

1 **Peripheral neuropathy linked mRNA export factor GANP**

2 **reshapes gene regulation in human motor neurons**

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53 **SUMMARY**

54 Loss-of-function of the mRNA export protein GANP (*MCM3AP* gene) cause early-onset
55 sensorimotor neuropathy, characterised by axonal degeneration in long peripheral nerves.
56 GANP functions as a scaffold at nuclear pore complexes, contributing to selective nuclear
57 export of mRNAs. Here, we aimed to identify motor neuron specific transcripts that are
58 regulated by GANP and may be limiting for local protein synthesis in motor neuron axons. We
59 compared motor neurons with a gene edited mutation in the Sac3 mRNA binding domain of
60 GANP to isogenic controls. We also examined patient-derived motor neurons. RNA
61 sequencing of motor neurons as well as nuclear and axonal subcompartments showed that
62 mutant GANP had a profound effect on motor neuron transcriptomes, with alterations in nearly
63 40 percent of all expressed genes and broad changes in splicing. Expression changes in multiple
64 genes critical for neuronal functions, combined with compensatory upregulation of protein
65 synthesis and early-stage metabolic stress genes, indicated that RNA metabolism was abnormal
66 in GANP-deficient motor neurons. Surprisingly, limited evidence was found for large-scale
67 nuclear retention of mRNA. This first study of neuropathy-linked GANP defects in human
68 motor neurons shows that GANP has a wide gene regulatory role in a disease-relevant cell type
69 that requires long-distance mRNA transport.

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73 **Keywords:** peripheral neuropathy, Charcot-Marie Tooth disease, intellectual disability,
74 GANP, *MCM3AP*, mRNA export, isogenic, pluripotent stem cells, motor neurons, CRISPR-
75 Cas9, RNA metabolism

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82 INTRODUCTION

83 Gene expression - from mRNA synthesis to splicing, maturation and export from the nucleus
84 to the cytoplasm for translation - is an interconnected process mediated through large
85 macromolecular complexes [13,31,51]. For example, the nuclear pore complex (NPC), which
86 is a large protein assembly embedded in the nuclear membrane, is not merely a gateway
87 enabling nucleocytoplasmic transport but also affects upstream gene expression regulation
88 [17,54]. Associating with the nuclear basket of the NPC, germinal centre associated nuclear
89 protein (GANP) is involved in mRNA export acting as a large scaffold in the TRanscription
90 and EXport-2 complex (TREX-2) [20,59]. GANP, a 210 kDa protein, has N-terminal FG
91 (phenylalanine/glycine) repeats, a yeast Sac3 mRNA binding homology domain, and a C-
92 terminal acetyltransferase domain. GANP has been described as a mediator of selective mRNA
93 export, thus facilitating dynamic regulation of specific biological processes [57,58].

94 We and others recently identified GANP to underlie a childhood-onset neurological syndrome,
95 caused by biallelic mutations in its encoding gene *MCM3AP* (MIM #618124, PNRIID)
96 [23,24,49,61,63]. The disease affects peripheral nerves causing Charcot-Marie-Tooth
97 neuropathy, and results in gait difficulties, often with loss of ambulation. Many patients have
98 intellectual disability. Variable other central nervous system involvement such as
99 encephalopathy or epilepsy have been reported in some patients, as well as non-neurological
100 symptoms such as ovarian dysfunction [61,63]. Studies in patient fibroblasts showed that
101 pathological *MCM3AP* variants either result in a decrease in the amount of GANP at the nuclear
102 envelope or affect critical amino acids in the Sac3 mRNA binding domain of GANP [61].
103 Depletion of GANP was associated with more severe motor phenotypes than the Sac3 variants.

104 Our identification of GANP loss-of-function as a cause for human neurological disease
105 suggested a key role for GANP in neuronal cells, and particularly in motor neurons.
106 Interestingly, GANP was previously found to modify TDP-43 toxicity in fly motor neurons
107 [50]. However, the functions of GANP, and its roles in gene regulation and mRNA export
108 selectivity in human neurons remain unresolved. We hypothesised that inefficient mRNA
109 export limiting the availability of mRNAs for local protein synthesis in axon terminals could
110 be a pathogenic mechanism in GANP-associated disease. Here we set out to investigate the
111 effects of GANP variants in human induced pluripotent stem cell (hiPSC) derived motor
112 neurons. Our results show that GANP mutant motor neurons differentiate normally in culture
113 but have altered expression of nearly 40 percent of their genes, and major changes in gene

114 splicing. Thus, we suggest that GANP has a key gene regulatory role in human motor neurons
115 beyond scaffolding for mRNA export.

116

117 **RESULTS**

118 ***MCM3AP* gene edited and patient-derived hiPSC are pluripotent**

119 We used CRISPR-Cas9 gene editing to knock in different *MCM3AP* patient mutations in the
120 kolf2_c1 parental hiPSC line [26] by homology-directed repair [3]. 128 clones were screened
121 for mutations by MiSeq, and successful editing was detected for the patient mutation R878H
122 located in the Sac3 domain of GANP [23] (Figure 1A,B). Sanger sequencing of the edited
123 hiPSC clones showed that one clone was homozygous for the mutation. Karyotypes of the
124 homozygous edited and parental lines were as expected 46 XY by g-banding.

125 We also reprogrammed skin fibroblasts of patients FIN1 and FIN2, who carry V1272M and
126 P148*fs48 compound heterozygous variants, which result in depletion of GANP [61,63], and
127 of two healthy unrelated controls (Figure 1A,B).

128 Pluripotency of all the above mentioned hiPSC was confirmed with Nanog
129 immunocytochemistry (Figure 1C) and by qRT-PCR of pluripotency genes (Figure 1D). Tested
130 pluripotency genes had higher expression in edited cells compared to parental hiPSC (*NANOG*,
131 *OCT4*, and *C-MYC* $p<0.0001$; *SOX2* $p<0.05$). For pluripotency gene expression, we also
132 investigated two additional edited hiPSC clones with heterozygous R878H mutation in one
133 allele and an indel in the other allele (clones 138 and 245, Supplementary Figure 1), and found
134 increased pluripotency gene expression also in those (*NANOG*, $p<0.0001$). In patient-derived
135 hiPSC, pluripotency genes were similarly expressed as in control hiPSC, apart from *C-MYC*,
136 which was higher in the patient-derived cells ($p=0.0060$) (Figure 1D).

137 **Mutation type affects GANP levels in hiPSC**

138 We have previously shown in patient fibroblasts that homozygous missense variants affecting
139 the Sac3 domain of GANP do not alter *MCM3AP* mRNA or GANP protein levels, whereas
140 compound heterozygous variants outside the Sac3 domain typically lead to lowered *MCM3AP*
141 mRNA level and depletion of GANP at the nuclear membrane [61]. Here, we used
142 immunostaining to detect GANP in the hiPSC lines. We found that GANP localized to nuclear
143 pores in the R878H/R878H knock-in, parental and healthy control hiPSC lines, but was reduced

144 in hiPSC of patient FIN1 (Figure 1E), in line with our previous findings in fibroblasts.
145 *MCM3AP* mRNA levels were reduced by approximately 30% in patient hiPSC compared to
146 healthy controls, whereas we observed a 25% increase in the R878H/R878H knock-in line
147 compared to the parental line (Figure 1F). These results are consistent with the two types of
148 disease-causing *MCM3AP* variants having different effects on the level of GANP at the nuclear
149 envelope.

150 **Patient-derived and knock-in hiPSC differentiate into motor neurons**

151 To determine the effects of GANP variants in a disease-relevant cell type, we next
152 differentiated hiPSC lines into motor neurons [15] (Figure 2A). Differentiation into a neuronal
153 lineage was validated by qRT-PCR analysis of the cytoskeletal markers neurofilament heavy
154 (*NEFH*) and β III-Tubulin (*TUBB3*), and into a motor neuron lineage by analysis of choline
155 acetyltransferase (*CHAT*) and ISL LIM homeobox 1 (*ISL1*) (Figure 2B). Immunofluorescence
156 analysis showed that differentiated cells from all hiPSC lines were positive for neurofilament
157 medium (NEFM), ISL1, and homeobox gene Hb9 (Figures 1C,D). ISL1 positivity was above
158 92% by the end of differentiation. In essence, we found that all hiPSCs differentiated similarly
159 into motor neurons.

160 **GANP is detected in patient-derived motor neurons**

161 We immunostained the motor neurons and found that GANP localized to the nuclear pores in
162 R878H/R878H knock-in, parental and healthy control motor neurons (Figure 3A). In contrast
163 with our previous observations in FIN patients' fibroblasts, in which GANP staining was
164 depleted at the nuclear envelope, we detected GANP staining in patient-derived motor neurons.
165 Nonetheless, also some abnormally staining nuclei were found particularly in FIN2 patient
166 samples (Figure 3A). Patient-derived motor neurons had approximately 30% reduced
167 *MCM3AP* mRNA levels in comparison to healthy controls ($p=0.0007$), whereas the levels were
168 unchanged in R878H/R878H knock-in motor neurons (Figure 3B). Staining with NPC marker
169 Mab414 appeared normal, suggesting that the nuclear envelope was intact in mutant and
170 control motor neurons (Figure 3C).

171 **GANP regulates gene expression in motor neurons**

172 Given the roles of GANP in RNA processing, we next assessed whether mutant GANP affected
173 gene expression in differentiated motor neurons by sequencing total RNA isolated from
174 R878H/R878H knock-in neurons and isogenic control cells (Figure 4A). The motor neuron

175 differentiation protocol we utilized yielded mostly homogeneous neuronal cultures, although
176 as differentiation progressed, mitotic progenitors were also occasionally seen. For RNA
177 sequencing we thus collected the neurons on day 22 of differentiation, and only from wells
178 which did not have progenitor cells.

179 Since GANP has roles in the nuclear export of RNA we also performed RNA sequencing on
180 separate nuclear and axonal RNA fractions (Figure 4A). For the nuclear subcompartment,
181 validation of the purity of the isolated nuclear fraction by western blotting showed highly
182 enriched histone H3 protein (Figure 4B). To analyse transcripts specific to axons, we cultured
183 motor neurons in Xona Chip devices consisting of microfluidic axon isolation technology. We
184 then dissociated motor neurons from porous 1 μm filter inserts to obtain an isolated axon
185 compartment [4]. The coating of the filter bottom with laminin encourages the attachment of
186 axons, whilst the somatodendritic compartment grows on top of the filter. We validated the
187 purity of our isolation of axons by western blotting, which showed low histone H3 levels
188 (Figure 4B). To collect sufficient RNA for axon-Seq, samples from 6 separate wells for each
189 replicate were combined, and in addition ribosomal RNA was depleted.

190 Principal component analysis of the RNA sequencing data showed that the sample types (total,
191 nuclear, and axonal) clustered away from each other, indicating successful subcompartment
192 isolation. Axon samples separated more from total and nuclear samples, demonstrating the
193 uniqueness of the axonal transcriptome [38]. The overall motor neuron axon transcriptome was
194 similar to previously reported datasets demonstrating enrichment in mtDNA- and nuclear-
195 encoded mitochondrial respiratory chain genes, neurofilaments and expression of *TMSB10*,
196 *YBX1* and *STMN2* [27,36,38]. Samples of the same genotype clustered together based on their
197 gene expression, particularly for total and nuclear RNA, whereas axon samples showed more
198 variation (Figure 4C).

199 Total RNA sequencing of R878H/R878H knock-in motor neurons showed that GANP has a
200 major role in gene expression regulation in human motor neurons. Out of the 18,146 genes that
201 we detected to be expressed in motor neurons, 7,044 (~39%) were differentially expressed (DE)
202 between R878H/R878H and parental motor neurons (adjusted p-value <0.01) (Figure 4D). The
203 results for nuclear RNA were similar, showing 5,612 DE genes. More than 50% of the DE
204 genes were the same in total and nuclear RNA (Figure 4E). Axon-Seq, by contrast, showed
205 only 297 altered genes.

206 Overall pathway analysis of DE genes by Reactome enrichment indicated downregulation of
207 genes related to neuronal system and extracellular matrix organization (Figure 4F). Several
208 crucial axon guidance and maintenance genes were downregulated in GANP-deficient motor
209 neurons including *SLITRK1*, *SLITRK3*, *DRAXIN*, *LSAMP* and axon repulsive genes involved
210 in glutamatergic synapses (*NETO1*, *NETO2*, *SHISA9*, *GRIN1*, *NPTX1*) and neuronal system
211 genes (*GABRG2*, *SYN2*, *SYT9*) (Figure 5).

212 The overall upregulated genes in GANP-deficient motor neurons were associated with
213 pathways involved in mitochondrial respiration, and in protein synthesis such as ribosomal
214 subunits and translation elongation. This could indicate compensatory effects in response to
215 altered mRNA export from the nucleus (Figure 4F, Figure 5). Interestingly, mtDNA-encoded
216 transcripts were downregulated, whereas nuclear-encoded mitochondrial transcripts were
217 upregulated, suggesting a nuclear response to mitochondrial dysfunction. Related to energy
218 metabolic changes, CKB (brain-type creatine kinase) involved in compartmentalized ATP
219 production and consumption was also upregulated [47]. Furthermore, upregulation of the serine
220 and one-carbon biosynthesis pathway (*PHGDH*, *PSAT1*, *SHMT2*, *MTHFD2*) was detected,
221 which is commonly observed in mitochondrial defects and as part of the integrated stress
222 response [11,16].

223 The axonal transcriptome of GANP mutant motor neurons showed decreased levels of
224 transcripts related to synaptic function such as glutamate ionotropic receptor subunits (Figure
225 5). Increased levels of a number of histone genes (for example *HIST2H2AC*, *HIST1H2BN*,
226 *HIST1H1B*) were observed in mutant axons (Figure 5). As histone mRNAs are not expected to
227 be translated in axons, this finding may have resulted from enrichment of histone mRNAs
228 lacking polyA tails. However, we previously found in patient fibroblasts that GANP may
229 specifically influence the export of intronless gene mRNAs [61], and it is possible that histone
230 genes, which are intronless, escape the regulation by mutant GANP in neurons. Indeed, we
231 identified a subset of intronless genes that were differentially expressed in motor neurons and
232 in the nuclear subcompartment (*ADRA2A*, *PIGM*, *NORAD*, *ATXN7L3B*).

233 Although genes related to neuronal functions were generally downregulated in knock-in motor
234 neurons, neurofilament genes *NEFL* and *NEFM* were increased ($p < 0.0001$) (Figure 5).
235 Interestingly, by using qRT-PCR we found that *NEFM* expression was also increased in
236 patient-derived motor neurons compared to controls (Figure 6A). The upregulation of
237 neurofilament genes may be linked to our previous observation that *NEFL* expression is

238 regulated by changes in protein synthesis [45]. Another possibility is that neurofilament gene
239 expression was increased as a response to axonal injury. Indeed, neurofilaments are released
240 by neurons in neurodegenerative diseases [25]. We therefore measured NEFL protein in the
241 culture media but found that GANP knock-in or patient-derived motor neurons did not release
242 more NEFL than control cells (Figure 6B). We also used microfluidic chips to test if the altered
243 neurofilament gene expression affected the regeneration capacity following axotomy, but we
244 did not detect defective axon regrowth in GANP knock-in motor neurons (Figure 6C).

245 We hypothesized that a defect in selective mRNA export should lead to nuclear mRNA
246 retention and thus axonal depletion of a set of mRNAs. Thus, we analysed the RNASeq data
247 for transcripts that were upregulated in GANP knock-in nuclei and downregulated in axons.
248 However, only few such genes were detected (Figure 6D). One of those was *PRIMAI*, involved
249 in organizing and anchoring acetylcholinesterase at neural cell membranes [41]. We studied
250 the localization of *PRIMAI* mRNA by RNAScope but did not observe any obvious nuclear
251 retention in the GANP mutant neurons (Figure 6E). Thus, we conclude that the R878H Sac3
252 mutation in GANP does not cause large-scale nuclear retention of mRNAs in human motor
253 neurons but has a major effect on motor neuronal gene regulation.

254 **GANP regulates gene splicing in motor neurons**

255 Finally, we analysed the effects of the R878H mutation on mRNA splicing in human motor
256 neurons by KissSplice analysis. We observed a large number of significant alterations in
257 splicing in motor neurons, in 421 genes, and fivefold more (in 2224 genes) in nuclear RNA
258 sequencing (Figure 7A). The majority of these involved the usage of alternative splice sites, or
259 were exon skipping or intron retention events. Splicing changes were observed both in genes
260 that had an overall decreased mRNA expression, and in genes that were not differentially
261 expressed (Figure 7B,C). As an example, a higher percentage of exon skipping was detected
262 for *GABRG2*, inhibitory gamma-aminobutyric acid receptor subunit, which is alternatively
263 regulated in neuronal development [56] (Figure 7D).

264

265 **DISCUSSION**

266 GANP was initially identified as an abundant protein in splenic germinal centers, which are
267 responsible for development of B cells [30], and subsequently the role of GANP has been
268 extensively characterized in the context of B cell affinity maturation [46]. The recognition of

269 GANP as a mammalian mRNA export factor [59] was preceded by the identification of the
270 yeast homologue of GANP, Sac3 [9,12,42], and the Drosophila homologue Xmas-2 [29].
271 Depletion of both Sac3 and Xmas-2 results in nuclear poly(A)⁺ RNA accumulation. Later
272 studies showed that GANP stably associates with the nuclear pore basket [54], forming a
273 scaffold for ENY2, PCID2 and centrin binding at TREX-2, thus firmly linking GANP to
274 mRNA export [20]. Interestingly, GANP was then shown in HCT116 colon carcinoma cells to
275 promote the export of highly expressed and short-lived transcripts that function particularly in
276 RNA synthesis and processing, indicating that GANP can enable rapid changes in gene
277 expression [58].

278 The identification of *MCM3AP* as a disease-linked gene causing a peripheral neuropathy
279 syndrome prompted us to study GANP's role in a disease relevant cell type. Although the
280 phenotypes associated with GANP mutations are not restricted to peripheral nerves, motor
281 neurons, which have a requirement for mRNAs to be transported extremely long distances into
282 axon terminals, are particularly affected. Pathogenic variants in the mRNA export factor GLE1
283 also underlie a motor neuron disease, suggesting that motor neurons are vulnerable to
284 inefficient mRNA export [10,21,39]. Reduced mRNA availability could have a major effect on
285 local protein synthesis in axons, which is now recognized to be important for axon maintenance
286 and synaptic function [34].

287 We aimed to identify human motor neuron specific genes that are regulated by GANP by
288 utilizing a neuropathy-causing Sac3 domain mutant, which we generated by CRISPR/Cas9
289 gene editing in hiPSC. The homozygous missense variant R878H does not change the
290 localization or the amount of GANP at the nuclear envelope but introduces local structural
291 changes in the winged helix region of Sac3, thus impairing RNA binding [61]. We previously
292 showed that GANP variants can be divided into two groups – compound heterozygous variants
293 leading to GANP depletion, and homozygous Sac3 domain missense variants. Both cause loss-
294 of-function, but notably the Sac3 variants associate with milder motor phenotypes than the
295 GANP depletion variants [61-63]. This suggests that R878H and other Sac3 mutants cause
296 partial impairment of GANP function. Unexpectedly, in the patient-derived motor neurons
297 GANP was not strongly depleted, in contrast to what we observed in fibroblasts and iPSC from
298 the same individuals [61]. The reason for this discrepancy is not known, but since the
299 compound heterozygous mutations carried by these individuals include a frameshift variant in
300 the first exon of *MCM3AP*, we speculate that the downregulation of nonsense-mediated decay

301 response, which has been shown to be required for neuronal differentiation [19], may have
302 resulted in higher amount of residual GANP.

303 Our results showed that motor neurons deficient for the RNA binding function of GANP
304 differentiated normally but had major changes in gene regulation, including abnormal gene
305 expression and splicing. Mutant GANP deregulated a large number of genes required for motor
306 neuron functionality, whereas transcripts required for protein synthesis were upregulated,
307 which we suggest is a compensatory response to abnormal mRNA availability. Expression of
308 nuclear-encoded mitochondrial genes was also upregulated, which may compensate for the
309 reduced availability of those transcripts in axonal protein synthesis that is needed to maintain
310 functional mitochondria. Mitochondrial transcripts are among the most abundant mRNAs in
311 axons [2,5,14,40,52,60], indicating their importance in local maintenance of axonal and
312 synaptic mitochondria. We also observed a transcriptional upregulation of the serine and one
313 carbon biosynthesis, an early-stage of integrated stress response, which may be caused by
314 mitochondrial dysfunction [7,11,16]. These neuronal compensatory responses may be of
315 relevance in patients who have the permanent impairment of GANP but have not been observed
316 in studies of transient GANP depletion [1,58].

317 Previous studies of GANP depletion in diverse non-neuronal cell types have resulted in
318 expression changes in genes that were largely distinct from those we detected here in GANP
319 mutant motor neurons. However, one of the rare common findings in all studies is the
320 downregulation of the intronless gene *ATXN7L3B*, encoding an adaptor protein in the TREX-
321 2 complex. It was downregulated by GANP siRNA knockdown in human colon carcinoma
322 cells [58], in our study of patient fibroblast gene expression [61], in a recent study of a degron
323 system induced GANP knockdown in a colorectal cancer line [1], and in the GANP mutant
324 motor neurons. Together, these findings suggest that *ATXN7L3B* is a specific target of GANP
325 regulation regardless of cell type. Interestingly, *ATXN7L3B* is also a subunit of the Spt-Ada-
326 Gcn5 acetyltransferase (SAGA) complex, involved in histone modifications and thus in gene
327 expression regulation [28]. The multiple effects that GANP has on gene regulation may thus
328 be mediated by the SAGA complex, or through the interactions of GANP with the other large
329 complexes that are interconnected from transcription to splicing and mRNA export.

330 In conclusion, we have shown that GANP is a major gene regulator in human motor neurons,
331 with effects beyond its role in mRNA export. Large-scale nuclear mRNA retention was not
332 observed in our neuronal model with the GANP Sac3 missense mutation, which suggests that

333 the disease-associated impairment is moderate enough to enable the export of most mRNAs,
334 but nevertheless limits mRNA availability, resulting in compensatory responses in protein
335 synthesis and metabolism. Our findings are consistent with GANP-associated disease effects
336 on projection neurons with long axons in human patients.

337

338 **MATERIALS AND METHODS**

339 **hiPSC culture and maintenance**

340 hiPSC were expanded and grown for experiments in E8/E8 Flex basal media with 50X
341 supplement (Thermo) and Primocin (1:500). Cells were routinely passaged with Versene
342 (Thermo) or EDTA and grown on Vitronectin (Thermo). When required, Revitacell/Y-27632
343 was added (1:1000). All cells used in this study were culture at 37°C, in a humidified
344 atmosphere, normoxia and 5% CO₂.

345 **Reprogramming**

346 Human skin fibroblasts from two healthy controls and from two patients (P148fs*/V1272M)
347 [63] were reprogrammed into pluripotent stem cells at Biomedicum Stem Cell Center
348 (University of Helsinki, Finland). Patient cells were reprogrammed in oriP/EBNA1 backbone
349 and expression of OCT3/4, SOX2, KLF4, MYC, LIN28 and shRNA against p53. All cell lines
350 were validated for cell growth, hiPSC morphology and pluripotency gene expression by
351 immunocytochemistry, RT-PCR and qPCR. hiPSC were cultured in Matrigel-coated (Corning)
352 plates with E8-medium (Gibco) supplemented with E8-supplement (Gibco). Cells were
353 passaged when confluent with 0.5 mM EDTA (Invitrogen) in phosphate-buffer saline (PBS).

354 **Gene editing**

355 Gene editing was done following a previously published protocol [3]. Kolf2_C1 p.13 human
356 induced pluripotent stem cells (hiPSCs) characterised by the HipSci consortium [32] were
357 cultured in a humidified incubator at 37°C and 5% CO₂. The cells were cultured in feeder-free
358 conditions in 10 cm culture dishes in TeSR-E8 (Stem Cell Tech) medium on SyntheMAX
359 (2µg/cm²) (Corning #3535). For passaging cells were washed with PBS (no Mg/Ca²), incubated
360 with Gentle Cell Dissociation Reagent (Stem Cell Tech) at 37°C for 3 min and added TesR-E8
361 media.

362 The synthetic guide RNA (sgRNA) harbouring the R878H mutation (clones R878H/R878H
363 and R878H/indel-138), AGCATCCTTGCGGATCTGAG, was ordered from Synthego
364 following design of effective guides (<https://wge.stemcell.sanger.ac.uk>). A single stranded
365 oligonucleotide(ssODN), sequence: 5-
366 GCTGTGTGCTCACCGTGTACGCAAAGTTGAGCGCCCGGAGAGCATCCTTGTGGAT
367 CTGAGAGGAGGAGCGAAATCACTGCAGTCTCAGACGAAGGCCAGT-3, was
368 ordered from IDT. sgRNA GATCCGCAAGGATGCTCTCC GGG (reversed) and ssODN 5-
369 ACTGGGCCTTCGTCTGAGACTGCAGTGATTCGCTCCTCCTCTCAGATCCACAAG
370 GATGCTCTCCGGGCGCTCAACTTTGCGTACACGGTGAGCACACAGC-3 was used to
371 produce clone: R878H/indel-245. Desalted ssODN (IDT, Ultramer DNA oligo) had equal
372 homology arms of 50 bases around the mutation.

373 Confluent 10 cm petri dish was dissociated with Accutase into single cells. 1 million cells were
374 used for electroporation. *SpCas9* ribonucleoprotein complexes (RNP) were formed with
375 sgRNA and ssODN. sgRNA oligos were suspended in IDT duplex buffer solution at
376 concentration 200µM and 500 pmol ssODN templates at final concentration of 100µM. Cas9
377 (purified from *E.coli*) suspended in Cas9 storage buffer and RNP was reconstituted to a final
378 concentration of 4µg/µl. Cells were electroporated with Amaxa 4D using (Lonza #AAF-
379 1002B, #AAF-1002x) suspended in P3 Primary cell 4D-Nucleofector X Kit L (Lonza, V4XP-
380 3012) and CA137 program. Final concentration of Cas9 per 1 M cells was 20 µg and 20 µg
381 sgRNA. Cells were processed and plated in TeSR-E8 media with Rock inhibitor (Y-27632) on
382 SyntheMAX coated (5µg/cm²) 6-well plates. Cells were fed daily until 70% confluent.
383 Recovered cells were dissociated three days later from semi-confluent wells with Gentle Cell
384 Dissociation Reagent and frozen in 90% knock-out serum replacement with 10% DMSO.

385 **Karyotyping**

386 To process cells for karyotyping, hiPSC were grown on 6 cm dishes until semi-confluent.
387 Colcemid 10 µg/mL was added to the cells and placed in the incubator for 4 hrs. After two
388 washes with PBS, the cells were dissociated with Tryple Select (Gibco). DMEM was added
389 and cells were centrifuged 200 g for 5 min. The cells were incubated in KCl at +37°C. Cells
390 were fixed with 25% Acetic acid in methanol and centrifuged in 200 g 5 min, and this was
391 repeated in total three times. Karyotype analyses based on chromosomal G-banding were
392 performed at Analisis Mediques Barcelona.

393 **Motor neuron differentiation**

394 To differentiate hiPSC into motor neurons, we used a previous described protocol [55] with
395 small modifications. In summary, confluent hiPSC were dissociated into low-attachment flasks
396 with 0.5 mM EDTA and cultured in Neuronal basal medium (DMEM/F12/Glutamax,
397 Neurobasal vol:vol, with N2 (Life Technologies), B27 (Life technologies), L-ascorbic acid 0.1
398 mM (Santa Cruz) and Primocin supplemented with 5 μ M Y-27632 (Selleckchem)/40 μ M
399 SB43154 (Merck)/ 0.2 μ M LDN-193189 (Merck/Sigma)/3 μ M CHIR99021 (Selleckchem) for
400 2 days. Following five days the cells were cultured in basal media and 0.1 μ M retinoic acid
401 (Fisher)/0.5 μ M SAG (Calbiochem) and for the next 7 days, BDNF and GDNF (10 ng/ml,
402 Peprotech) were added. 20 μ M DAPT (Calbiochem) was added for days 9-17. Day 10 neurons
403 grown in spheroids were dissociated with Accumax (Invitrogen) on poly-D-lysine 50 μ g/ml
404 (Merck Millipore) and laminin 10 μ g/ml (SigmaAldrich). Day 17 onwards until analysis,
405 neurons were kept in BDNF, GDNF and CNTF. Media were changed every 2-3 days by
406 replacing half of the medium. Developing motor neurons were analysed for experiments from
407 day 22 onwards.

408 **cDNA synthesis and RT-PCR**

409 iPSC and neuron total RNA was isolated with Nucleospin RNA kit (#740955.50, Macherey-
410 Nagel). cDNA was synthesized according to manufacturer's instructions with Maxima First
411 Strand cDNA Synthesis Kit (#K1671, Thermo Fisher Scientific).

412 **qRT-PCR**

413 qPCR was run on a CFX96 Touch Real-Time PCR detection system (Bio-Rad) with SYBR
414 Green qPCR Kit (#F415S, Thermo Fisher Scientific) on a 96-well plate with gene-specific
415 primers 10 μ M and H₂O. The cycling protocol was 95°C for 7 min, then 40 cycles of 95°C for
416 10 s and 60°C for 30 s. Normalization was done with *GAPDH* as a housekeeping gene using
417 $\Delta\Delta$ cT method. Minimum of 3-4 technical samples were used for all studies. 12.5 ng of cDNA
418 was used for hiPSC and 6.25 ng for neurons.

419 **Immunocytochemistry**

420 Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 15 min followed
421 by three PBS washes. Cells were then permeabilized in 0.2% Triton X-100/PBS for 15 min at
422 RT. Coverslips were washed three times in PBST (0.1% Tween-20) followed by block at RT
423 with 5% BSA/PBST (#001-000-162, Jackson Immuno Research) for two hours. Primary
424 antibody in 5% BSA/PBST was incubated overnight at 4°C. Following overnight incubation,

425 coverslips were washed with PBST for 15 min three times, followed by incubation in secondary
426 antibody in 5% BSA/PBST for one hour. The cells were finally washed three times for 10 min
427 and coverslips plated on glass with Vectashield containing DAPI (#H-1200, Vector
428 Laboratories).

429 **Antibodies**

430 Antibodies were used at the following dilutions: 1:200 α -rabbit N-terminal GANP antibody for
431 immunocytochemistry was (#198173, Abcam) with secondary antibody 1:1000 (488 goat-anti-
432 rabbit #A11008, Alexa or 594 goat-anti-rabbit #110012, Alexa). To detect α -rabbit Nanog
433 1:200 (#4903, Cell Signalling), secondary 488 goat-anti-rabbit 1:1000 (A11008, Alexa) was
434 used. Neuronal antibodies used for immunocytochemistry used were: 1:200 mouse α -Hb9
435 (#81.5C10-C, DSHB) with secondary antibody 1:1000: (#A11007, Alexa), 1:200 chicken α -
436 MAP2 antibody (#AB5543, Millipore) with secondary antibody 1:1000 (#SA5-10070,
437 Thermo). 1:1000 rabbit α -histone H3 (#4499, Cell Signalling) with secondary antibody 1:1000:
438 1:700 rabbit α -NEFM antibody for immunocytochemistry and 1:1000 for Western blotting
439 (#20664-1-AP, Proteintech), with secondary antibodies 1:1000 (A11008, Alexa) and (#111-
440 035-144, Jackson), respectively. To visualize mouse α -Isl1 1:200 (#39-4D5, DSHB),
441 secondary 594 goat-anti-mouse was used 1:1000 (#A11007, Alexa). To visualize nuclear pores,
442 mouse α -Mab414 1:200 (#ab24609, Abcam) was used with secondary antibody 594 goat-anti-
443 mouse 1:1000 (#A11007, Alexa). For Western blotting, rabbit α -GAPDH 1:1000 (#14C10,
444 Cell Signalling) with secondary 1:5000 (#111-035-144, Jackson) and mouse α -NEFL 1:1000
445 (#sc39073, Santa Cruz) with secondary 1:5000 (#115-035-146, Jackson).

446 **Axon isolation**

447 Dissociated Accutase passaged single neurons d11 (400K) were plated in motor neuron
448 differentiation media in 6 well hanging inserts with Boyden chamber device (1 μ m pore, Falcon
449 Cell Culture Inserts, #10289270, Thermo) for axon isolation (d22) similar to recent protocols
450 [4,18]. Transmembrane filters were coated on both sides with poly-d-lysine and only on bottom
451 side with laminin. Somatodendritic compartment was removed by scraping with PBS. The filter
452 top was then washed 10 times with PBS RT strongly pipetting and then filter containing axons
453 on bottom side was cut from the insert and washed 1 time with cold PBS. For RNA, PBS was
454 removed by aspiration after spinning down and continued to RNA extraction with Nucleospin
455 RNA kit (#740955.50, Macherey-Nagel) (incubated in the first buffer for 15 min after

456 vortexing). Six filters combined into same conical tube for one technical replicate for RNA
457 sequencing.

458 For isolating protein for validation by immunoblotting, the filter was then placed on ice
459 following the washing steps and lysed in 30 uL RIPA containing Halt protease inhibitor
460 (#1861284, Thermo) was added. All samples were vortexed and incubated on ice for 15 min.
461 The samples were then centrifuged for 10 min at 14 000 x g in a cold centrifuge and supernatant
462 was removed for protein. Three wells combined into same conical tube. Protein concentration
463 was measured with BCA (#23227, Thermo).

464 **Nuclei isolation for RNA and protein**

465 Neurons d22 (500K) were washed once with PBS RT, lysed in homogenizing buffer (0.3 M
466 sucrose, 1 mM EGTA, 5 mM MOPS, 5 mM KH₂PO₄, pH 7.4). Cells were scraped and an
467 aliquot was removed for total cell extract for protein samples. Cells were run through 22 G
468 syringe and needle 8 times, an aliquot was taken for trypan blue staining and cells were
469 observed under microscope for lysis. Cells were centrifuged 15 minutes at 6500 rpm at 4°C.
470 Supernatant formed the cytoplasmic extract and pellet the nuclear extract. RNA was isolated
471 with Nucleospin RNA kit (#740955.50, Macherey-Nagel) and RNA concentrations were
472 measured with Denovix and TapeStation. For protein, nuclear and total cell extracts were lysed
473 in 1 x RIPA with protease inhibitor, incubated on ice 5 min, centrifuged 14 000 x g for 10 min,
474 removed supernatant for protein.

475 **Protein extraction and Western blotting**

476 The cells were isolated with 1X RIPA (#9806, Cell Signalling) for 5 min on ice, scraped and
477 centrifuged at 14 000 x g for 10 min at 4°C. Protein concentrations were measured with BCA
478 (#23227, Thermo). For SDS-PAGE analysis, 5 µg of protein was loaded per well on a 10%
479 TGX 10-well 30µl/well gel (456-1033, Bio-Rad) for 1 hr. at 150V. Molecular size of bands
480 was detected with Protein Standard (#161-0374, Bio-Rad) and proteins loaded together with
481 4x Laemmli loading buffer (#161-0747, Bio-Rad) in 2-mercaptoethanol (#1610710, Bio-Rad).
482 Gel was transferred into a nitrocellulose membrane (#1704158, Bio-Rad) and transferred using
483 Mixed Molecular Weight protocol in Trans-Blot Turbo (Bio-Rad). The blot was washed
484 blocked in 5% milk/TBST for 1 hr in RT. For immunoblotting analysis, antibodies were
485 incubated overnight in 4°C in 2,5% milk/BSA in TBST. The blot was washed three times for
486 five min in TBST. Secondary HRP antibodies (1:5000) in 1% BSA/TBST was incubated for 1
487 hr at RT on a shaker and washed three times 10 min in TBST. The blot was visualized with

488 ECL reaction (#K-12049-D50, Advansta). The images were obtained with a Chemidoc imager
489 (Bio Rad) and quantified on Image Lab software (Bio-Rad). Results were visualized on Graph
490 Pad Prism Software.

491 **RNA sequencing and bioinformatics**

492 For total neuron population, RNA was isolated from 6-well plates of Accumax dissociated and
493 cultured single neurons (500K) four independent wells grown in poly d lysine and laminin
494 grown in culture for 22 days. Samples were sequenced and analysed for Kolf2_c1 parental wild
495 type and R878H/R878H knock-in line. Cells were washed with PBS once and RNA isolated
496 from lysed cells Nucleospin RNA kit (#740955.50, Macherey-Nagel) according to
497 manufacturer's instructions including rDNAse treatment. RNA concentrations were measured
498 with Agilent TapeStation and Nanodrop, RNA integrity was checked on a gel with Agilent
499 TapeStation.

500 The RNA-seq libraries were made with NEBNext Ultra II Directional (PolyA) kit and
501 sequenced with NovaSeq SP 2x150bp (Illumina) at Biomedicum Functional Genomics Unit
502 (FuGU). The raw data was filtered with cutadapt [37] to remove adapter sequences, ambiguous
503 (N) and low quality bases (Phred score < 25). We also excluded read pairs that were too short
504 (<25bp) after trimming. The filtered read pair were mapped to the human reference genome
505 (GRCh38) with STAR aligner [6]. Gene expression was counted from read pairs mapping to
506 exons using featureCount in Rsubreads [33]. Duplicates, chimeric and multimapping reads
507 were excluded, as well as reads with low mapping score (MAPQ < 10). The read count data
508 was analyzed with DESeq2 [35]. We analyzed the effect of R878H/R878H mutation in
509 *MCM3AP* by comparing the mutant samples to the parental cell line, separately for each sample
510 type (total RNA, nuclear RNA and axonal RNA). We removed genes with low expression
511 levels from the analysis (<50 reads across all samples). PCA was calculated with prcomp using
512 variance stabilizing transformation (vst) on the read counts. The differentially expressed genes
513 (FDR<0.01) were analyzed for enrichment separately for the up- and down-regulated genes
514 using clusterProfiler [64] against the Reactome pathways [8], KEGG [22], Gene ontology [53]
515 and Disease ontology [48]. For background genes we used all the genes that were detected as
516 expressed in the samples (above the low coverage cutoff, >50 reads). We analyzed alternative
517 splicing dynamics with KisSplice [44]. The results from the gene expression analysis together
518 with the raw sequences were deposited to GEO, under accession GSE162781.

519 **RNAScope**

520 RNA *in situ* hybridization was performed on fixed adherent hiPSCs grown on coverslips using
521 RNAscope Multiplex Fluorescent Reagent Kit V2 (#323100, Advanced Cell Diagnostics) for
522 target detection according to the manual. Briefly, 4% paraformaldehyde fixed cells were
523 dehydrated in increasing ethanol concentrations (50%, 70%), and stored in 100% EtOH at -
524 20°C. The day of RNA *in situ* hybridization, were cells rehydrated in decreasing concentrations
525 of ethanol (70%, 50%) and in PBS. Then treated with hydrogen peroxide for 10 min at RT,
526 washed with distilled water, and treated with 1:30 diluted protease III for 10 min at RT, and
527 washed with PBS. Probes Hs PRIMA1 (ACD, #900601) were hybridized for 2 h at 40°C
528 followed by signal amplification and developing the HRP channel with TSA Plus fluorophore
529 Cyanine 3 (1:1500) (NEL744001KT, Perkin Elmer) according to the manual. Cells were
530 counterstained with DAPI and mounted with ProLong Gold Antifade Mountant (#P36930,
531 Invitrogen). Cells were imaged with Zeiss Axio Observer microscope (Zeiss, Germany) with a
532 20X air objective and Apotome.2.

533 **Axon regeneration assay**

534 Neurons were plated and axons grown on microfluidic chips (#XC450, Xona microfluidics).
535 To dissociate, axons were washed with media until they detached. Axon growth was visualized
536 by light microscopy on days 21-23 of differentiation, before and after axotomy. Axon masks
537 from images were generated with Fiji.

538 **Measurement of NEFL from culture medium**

539 For measurement of NEFL, 1 mL of culture medium was collected from single 6-well neuronal
540 cultures (4 technical replicates) at day 23 of neuronal differentiation. Samples were centrifuged
541 at 13000 x g for 10 min in a cold centrifuge and supernatant frozen in -20°C. NEFL levels were
542 quantified using the Quanterix single molecule array [43] (Simoa, Billerica, MA, USA) HD-1
543 analyzer and Quanterix Simoa NF-Light Advantage Kit (ref. 103186) according to
544 manufacturer's instructions. Briefly, frozen medium samples were slowly thawed on ice, mixed,
545 and centrifuged at 10000g for 5 min at room temperature and diluted 1:50 in sample diluent
546 prior to loading to a 96-well plate. Samples were measured in duplicate. All measured
547 concentrations were within the calibration range and the mean coefficient of variation (CV) of
548 sample replicates was 4.5%. A CV of <15% was considered acceptable between the replicates.
549 The data are shown as mean of the replicate samples \pm SD (pg/mL).

550 **Statistics**

551 One-way ANOVA was used for comparing means between different groups and unpaired two-
552 tailed t-test for comparisons of two groups. Significance was measured by $p < 0.05$ in qRT-PCR
553 and axon microfluidics experiments. Statistical tests were performed in Graph Pad Prism
554 7.04/8.03. All measured data points were included in the analysis.

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565

566 **AUTHOR CONTRIBUTIONS**

567 RW, EY, JS and HT conceived the ideas and planned the experiments. RW, MS, MTS, SH
568 developed and modified the methodologies used. JK did the bioinformatics analysis and
569 implemented the codes. RW, MS and NH completed and validated the experiments and did the
570 formal analyses. NH, S-K.H and AH produced the NEFL data. SHä conducted RNAScope
571 experiment which OC supervised. RT-M participated in AxonSeq experiment. RW gene edited
572 hiPSC, which JS, MW and AB supervised. RW, MS, JK prepared the figures and visualization
573 of the data. RW wrote the initial manuscript and HT and JS reviewed and edited the draft. All
574 authors reviewed the final manuscript.

575

576 **CONFLICTS OF INTEREST STATEMENT**

577 The authors report no conflicts of interest.

578 **SUPPLEMENTAL INFORMATION**

579 **Supplemental table 1.**

580 Differential expression analysis results.

581 **Supplemental table 2.**

582 Gene-set enrichment analysis results.

583 **Supplemental table 3.**

584 Splicing analysis results.

585 **Supplemental table 4.**

586 Gene-set enrichment analysis results for splicing changes.

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824 LEGENDS TO FIGURES

825 **Figure 1. GANP-deficient hiPSC are pluripotent.**

826 a. Cell lines used in this study. Gene editing was used to generate the homozygous R878H Sac3
827 domain mutation in *MCM3AP* (GANP) on Kolf2_c1 parental hiPSC line, which was used as
828 the isogenic control. In addition, fibroblasts from two FIN patients carrying P148fs*/V1272M
829 were reprogrammed, and compared to healthy control hiPSC. b. Localization of the modelled
830 disease variants in GANP domains. c. Immunocytochemistry to validate pluripotency with
831 Nanog. DAPI indicates nuclei. Scale bar 50 μ m. d. qRT-PCR of pluripotency markers *OCT4*,
832 *NANOG*, *C-MYC* and *SOX2* relative to *GAPDH* expression (n=4-6). Fibroblasts are shown as
833 a negative control. Patient values are relative to controls' and R878H/R878H to parental
834 average. e. Immunocytochemistry of GANP in hiPSC lines. Scale bar 50 μ m. f. qRT-PCR of
835 *MCM3AP* expression relative to *GAPDH* (n=3-6) in hiPSC. * p<0.05, *** p<0.0001.

836 **Figure 2. GANP-deficient hiPSC differentiate normally into motor neurons.**

837 a. hiPSC lines were differentiated into motor neurons. b. Gene expression validation of motor
838 neuron transcripts *CHAT* and *ISL1* and neural transcript *NEFH* and *TUBB3* relative to *GAPDH*
839 by qRT-PCR on day 23 of motor neuron differentiation (n=6 per clone). Patient lines compared
840 to controls' average and R878H/R878H to parental average. c. Immunocytochemistry of

841 ISL1/2 (red) and NEFM (green) in neuronal cultures on day 23. d. Immunostaining of HB9
842 (red) and MAP2 (green). Scale bars 50 μ m. ns = not significant.

843 **Figure 3. GANP localization and expression in human motor neurons**

844 a. Immunocytochemistry of GANP (red) in MAP2 (green) positive neuronal cultures. DAPI
845 stains for nuclei shown in blue. Scale bar 50 μ m. b. Expression of *MCM3AP* in motor neurons
846 analysed by qRT-PCR, relative to *GAPDH* (n=3-6 per clone). c. Immunocytochemistry of
847 nuclear pore marker Mab414 (red) and neuronal marker MAP2 (green). DAPI is shown in blue.
848 Scale bar 50 μ m. ** p<0.001.

849 **Figure 4. GANP is a major regulator of gene expression in human motor neurons.**

850 a. Schematic of workflow used for RNA sequencing experiments. b. Validation of nuclear and
851 axonal isolations by western blotting of histone H3 (20 kDa), GAPDH (37 kDa), and NEFM
852 (150 kDa). SD=somatodendritic fraction. c. Principal component analysis of RNA sequencing
853 datasets. d. Density plot of differentially expressed genes in total neuron population and nuclear
854 and axonal fractions. e. Venn diagram of the differentially expressed genes across the three
855 different RNA sequencing datasets. f. Gene-set enrichment analysis of altered Reactome
856 pathways.

857 **Figure 5. Heatmap of selected differentially expressed genes in GANP-deficient motor** 858 **neurons**

859 The heatmap shows representative genes from multiple enriched Reactome categories. To
860 highlight differences among samples, the expression values (log_{1p} FPKM) were scaled to gene
861 averages separately for each sample type (total, nucleus and axon).

862 **Figure 6. Nuclear mRNA retention in GANP-deficient motor neurons**

863 a. mRNA expression of *NEFL* and *NEFM* in motor neurons by qRT-PCR, relative to *GAPDH*.
864 b. NEFL measurement from neuronal culture medium. c. Axon regeneration assay. Images
865 shown from 24 hrs before axotomy, immediately following axotomy and 24/48 hrs post-
866 axotomy. One device analysed per line. Scale bar 100 μ m. c. Quantification of axon thickness
867 from light microscopy images 24 hrs before axotomy. d. Fold changes of genes that were
868 upregulated in nuclear fraction and downregulated in axons, suggestive of nuclear mRNA
869 retention e. RNA *in situ* hybridization of *PRIMA1* mRNA foci in motor neurons (orange).
870 DAPI (blue) stains the nuclei. * p<0.05, ** p<0.001, ***p<0.0001.

871 **Figure 7. GANP regulates gene splicing in motor neurons**

872 a. Volcano plot of splicing changes and types in total and nuclear RNA sequencing. b. Venn
873 diagram indicating the numbers of differentially expressed and spliced genes in total RNA
874 sequencing. c. Venn diagram indicating the numbers of differentially expressed and spliced
875 genes in the nuclear compartment. d. Box plot indicating exon inclusion percentages in
876 *GABRG2*. Sashimi plot around alternatively spliced exon. The values on the lines indicate the
877 read counts for junction reads (reads that maps partially to more than one exon). The location
878 of the splicing event is indicated with the refseq models.

879 **Supplementary Figure 1. Increased expression of pluripotency marker *NANOG* in iPSC.**

880 qRT-PCR of *MCM3AP* and pluripotency marker *NANOG* in different gene-edited iPSC lines.

881 * $p < 0.05$, *** $p < 0.0001$.

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