the BRG1 complex is bound to. C) Decreased binding at all 382 FDR
864 significant sites in control cells compared to *ACTL6Bext*33* cells (pink dots are
865 significant, while blue dots are not). D) Proportion of BRG1 binding sites found in relation
866 to their proximity to a gene. E) Gene ontology analysis of differentially bound regions. F)
867 Within-subjects differential binding across developmental stages (D0 and D5) showing
868 decreased binding in D0 compared to D5 in *ACTL6Bext*33* cells (pink dots are
869 significant, while blue dots are not). Genes showing a significant difference (FDR-
870 adjusted p-values (Benjamini-Hochberg) ≤0.05) in D5 relative to D0 using a GLM as
871 implemented in DESeq2.

872

873 **Figure 7. External validity in multiple *ACTL6B* mutant models in human neurons**

874 A) *TPPP* and *FSCN1* expression in initial RNA-Seq (n≥4) and qPCR (n≥3) data
875 (*ACTL6Bext*33* vs control); unrepaired (UR) *ACTL6Bext*33* vs *ACTL6Bext*33*
876 Successful Repair (SR) (n=6); and *ACTL6B* KO vs isogenic control cells (n=5). Results
877 are represented as mean ± SEM. Student's t-test *P<0.05, **P<0.01, ***P<0.001 B)
878 Experimental plan for generation of multiple human neuronal cell lines expressing
879 various mutant *ACTL6B* constructs. C) Brightfield and GFP images demonstrating high
880 transfection of *ACTL6B* constructs. D) mRNA expression in transfected *ACTL6B* KO
881 NPCs at D5 timepoints of *ACTL6B*, *TPPP*, and *FSCN1* (E) (n=3). Results are
882 represented as mean ± SEM. Student's t-test *P<0.05, **P<0.01, ***P<0.001
883

884 **Figure 8. Neurons derived from an individual with a compound mutation in**
885 ***ACTL6B* show a similar phenotype to *ACTL6Bext33* and *ACTL6B* KO neurons.**

886 A) Schematic showing generation of ACTL6Bcompoundmutant NPCs B) Sanger
887 sequencing traces of ACTL6Bcompoundmutant and control cell line at both identified
888 point mutations in the *ACTL6B* gene C) Representative TUJ1 and MAP2 staining of
889 control and ACTL6Bcompoundmutation immature forebrain neurons. D) Quantification of
890 the surface area of the nucleus in the cell lines shown in E. E) *TPPP* and *FSCN1*
891 expression in ACTL6Bcompoundmutant vs control cells at mitotic (D0) and post-mitotic
892 (D5) timepoints (n>50). Student's t-test, *P<0.05, **P<0.01.

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898 Table 1. Pathogenic variants and key clinical information of individuals with bi-allelic
899 mutations in *ACTL6B*.

Individual	R1	R2	R3	R4	R5	R6	R7	R8a	R8b	R9	R10					
Inheritance	Recessive, homozygous	Recessive, Compound heterozygous. Similarly affected brother passed away at 5y	Recessive, homozygous. Similarly affected brother passed away at 4y	Recessive, Compound heterozygous	Recessive, Compound heterozygous	Recessive, Compound heterozygous	Recessive, homozygous	Recessive, homozygous	Recessive, homozygous. Sister of R8a	Recessive, Compound heterozygous	Recessive, homozygous					
Coding Change (NM_016188.4)	c.441_443del CTT	c.695del C	c.1275C>A	c.1279del	c.389G>A	c.556C>T	c.852C>G	c.740G>A	c.1231C>T	c.669+1G>A	c.289C>T	c.1045G>A	c.1045G>A	c.724C>T	c.617T>C	c.1279del
Protein Change (NP_057272.1)	p.Phe147del	p.Pro232Glnfs*24	p.Cys425*	p.*427A spext*33	p.Arg130Gln	p.Gln186*	p.Tyr284*	p.Trp247*	p.Gln411*	splitting	p.Arg97*	p.Gly349Ser	p.Gly349Ser	p.Gln42*	p.Leu206Pro	p.*427A spext*33
gnomAD MAF	0.000144, no homozygotes	Absent	Absent	Absent	0.00008132, no homozyg	Absent	Absent	Absent	Absent	Absent	0.00004064, no homozyg	0.0000001219, no homo	0.0000001219, no homo	Absent	Absent	Absent

				otes				otes	zygotes	zygotes		
Age at assessment	3y F	5y M (passed away at age 5)	11m M (passed away age 5)	8y F	5m F	12m M (passed away age 2)	4y F	6y F	5y F	14m F	4.5y F	
Head circum. In cm	43 (-3.5 SD)	NA	44 (3 rd)	50.3 (10 th %ile)	38,4 (-3.0 SD)	42 (-2.4 SD)	NI	18m 41.5 (-3.8 SD)	4m 39 (7 th %ile)	43 (-2,5 SD)	47 (-2.8 SD)	
ID, DD	+, Severe	+	+	+, Severe	+, Severe	+, Severe	+, Severe	+, Severe	+, Severe	+, Severe	+	
Speech	-	-	NA	-	NA	-	NA	-	-	-	-	
Ambulation	-	-	NA	-	NA	-	NA	-	-	-	+, with support	
Axial hypotonia	+	+	+	+	+	+	+	+	+	+	+	
Limb spasticity	+	+	+	+	+	+	+	+	+	+	+	
Feeding difficulties	+	+	+	-	+	+	+	+	+	+	-	
Epilepsy	+	+	+	+	+	+	+	+	+	+	+	
Seizures (age at beginning)	3 months	3y	NA	NA	2 months	Neonatal (25 days)	Infancy	Infancy	Infancy	Antenatal	9 months, infantile	

											spasms
Seizure types	Myoclonias 2-6 per day	Complex partial	NA	NA	Tonic and myoclonic	Focal onset epilepsy, progressed to infantile spasms	NA	NA	NA	myoclonic seizures AND tonic seizures	Tonic and myoclonic
EEG anomalies	Multifocal epileptic activity	NA	Multifocal epileptic activity	NA	Multifocal epileptic activity	Multifocal interictal epileptiform spike discharges, lack of posterior dominant rhythm	Multifocal EEG abnormalities	generalized slowing of background rhythms	generalized slowing of background rhythms	Multifocal epileptic activity, esp. left hemisphere	Multifocal epileptic activity, esp. left hemisphere
MRI	Prominent subarachnoid spaces and small corpus callosum	Normal	Mild T2 hyperintensity in frontal periventricular white matter	Mild T2 hyperintensity in frontal periventricular white matter	2 months of age showed symmetric signal changes in the brainstem and in the dorsal medulla oblongata, possibly also around	3 w.o.: asymmetric ventricles, cortical dysplasia right parietal lobe. 9 m: cerebral atrophy, hypoplasia of corpus callosum	NA	MR I at 5mo: Significantly decreased white matter throughout, extr	10 mo: Periventricular leukomalacia with white matter volume loss,	Thin corpus callosum High signal intensity dorsal globus pallidus/putamen Some asymmetric gyral pattern	At 3.5yo: Cerebral and cerebellar atrophy, thin corpus callosum

					the dentate nucleus			emely thin corpus callosum. Normal MR spectroscopy	overall brain volume loss, delayed myelination and thinning of corpus callosum. Normal MR spectroscopy		
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909 Table 2. Pathogenic variants and key clinical information of individuals with *de novo*910 mutations in *ACTL6B*.

Individual	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Coding Change (NM_016188.4)	c.1027G>A	c.1027G>A	c.1027G>A	c.1027G>A	c.1027G>A	c.1027G>A	c.1027G>A	c.1027G>A	c.230A>G	c.1027G>C
Protein Change (NP_057272.1)	p.Gly343Arg	p.Gly343Arg	p.Gly343Arg	p.Gly343Arg	p.Gly343Arg	p.Gly343Arg	p.Gly343Arg	p.Gly343Arg	p.Asp77Gly	p.Gly343Arg
gnomAD MAF	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Age at assessment	5y6m M	29y F	6y6m M	5y9m F	4y6m F	3y F	21y F	2y 6m F	8y F	12yF
Head circum. In cm	49 (-2.1 SD)	53 cm (11 th %ile)	51 cm at age 5 yr (50 th centile)	48.6 (-2.2 SD)	48 cm (2 nd %ile)	48.0 cm (20 th %ile)	52.2 cm (-2.0 SD)	45.5 cm (-0.1 SD)	50 th -75 th %ile	52 (-1,-2SD)
Degree of	Seve	Seve	Seve	Seve	Seve	Seve	Sever	Sever	Seve	Seve

ID/DD	re	re	re	re	re	re	e	e	re	re
Speech	-	-	-	10-20 words, Receptive skills better	-	-	-	-	-	One word
Ambulation	-	+	limited	Delayed. Wide based gait	-	-	-	-	NA	Delayed. Wide based gait
Hypotonia	NA	+	+	NA	+	+	NA	-	NA	+
Autism spectrum disorder	NA	+	Unknown	+	NA	NA	NA	-	+	-
Features of ASD	NA	NA	+	NA	stereotypes	NA	handwriting	-	NA	stereotypes
Seizure disorder	-	-	-	-	-	-	Infantile spasms and GTC S	-	NA	-
MRI	NA	NA	NA	NA	NA	thinning of the corpus callosum	Generalised atrophy at 2y	mild periventricular gliosis	NA	NI

Wide or prominent forehead	+	+	+	+	-	-	+	+	+	-
Hypertelorism	+	+	+	-	-	+	-	+	+	-
Wide mouth	+	+	+	-	-	-	+	+	NA	+
Short phalanges or nails	NA	+	-	+	-	-	+	-	+	-

911

912 **Web Resources**

913 BWA: <http://bio-bwa.sourceforge.net/>

914 GATK: <https://software.broadinstitute.org/gatk/>

915 MACS2: <https://github.com/taoliu/MACS/wiki/Install-macs2>

916 Trim Galore: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

917 Bioconductor: <http://bioconductor.org/packages/release/bioc/html/DiffBind.html>

918 HOMER: <http://homer.ucsd.edu/homer/>

919 Neurolucida: <https://www.mbfioscience.com/neurolucida>

920 Imagemlab: <http://www.bio-rad.com/en-ca/product/image-lab-software6>

921 Online Mendelian Inheritance in Man: <http://www.omim.org>

922 VarSome: <https://varsome.com/>

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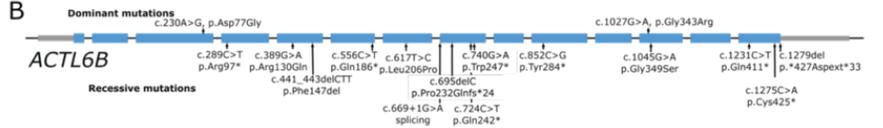
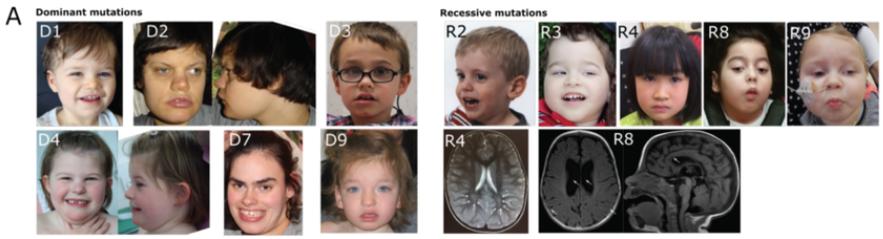
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1061



C

Dominant mutations

	Asp77	Gln130
Human	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V
Rhesus	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V
Mouse	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V
Dog	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V
Elephant	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V
Xenopus	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V
Zebrafish	H V P F D G A E V P G L V G S V I V	H V P F D G A E V P G L V G S V I V

Recessive mutations

	Phe147	Arg130	Leu206	Gly349
Human	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T
Rhesus	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T
Mouse	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T
Dog	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T
Elephant	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T
Xenopus	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T
Zebrafish	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T	I P A F T L C K T T R A A E K L T M E Q F L E R C Q T L K I R K A R T



Figure 1

