

1 **CryoET Reveals Organelle Phenotypes in Huntington Disease Patient iPSC-Derived and Mouse**
2 **Primary Neurons**

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28

29 **Abstract**

30 Huntington's Disease (HD) is caused by an expanded CAG repeat in the huntingtin gene, yielding
31 a Huntingtin protein with an expanded polyglutamine tract. Patient-derived induced pluripotent
32 stem cells (iPSCs) can help understand disease; however, defining pathological biomarkers is
33 challenging. Here, we used cryogenic electron tomography to visualize neurites in HD patient
34 iPSC-derived neurons with varying CAG repeats, and primary cortical neurons from BACHD,
35 deltaN17-BACHD, and wild-type mice. In HD models, we discovered mitochondria with enlarged
36 granules and distorted cristae, and thin sheet aggregates in double membrane-bound organelles.
37 We used artificial intelligence to quantify mitochondrial granules, and proteomics to show
38 differential protein content in HD mitochondria. Knockdown of Protein Inhibitor of Activated
39 STAT1 ameliorated aberrant phenotypes in iPSC-neurons and reduced phenotypes in BACHD
40 neurons. We show that integrated ultrastructural and proteomic approaches may uncover early
41 HD phenotypes to accelerate diagnostics and the development of targeted therapeutics for HD.

42 **Key Words:** Huntington's Disease, cryoET, iPSC, BACHD, mitochondria, aggregates, PIAS1

43 **INTRODUCTION**

44 Huntington's disease (HD) is a progressive, fatal neurodegenerative disorder caused by a genetic
45 mutation in the huntingtin gene (*HTT*)¹. The mutation is an expansion of an N-terminal CAG
46 repeat to 40 and above. This yields a mutated Huntingtin protein (mHTT) with an expanded
47 polyglutamine (polyQ) tract that is pathogenic. Disease typically strikes in mid-life, lasting ~10-15
48 years with ongoing progression of symptoms, which include cognitive decline, mood and
49 personality disorders, and loss of motor control². CAG length is correlated inversely with age of
50 disease onset with repeats longer than ~60 causing a juvenile form of HD^{3,4}. No disease-modifying
51 treatments are available. Neuropathologically, degeneration of medium spiny neurons in the
52 striatum and cortical atrophy serve as prominent manifestations⁵.

53 Methods to decipher the pathogenesis of neurodegenerative disorders are needed to identify
54 biomarkers sensitive to clinical progression and to inform therapeutic trials. A potential source
55 of such insights are patient-derived induced pluripotent stem cells (iPSCs)^{6,7}, which can be

56 differentiated into multiple cell types, including neurons⁸ exhibiting disease phenotypes. Indeed,
57 a number of HD-associated phenotypes have been recapitulated in neurons differentiated from
58 HD iPSCs, including transcriptional dysregulation^{4,9–12}, bioenergetic deficits¹³, impaired
59 neurodevelopment^{9,14,15}, altered cell adhesion^{10,12}, impaired nucleocytoplasmic trafficking^{16,17}
60 and increased susceptibility to cell stressors¹⁸, among others¹⁸.

61 The propensity of mHTT to aggregate in neuronal cells is a hallmark of HD and leads to the
62 appearance of large (micrometer scale) nuclear and neuritic inclusions¹⁹, as seen in mouse
63 models²⁰ and human²¹ post-mortem brains^{19,22}. Mutant HTT's role in the potential impairment of
64 autophagy may contribute to aberrant protein accumulation²³. However, neither protein
65 aggregation nor disruptions to protein homeostasis have been observed in iPSC-derived HD
66 models unless treated with an inhibitor of the proteasome²⁴, likely because these models
67 represent early developmental stages where overt disease phenotypes are challenging to detect.

68 In parallel, other technological advances in cell, molecular and structural biology are poised to
69 contribute to the goal of identifying early pathology biomarkers. For example, advances in
70 cryogenic electron microscopy (cryoEM) and tomography (cryoET) have recently elucidated the
71 structure of soluble HTT in complex with HAP40 at near atomic resolution²⁵, the topology of
72 mHTT-exon 1 and polyQ *in vitro* aggregates at nanometer resolution²⁶, and have enabled
73 visualization of the interactions between mHTT-exon 1 aggregates and other proteins and cellular
74 compartments in transfected yeast²⁷ and HeLa²⁸ cells, and with molecular chaperones *in*
75 *vitro*^{29,30}. Correlative light and traditional EM microscopy has also been used to image
76 recruitment of mHTT-exon 1 to cytoplasmic aggregates within single membrane, vesicle-rich
77 endolysosomal organelles³¹.

78 Herein, we used cryoET to visualize neurites from five human iPSC-derived neuronal cell lines
79 endogenously expressing full-length mHTT with a range of CAG repeat lengths (Q18, Q53, Q66,
80 Q77 and Q109). Using the same methods, we also studied mouse primary neurons from the
81 BACHD model³² expressing full-length human mHTT, full-length human mHTT lacking the first 17
82 amino acids (deltaN17; dN17)³³, and control WT mice. For all our samples, we examined
83 subcellular organelles previously implicated in HD, namely mitochondria³⁴ and

84 autophagosomes³⁵, and found marked changes in morphology as compared to controls. We then
85 coupled these ultrastructural observations with mitochondrial proteomics and found changes in
86 the levels of a number of mitochondrial proteins in HD samples. We also developed an artificial
87 intelligence-based semi-automated 3D segmentation method to quantify changes in
88 mitochondrial granule numbers and sizes.

89 Guided by combined ultrastructural and proteomic data, we explored the impact of genetic
90 knockdown (KD) of a SUMO E3 ligase Protein Inhibitor of Activated STAT1 (PIAS1), a protein linked
91 to maintenance of proteostasis and synaptic function in HD. In both systems, we observed rescue
92 of mitochondrial morphology. Additionally, PIAS1 reduction abrogated aberrant aggregates in
93 human HD iPSC-derived neuron autophagic organelles, findings consistent with prior studies
94 showing that PIAS1 inhibition ameliorates HD pathology in mice and human iPSCs³⁶.

95 Our investigations provide a platform with which to structurally evaluate *in situ* organelle
96 phenotypes in thin regions of intact cells at nanometer resolution, in the presence and absence
97 of potential therapeutics. The paradigm we propose emphasizes the ability to explore early
98 disease manifestations and mechanisms in neurites of intact patient and mouse model-derived
99 neurons, which serves as a proof of concept for the utility of cryoET as a structural readout to
100 assess early HD phenotypes and to support preclinical evaluation of potential therapies.

101 **RESULTS**

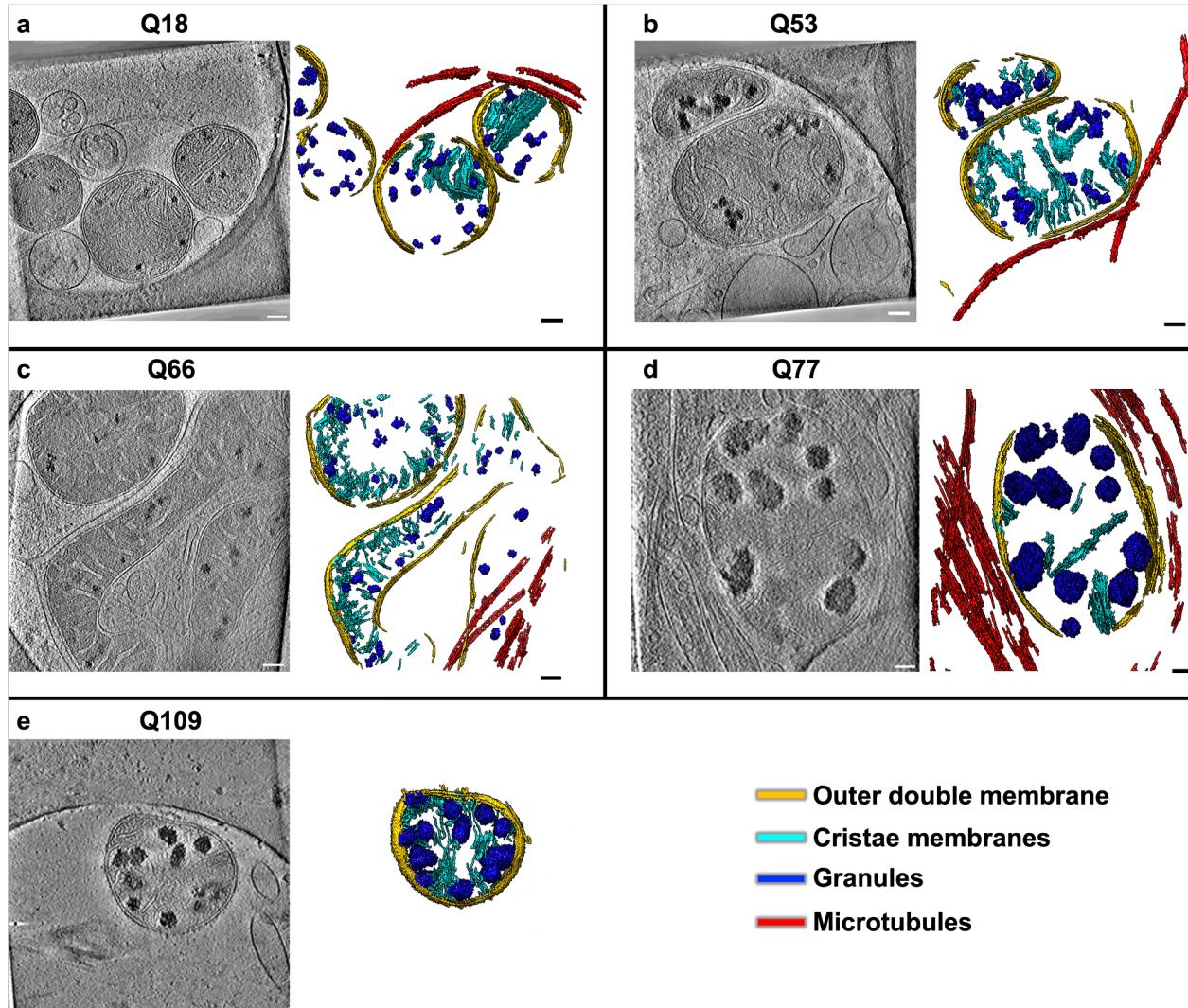
102 **Huntington's disease patient-derived iPSCs differentiated into mature neurons on electron**
103 **microscopy grids**

104 Neurons differentiated from human iPSCs with pathological- and normal-length polyQ tracts in
105 their *HTT* gene provide a platform to study different HD pathological states within their
106 endogenous genetic context⁴. Here, we developed a robust protocol to differentiate iPSC-derived
107 neurons with characteristics of medium spiny neurons on electron microscopy (EM) gold grids
108 (**Supplementary Fig. 1**).

109 We first differentiated iPSCs to neural progenitors and then adapted our prior maturation
110 protocol⁴ to allow the cells to grow directly on gold EM grids, eliminating the Matrigel matrix to
111 minimize background densities, thereby maximizing contrast in cryoET images (**Supplementary**
112 **Fig. 1a**). The cells survived, differentiated and matured without Matrigel, producing axons and
113 dendrites, and displaying normal neuronal morphology (**Supplementary Fig. 1b,c**).
114 Differentiation of cells was also performed at half density (see Methods) to increase the
115 likelihood of obtaining grids with only one cell per grid square, minimizing the potential for
116 overlaps between cells while maximizing the number of areas suitable for cryoET imaging
117 (**Supplementary Fig. 1c**). These modifications allowed iPSCs to differentiate into cells with
118 medium spiny neuron-like characteristics, as validated by DARPP32 and CTIP2 co-staining¹²
119 (**Supplementary Fig. 1d**). Cells were differentiated for 16 days, then plated on gold EM grids for
120 terminal differentiation and maturation. After three more weeks (21 ± 2 days) of terminal
121 differentiation, grids were vitrified by rapid plunging into liquid propane on day ~37-39^{37,38} to
122 preserve the neurons on them in a near-native state without chemical fixative or metal stain.

123 **CryoET data showed mitochondria with abnormal cristae and enlarged granules in neurites of**
124 **HD patient iPSC-derived neurons**

125 The initial motivation to image intact human HD neurons using cryoET was to determine whether
126 we could directly visualize at nanometer scale *in situ* aggregates of native mHTT (endogenous
127 and untagged) that are not visible using other microscopy methods, as well as their surrounding
128 subcellular components. A single iPSC-derived neuron is a micrometer-sized cell with a thick cell
129 body and long, thin neurites. Since the electron beam cannot penetrate through the cell body of
130 neurons, we extensively surveyed the structural features in the neurites of our HD cells by
131 recording 2D cryoEM low-magnification projection images, from which we were able to identify
132 potential regions of interest for subsequent higher magnification cryoET data collection
133 (**Supplementary Fig. 2**).



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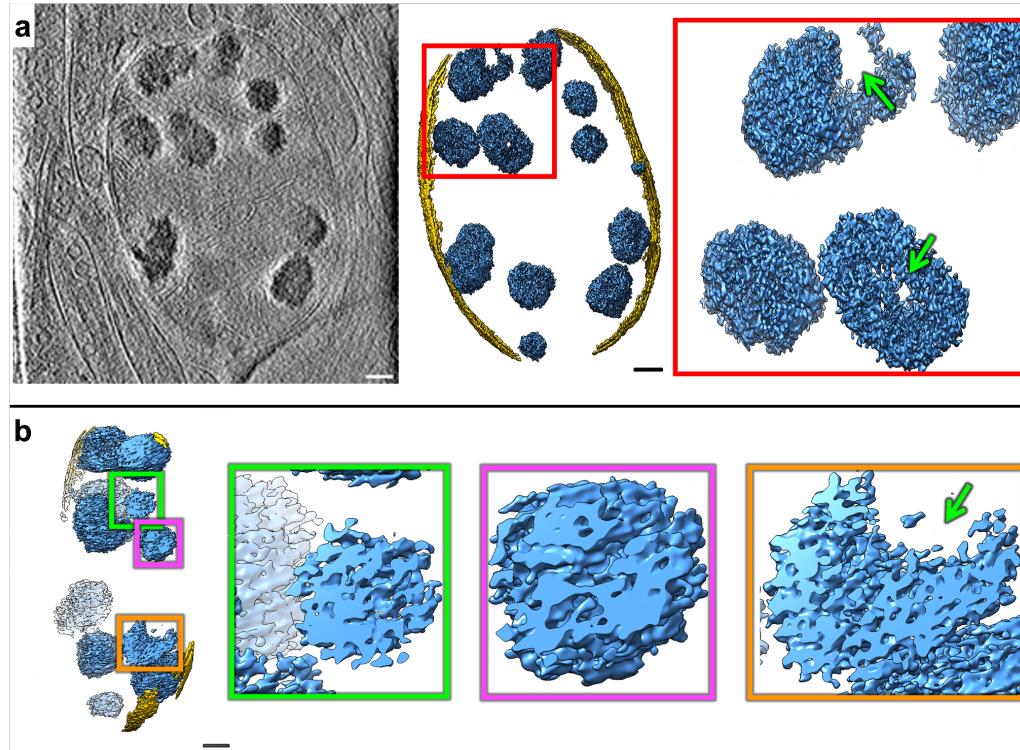
135 **Fig. 1. Mitochondria in neurites of HD patient iPSC-derived neurons exhibit altered morphology and**
136 **contain enriched granules of varying size.** Slices (~1.4 nm thick) through selected regions of
137 representative cryoET tomograms and corresponding segmentations of local features for **a** Q18, **b** Q53, **c**
138 Q66, **d** Q77 and **e** Q109 human iPSC-derived neurons. Q53-Q109 reveal that mitochondria in HD patient-
139 derived neurons have swollen cristae and contain enlarged granules compared to controls (Q18).
140 Tomogram numbers: Q18=21, Q53=14, Q66=10, Q77=5 and Q109=37. Segmentation colors:
141 red:microtubules, yellow:mitochondrial outer double-membranes, dark blue:granules, and cyan:cristae
142 membranes. Scale bars = 100 nm.

143

144 Following an iterative search in many different areas on the cryoEM grids for all HD patient iPSC-
145 derived (Q53, Q66, Q77 and Q109) neurons, we failed to detect large cytoplasmic inclusions or

146 aggregates such as those formed by mHTT-exon 1 previously observed by cryoEM/cryoET *in*
147 *vitro*^{26,29,30,39,40} and in transfected cells^{28,41}. However, this exhaustive examination did detect in
148 numerous regions in the neurites of all samples abnormally large and dense, discrete, granular
149 features as well as tangled aggregates, both within double membrane-bound compartments.
150 Some of these compartments showed classic features of mitochondria (**Fig. 1**), as marked by an
151 outer double membrane and the appearance of interior folded cristae, continuous with the inner
152 membrane of the compartment (**Supplementary Video 1**).

153 The large and dense granular features inside mitochondria were consistently present in tens of
154 tomograms of all HD cell lines (Q53, Q66, Q77 and Q109), and cristae were abnormal in most
155 mitochondria in higher polyQ lines (Q66, Q77 and Q109) (**Fig. 1c-e**). In general, both phenotypes
156 (namely, enlarged granules and disrupted cristae) became more pronounced with higher polyQ
157 length. Importantly, these aberrant features were absent from mitochondria of control iPSC-
158 derived neurons (Q18), though smaller features, consistent with normal mitochondrial granules,
159 were readily observed (**Fig. 1a**). To facilitate 3D visualization, we used the convolutional neural
160 network-based algorithm in EMAN2⁴² to annotate and segment these dense structures as well as
161 other mitochondrial and subcellular features in surrounding areas, such as microtubules (**Fig. 1a-**
162 **e, Supplementary video 1**). Of note, the enlarged granules in mitochondria of HD cells did not
163 comprise homogeneous, smooth densities, but rather exhibited complex interwoven textures,
164 occasionally displaying lower-density regions or voids (**Fig. 2**).



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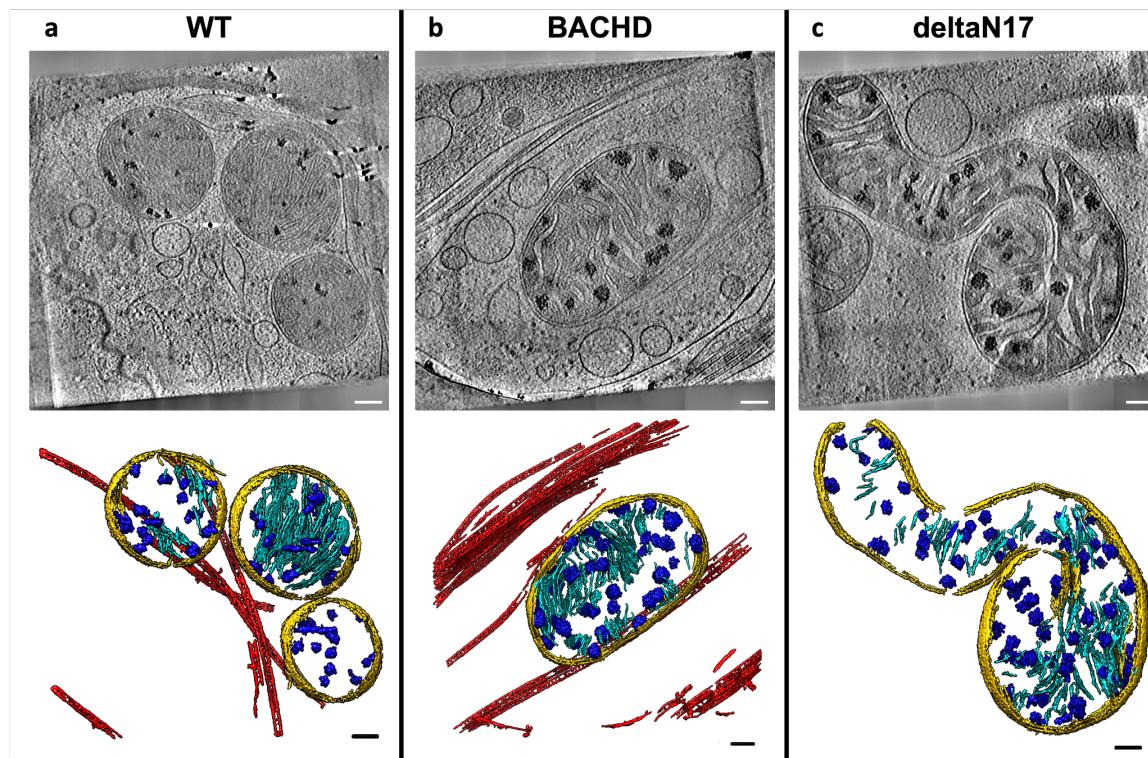
166 **Fig. 2. Mitochondria in HD neurites contain enlarged granules composed of tightly packed,**
167 **heterogeneous densities.** **a** Z-slice (~1.4 nm thick) through a representative cryoET tomogram of a neurite
168 in an HD patient iPSC-derived neuron (Q77) and corresponding segmentation of double membranes
169 (yellow) and dense, granular densities inside (light blue). **b** Cutaway, oblique view of the segmentation in
170 **a** and zoomed-in views of selected regions showing examples of interwoven densities in enlarged
171 mitochondrial granules. Green arrows point to void regions. Scale bars = 100 nm. Segmentation colors:
172 yellow:double membrane, light blue:mitochondrial granules.

173

174 **CryoET data showed mitochondria with abnormal cristae and enlarged granules in neurites of**
175 **HD mouse model primary neurons**

176 Next, we tested whether the abnormal ultrastructural features we observed in iPSC-neurons
177 were also present in neurons derived from the well-studied BACHD mouse model³², which
178 expresses full-length human mHTT with an expanded polyQ tract comprised of 97 mixed CAG-
179 CAA repeats under the control of human regulatory sequences. We used cryoET to image
180 neurites in primary cortical BACHD neurons and again found abnormally enlarged granules within
181 mitochondria whose cristae were often disrupted compared to WT (**Fig. 3a,b**), similar to those
182 seen in neurites of HD patient iPSC-derived neurons (**Fig. 1b-e**). Our data are evidence that
183 neurons from both human and mouse HD models share the presence of enlarged granules and
184 other changes in mitochondria, supporting the view that mHTT is responsible and raising the

185 possibility that these morphological abnormalities could be used as diagnostic features. We also
186 evaluated primary cortical neurons from BACHD transgenic mice expressing human mHTT lacking
187 the first 17 N-terminal amino acids (dN17-BACHD)³³. The N-terminus of HTT contains a putative
188 mitochondrial membrane-targeting sequence that can form an amphipathic helix^{43,44}
189 characteristic of proteins transported into the mitochondria. This domain also promotes cytosolic
190 localization of HTT⁴⁵, potentially as a nuclear export sequence⁴⁶ and is required for interaction
191 with the subunit translocase of mitochondrial inner membrane 23 (Tim23), a mitochondrial
192 protein import complex essential for protein import⁴⁷. Phenotypes are more robust and progress
193 more rapidly for dN17-BACHD than for BACHD mice³³, with nuclear and endoplasmic reticulum
194 (ER) mHTT localization for the former, as well as large nuclear inclusions visible by
195 immunofluorescence, which are not observed in the BACHD model³³. CryoET of neurites in dN17-
196 BACHD neurons also showed severe distortions in most of their mitochondria (**Fig. 3c**). These
197 observations therefore suggest that mHTT can disrupt mitochondria via polyglutamine repeat-
198 dependent effects, such as those previously reported (using other techniques) to disrupt
199 mitochondrial membranes⁴⁸, independent of the N17 domain.



200
201 **Fig. 3. Mitochondria in neurites of HD mouse model neurons exhibit altered morphology and contain**
202 **enlarged granules of varying size.** Slices (~1.4 nm thick) through selected regions of representative cryoET
203 tomograms and corresponding segmentations of local features for **a** WT, **b** BACHD and **c** dN17 BACHD
204 primary neurons reveal that neuronal mitochondria in HD mice have swollen cristae and contain enlarged
205 granules compared to controls (WT). Tomogram numbers: WT=31, BACHD=22 and deltaN17 BACHD=15.

206 Segmentation colors: red:microtubules, yellow:mitochondrial double-membranes, dark blue:granules,
207 and cyan:cristae. Scale bars = 100 nm.

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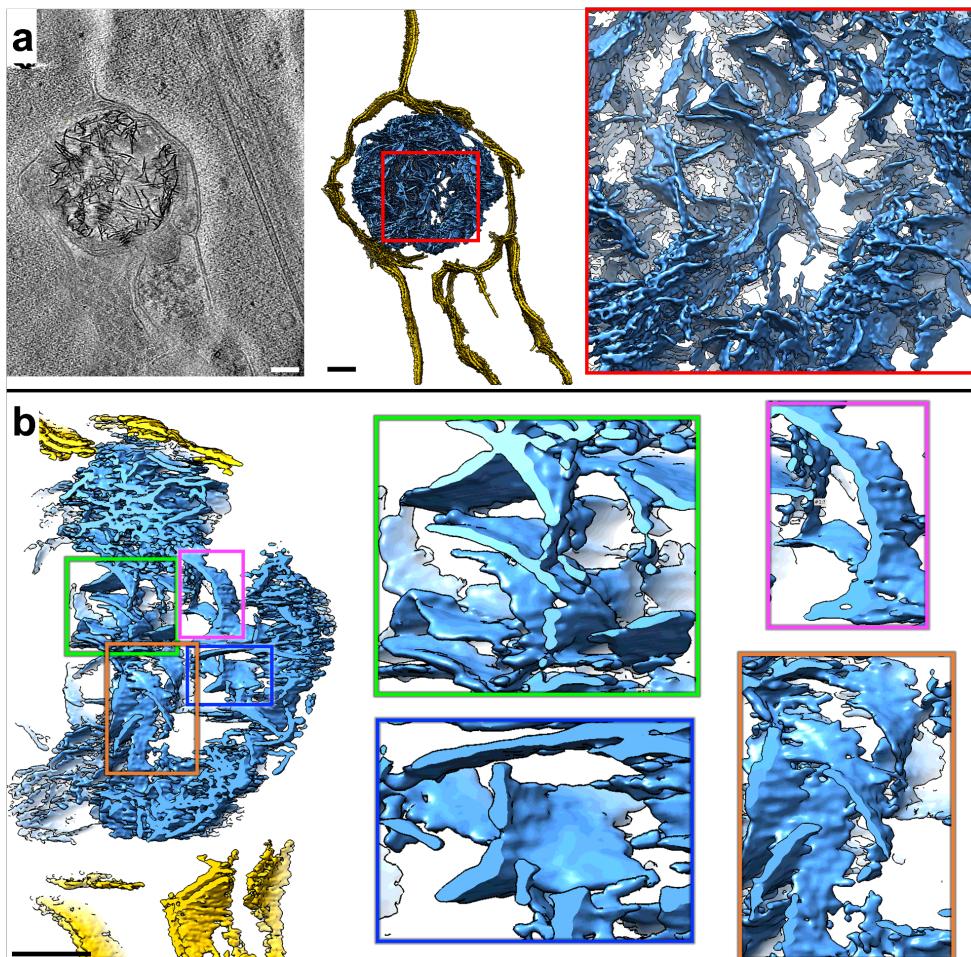
209 **Neurites in HD patient iPSC-derived and mouse model neurons contain sheet-like aggregates**
210 **in autophagic organelles**

211 In addition to the dense, enlarged granules observed in mitochondria, we observed numerous,
212 much larger aggregates in other membrane-bound compartments in both HD patient iPSC-
213 derived and mouse primary neurons. While they appeared to be filamentous when viewed in
214 two-dimensional (2D) z-slices through the tomograms, closer three-dimensional (3D) inspection,
215 including the use of UCSF ChimeraX's virtual reality (VR) graphics⁴⁹, revealed that they are
216 composed of densely interwoven slab-shaped, and sheet-like aggregates (**Fig. 4, Supplementary**
217 **Video 2**). Indeed, these aggregates had a distinct sheet morphology, similar to that of intricate
218 desert rose-like crystal of selenite, gypsum or barite found in the desert (hereafter called sheet
219 aggregates) [[https://en.wikipedia.org/wiki/Desert_rose_\(crystal\)](https://en.wikipedia.org/wiki/Desert_rose_(crystal))].

220 Importantly, these sheet aggregates were present in the neurites of all HD patient Qn neurons
221 (**Fig. 5a**)(Q53, Q66, Q77 and Q109) as well as in those of HD mouse model neurons (**Fig.**
222 **5b**)(BACHD and deltaN17 BACHD). On the other hand, double-membrane bound compartments
223 in neurites of control human iPSC-derived (Q18) (**Fig. 5c**) and mouse (WT) (**Fig. 5d**) neurons lacked
224 these sheet aggregates.

225 Of note, the sheets in these aggregates were reminiscent of some regions in mHTT exon 1 and
226 polyQ-only aggregates *in vitro*, shown to contain long, relatively flat or slightly curved ribbon-
227 like⁵⁰ or sheet-like²⁶ morphologies as visualized by negative stain electron microscopy and
228 cryoET, respectively. Interestingly, their thickness appeared relatively uniform, ~2 nm, when
229 visualized in our 3D tomograms with contrast transfer function correction and without
230 downsampling or low-pass filtration. Importantly, we also collected higher-magnification cryoEM
231 images of neurite regions with sheet aggregates and analyzed their Fourier transforms but did
232 not detect any periodic arrangement in them.

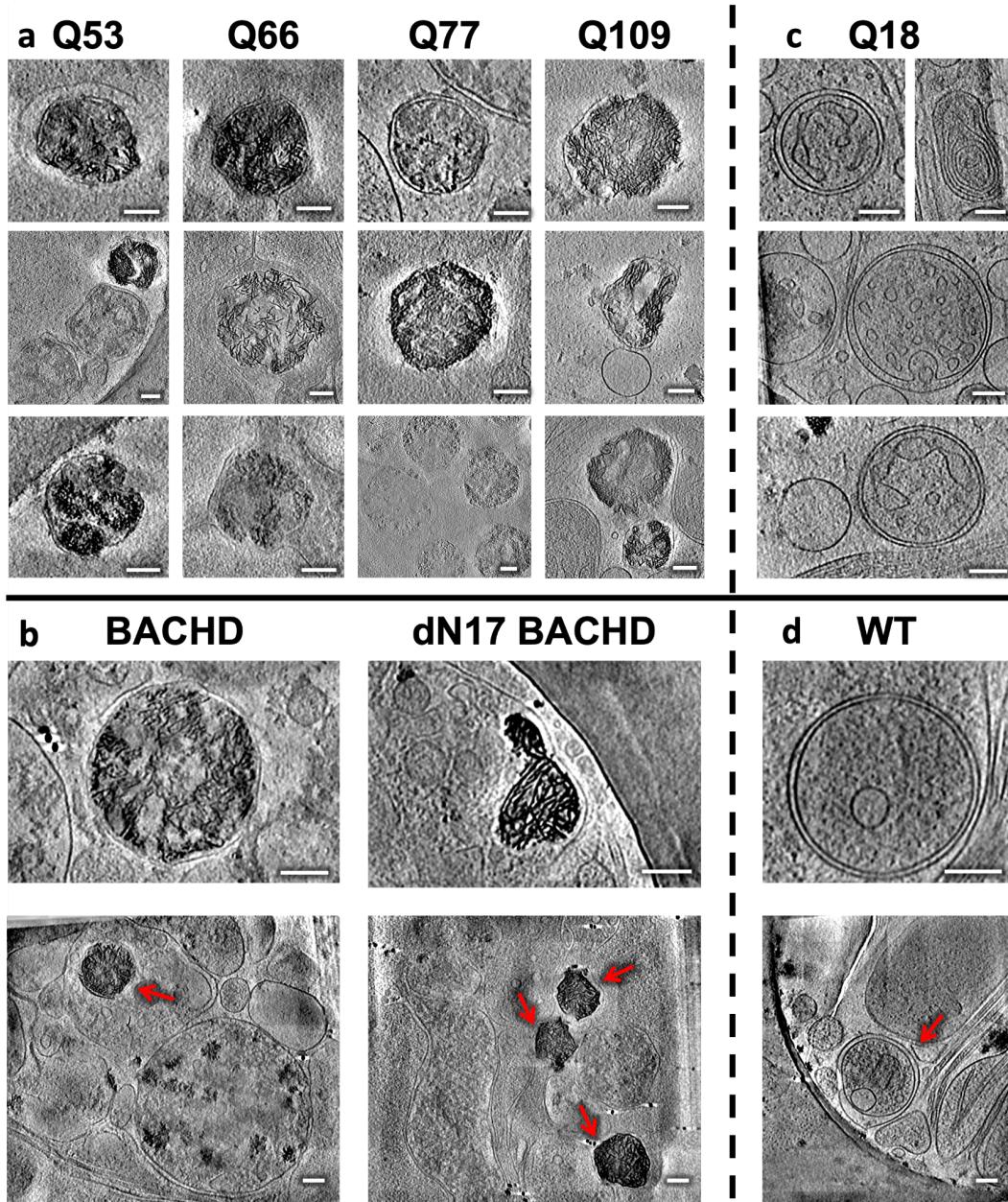
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235 **Fig. 4. Neurites in HD cells contain double membrane-bound compartments with sheet aggregates**
236 **composed of interwoven slabs and sheets.** a Z-slice (~1.4 nm thick) through a selected region showing a
237 sheet aggregate in a representative cryoET tomogram of a neurite of a HD patient iPSC-derived neuron
238 (Q66) and corresponding segmentation of double membranes (yellow) and aggregated densities inside
239 (light blue). b Cutaway, oblique view of an enlarged region from the segmentation in a and further
240 zoomed-in views of selected subregions showing examples of sheet-like areas within the aggregate.
241 Scale bars = 100 nm. Segmentation colors: yellow:double membrane, light blue:sheet aggregate.

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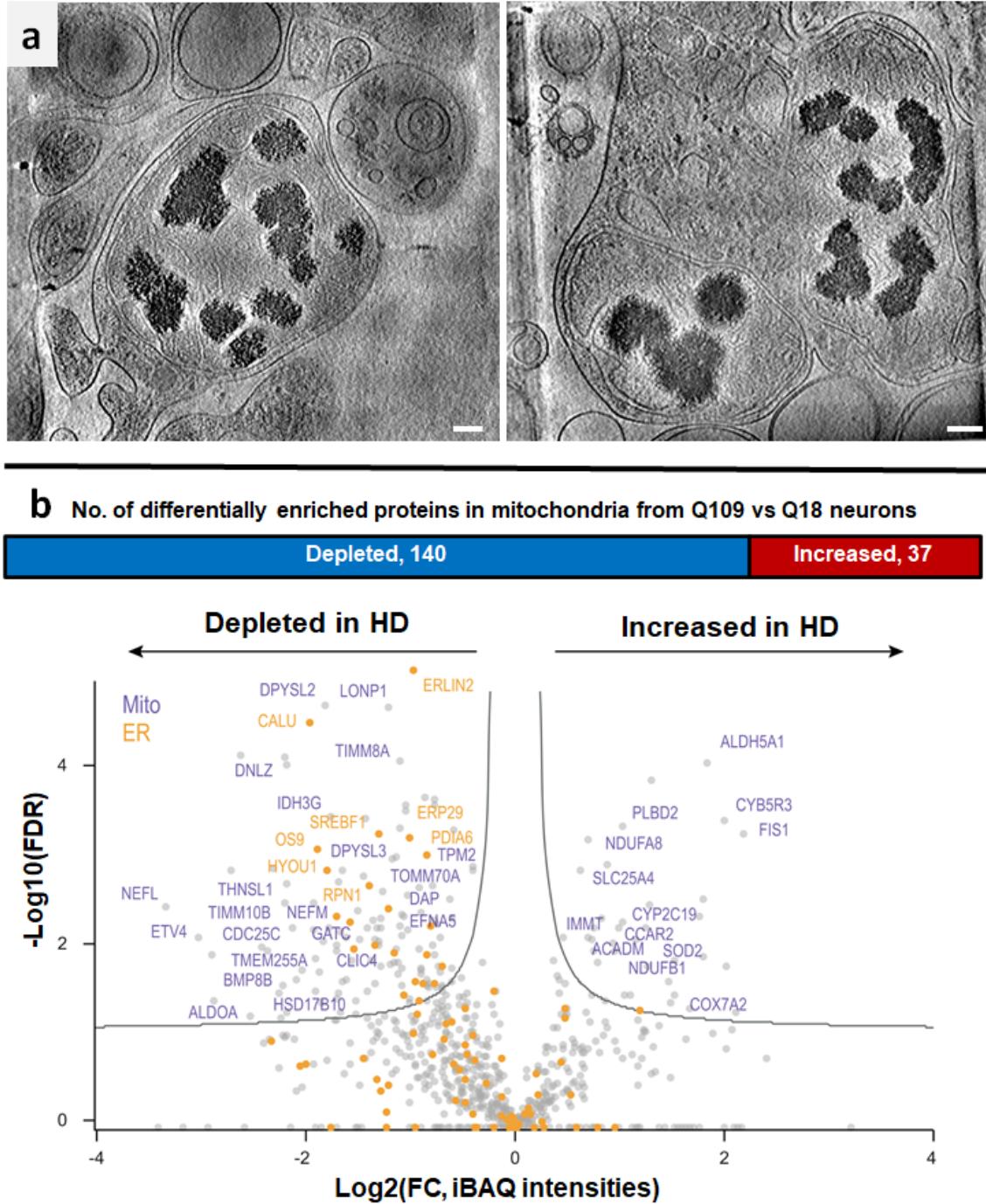
244 **Fig 5. Neurites in HD patient iPSC-derived and mouse model primary neurons exhibit sheet aggregates**
245 **within double membrane-bound organelles.** Slices (~1.4 nm thick) through selected regions of
246 representative cryoET tomograms showing double membrane-bound compartments in neurites of HD **a**
247 patient iPSC-derived (Q53, n=5; Q66, n=8; Q77, n=6; Q109, n=6) and **b** mouse model (BACHD, n=14; and
248 dN17-BACHD, n=6) primary neurons, as well as normal **c** Q18 (n=4) and **d** WT (n=3) controls. Scale bars =
249 100 nm. The red arrows in full-frame views in **b** indicate double membrane-bound organelles.

250

251 Eukaryotic cells contain several characteristic double membrane-bound compartments including
252 mitochondria, nucleus, and organelles in the autophagy pathway such as mitochondria-derived
253 vesicles⁵¹, autophagosomes⁵², and amphisomes⁵². Here, the compartments containing the sheet
254 aggregates (~200 nm to ~500 nm range in longest span) were much smaller than the nucleus (~3-
255 18 µm in diameter), and sometimes seemed to bind or merge with one another (**Supplementary**
256 **Fig. 3a**). While they were most often similar in size to small mitochondria, and could possibly
257 correspond to degenerating versions of this organelle or mitochondria-derived vesicles⁵¹, there
258 is a possibility they may also correspond to other autophagic organelles with no visible cristae or
259 to other molecular components targeted for autophagy⁵², suggesting alternative and possibly
260 complementary or parallel biogenesis origins. For instance, autophagosomes participate in
261 cellular pathways for degradation and clearance, and thus are possible candidates to contain
262 these sheet aggregates. Supporting this interpretation, we found instances of sheet aggregates
263 within double membrane-bound compartments fused with single membrane-bound
264 compartments (**Supplementary Fig. 3b-d**), reminiscent of lysosomes, a picture strikingly similar
265 to amphisomes, which result from fusion of autophagosomes with lysosomes⁵². On the other
266 hand, in support of the degenerating mitochondria assignment, we observed features suggestive
267 of nascent sheet aggregates in what appeared to be degenerating mitochondria (**Supplementary**
268 **Fig. 3e**). To further characterize the nature of these features, we again used semi-automated,
269 neural-networks-based annotation of the corresponding tomogram with EMAN2⁴², training on a
270 few positive references (n=10) from a mature sheet aggregate only. Strikingly, the algorithm
271 assigned the putative nascent sheet aggregate features in what appears to be a mitochondrion
272 as belonging to the same type of feature as mature sheet aggregates, even though the networks
273 were trained exclusively with mature sheet aggregate references (**Supplementary Fig. 3f**). This
274 organelle could represent a degenerated mitochondrion bound with lysosomes, as these two
275 organelles were recently discovered to interact directly via their membranes⁵³. Indeed, this
276 mitochondrion and a similar neighboring organelle are seen interacting with single membrane
277 bound compartments (**Supplementary Fig. 3g**).

278 **Mitochondrial proteomics of human iPSC-derived neurons identified differentially expressed**
279 **proteins, including those engaged in RNA binding**

280 The accumulation of mitochondrial granules (**Fig. 2**), distinctly different from the sheet
281 aggregates (**Fig. 4**), and the disruption of cristae observed in the neurites of HD neurons, are
282 consistent with impaired mitochondrial function and bioenergetics previously described for
283 HD^{34,54}. This phenotype could represent aberrant accumulation of RNA granules⁵⁵, suggesting
284 RNA processing and RNA quality control deficits, and/or aberrant protein quality control due to
285 disrupted protein import arising from the presence of mHTT⁴⁷. To investigate potential
286 mechanisms underlying the abnormal enlargement of mitochondrial granules, we performed
287 liquid chromatography tandem mass spectrometry-based proteomic analysis on mitochondria
288 isolated from HD patient iPSC-derived neurons (Q109) and controls (Q18) since the former
289 represents the most aggressive variant among our HD patient iPSC-derived samples (**Fig. 6**).
290 Mitochondria were isolated by using magnetically labeled anti-TOM22 microbeads⁵⁶. Isolated HD
291 mitochondria looked similar in cryoEM projection images and reconstructed cryoET tomograms
292 (**Fig. 6a**) to those in neurites containing enlarged granules (**Fig. 1e**). In quality control
293 experiments, the mitochondrial isolation method was assessed via Western analysis on each cell
294 fraction; this yielded a fraction with enrichment of mitochondria (using ATPB protein levels as a
295 proxy) with some minor contamination from other organelles (LC3 levels were used as a proxy
296 for autophagosomes, and CTIP2 levels as a proxy for nuclei) (**Supplementary Fig. 4a**). Consistent
297 with successful isolation of mitochondria previously, our proteomic datasets were enriched for
298 Gene Ontology (GO) terms related to mitochondrial functions (**Supplementary Fig. 4b**).
299 Comparing the mitochondrial proteome of Q109 HD patient iPSC-derived neurons vs controls
300 (Q18) revealed a total of 177 differentially enriched peptides (DEPs), with a potential 236
301 identities, the majority of which were depleted in HD (**Fig 6b & Supplementary Table 3**).
302 Differentially enriched proteins included depletion of mitochondrial proteins (purple in **Fig. 6b**)
303 such as TOMM70A, a mitochondrial import receptor involved in the translocation of preproteins
304 into mitochondria, which is impaired in HD⁴⁸. We also detected a depletion of ER proteins in HD
305 mitochondria (orange in **Fig. 6b**), which could reflect impaired mitochondria-ER interactions⁵⁷
306 and well-documented ER dysfunction in HD^{58,59}. Interestingly, FIS1 (mitochondrial fission protein)
307 levels were increased in HD (**Fig. 6b**), consistent with previous data showing altered regulation
308 of mitochondrial fission in HD^{60,57}.



309

310 **Fig. 6. Mass spectrometry of isolated mitochondria revealed differentially enriched proteins (DEPs) in**
311 **neurites of HD patient iPSC-derived neurons (Q109) vs controls with cryoET showing accumulation of**
312 **granules in such HD mitochondria. a. Z-slices (~1.4 nm thick) through representative cryoET tomograms**
313 **of isolated Q109 mitochondria showed abnormal accumulation and enlargement of mitochondrial**
314 **granules. Scale bars = 100 nm. b. Mass spectrometry of proteins in isolated mitochondria showed 177**
315 **differentially enriched peptides with a potential of 236 protein identities. The scatter plot highlights**

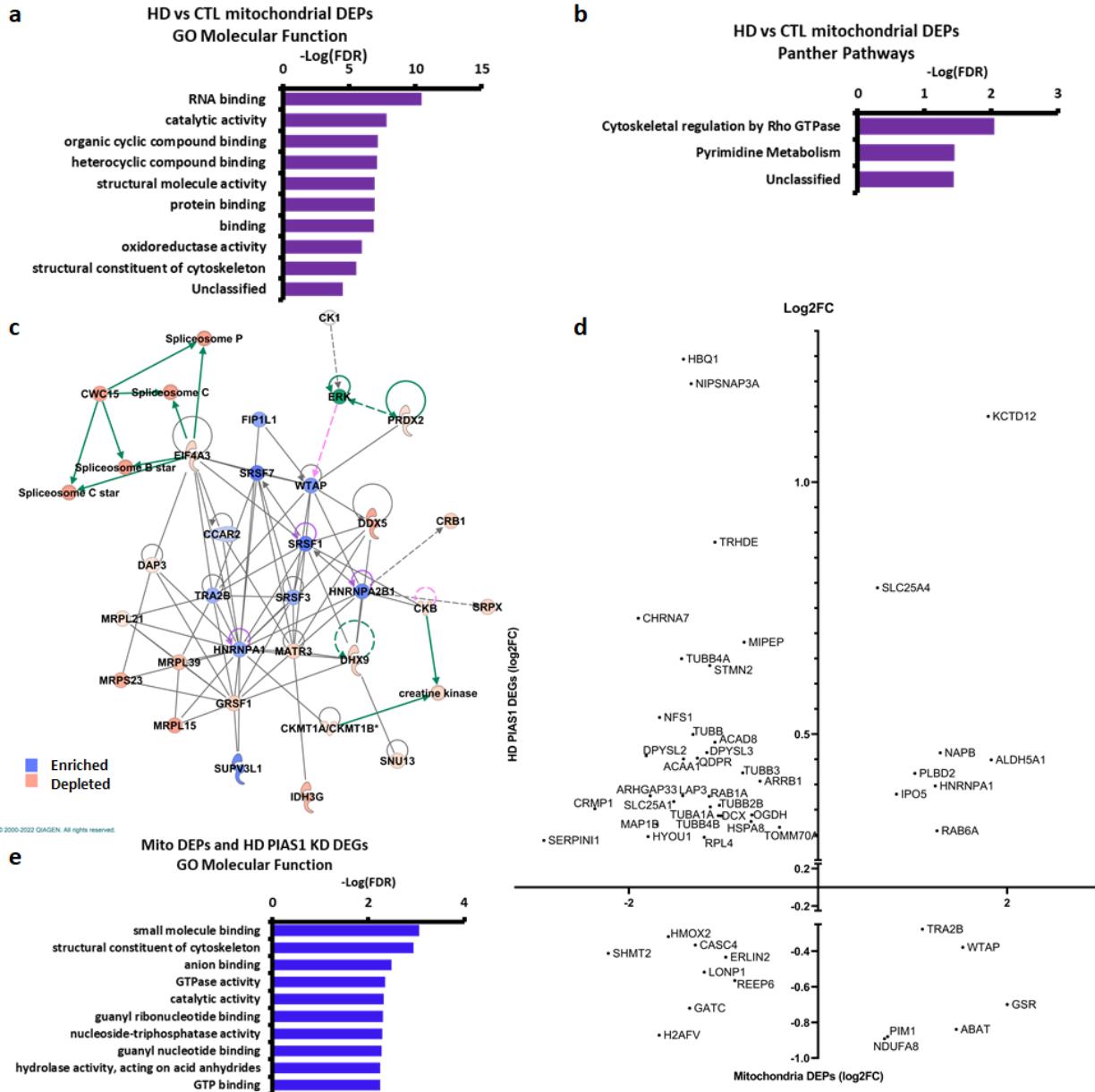
316 selected proteins that were depleted or increased in HD (Q109) mitochondria in comparison to controls
317 (Q18). Mitochondrial proteins are highlighted in purple and ER proteins in orange.

318

319 GO analysis of the differentially enriched peptides found RNA binding to be the most enriched
320 molecular function (**Fig. 7a**), as reflected by increased levels of various cytoplasmic RNA binding
321 proteins such as hnRNPA2B1, hnRNPA1 and hnRNPH1. On the other hand, GRSF1, a
322 mitochondrial RNA binding protein⁶¹, showed reduced levels in HD mitochondria. This protein is
323 essential for mitochondrial function and required for mitochondrial RNA processing⁶². Indeed,
324 loss of GRSF1 causes mitochondrial stress and can induce senescence phenotypes⁶³. Panther
325 pathway analysis identified cytoskeletal regulation by Rho GTPase and pyrimidine metabolism as
326 two other of the three most overrepresented pathways by the DEPs (**Fig. 7b**).

327 Ingenuity Pathway Analysis (IPA) of the mitochondrial DEPs identified a network representing
328 proteins involved in molecular transport, RNA post-transcriptional modification and RNA
329 trafficking, potentially further implicating these proteins in RNA biology and dynamics (**Fig. 7c**).
330 Upstream regulators identified by IPA included Amyloid Precursor Protein (APP) and
331 transforming growth factor β (TGF- β) as predicted inhibitors of the mitochondrial DEPs in HD. IPA
332 pathways included mitochondrial dysfunction, consistent with the mitochondrial deficits in HD⁶⁴
333 (**Supplementary Fig. 5c-f**).

334



335

336 **Fig. 7. Analysis of mass spectrometry data from isolated HD (Q109) vs control mitochondria showed**
337 **RNA binding and an overlap with PIAS1 knockdown differentially expressed genes (DEGs).** a Graph of
338 gene ontology (GO) analysis of the potential 236 identities of the DEPs in HD vs control mitochondria
339 showing molecular functions overrepresented by HD DEPs. b Graph of Panther analysis of the 236 DEPs
340 in HD vs control mitochondria showing Panther Pathways overrepresented by HD mitochondria DEPs. c
341 Ingenuity pathway analysis of the differentially enriched proteins between HD and control mitochondria
342 highlighted this as the top network (score, 55 Focus molecules: 28), and represents Molecular Transport,
343 RNA Post-Transcriptional Modification, and RNA Trafficking. Proteins in orange are depleted in HD while
344 proteins in blue are enriched. d Scatter plot of overlapping DEG log2 fold changes generated from PIAS1
345 knockdown in HD iPSC-derived neurons from previous work⁶⁵ plotted against the log2 fold enrichment of

346 mitochondrial DEPs. **e** GO analysis of molecular functions overrepresented by the 55 overlapping
347 DEPs/DEGs that are in **d**. N=3 per control and HD samples.

348

349 ***PIAS1* heterozygous knockout in HD patient iPSC-derived neurons and short-term *Pias1***
350 **knockdown in BACHD mouse neurons rescues aberrant mitochondrial granules and presence**
351 **of sheet aggregates in neurites**

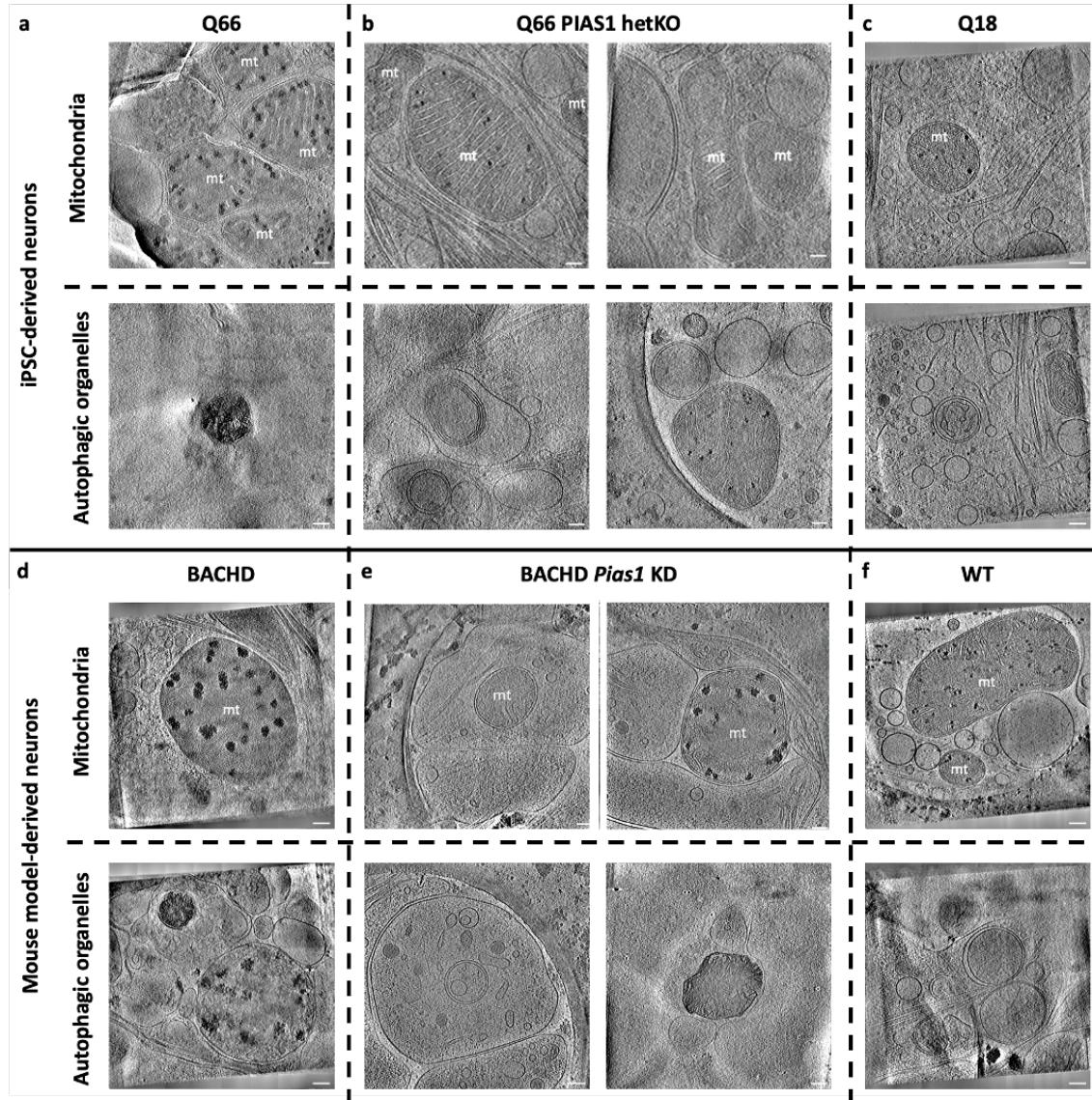
352 Mitochondrial dysfunction can be highly detrimental to neuronal function, particularly in light of
353 the extensive energetic requirements for synaptic function⁶⁶. To evaluate whether the observed
354 phenotypes can be ameliorated, we evaluated genetic reduction of an E3 SUMO ligase, PIAS1,
355 based on our previous data showing that reduced *Pias1* expression resulted in: increased
356 expression of the presynaptic protein synaptophysin in R6/2 HD mice, rescued transcriptional
357 synaptic deficits in zQ175 HD mice, and improved mitochondrial DNA integrity and synaptic gene
358 expression in iPSC-derived neurons^{65,36}. PIAS1 enhances SUMOylation of various proteins,
359 including HTT^{45,67}. To computationally determine whether targeting PIAS1 would predict changes
360 in the HD mitochondrial proteome, we first compared the changes in the mitochondrial
361 proteome of HD iPSC-derived neurons (Q109 vs Q18) described above with findings from our
362 prior study of gene expression in the same type of differentiated neurons following PIAS1
363 knockdown⁶⁵ (**Fig. 7d**). We found a significant overlap between mitochondrial DEPs and RNA
364 changes induced by siRNA depletion of PIAS1 in HD iPSC-derived neurons (representation
365 factor:1.9 p<2.081e-06, **Fig. 7d, Supplementary Fig. 5a, Supplementary Table 3**). This overlap of
366 genes and proteins included overrepresentation of GO terms including “GTP binding” and
367 “GTPase activity” as well as several RNA processing-related proteins and tubulins⁶⁵ (**Fig. 7e**).
368 These comparisons further supported investigating whether knockdown of PIAS1 could influence
369 the presence and/or size of the aberrant granules we observed within HD mitochondria.

370 We again used cryoET to visualize iPSC-derived neurons (Q66, representing an intermediate
371 range of phenotypes) with *PIAS1* heterozygous knockout (hetKO), using a CRISPR-Cas9-generated
372 heterozygous KO, which produces approximately 50% knockdown (**Supplementary Fig. 5e,f**). The
373 *PIAS1* KD iPSC neurons (Q66) differentiated well on EM gold grids in preparation for cryoET

374 experiments (**Supplementary Fig. 5g**). The Q66 *PIAS1* hetKO tomograms (**Fig. 8**) showed
375 seemingly healthy mitochondria and other double membrane-bound organelles (**Fig. 8b**), lacking
376 the abnormally enlarged granules and sheet aggregates, respectively, as had been observed in
377 HD patient iPSC-derived neurons for all Qns (**Fig. 1, 5 & 8a**). Indeed, the structural features of
378 Q66 *PIAS1* hetKO neurites resembled those from control Q18 neurons (**Fig. 8c**) rather than Q66
379 ones.

380 To determine if the rescue of abnormal morphologies observed in HD patient iPSC-derived
381 neurons (Q66) treated with *PIAS1* KD translates to mouse cortical neurons, we carried out a
382 short-term *Pias1* KD in mouse primary neuronal cultures derived from E18 BACHD cortical
383 neurons on EM grids and visualized cells with cryoET (**Fig. 8**). To reduce *Pias1* levels, Accell
384 (Dharmacon) siRNA smart pools against mouse *Pias1* were used. *Pias1* KD was initiated at day *in*
385 *vitro* 3 (DIV3) with one treatment and grown for 11 days. Cells were vitrified for cryoET analysis
386 at DIV14. Knockdown of *Pias1* in the BACHD neurons was successful according to qRT-PCR
387 analyses; 43% knockdown was achieved comparing control-siRNA-treated neurons to *Pias1*
388 knockdown (**Supplementary Fig. 5i,j**).

389 CryoET experiments showed that treating BACHD neurons with *PIAS1* siRNA resulted in partial
390 rescue. Indeed, while many mitochondria completely lacked detectable granules, visibly reduced
391 in comparison to the BACHD mitochondria, sheet aggregates in autophagic organelles were
392 present in comparable numbers to those in BACHD neurons without *Pias1* KD (**Fig. 8e**, bottom
393 right). Thus, the beneficial effects of *PIAS1* hetKO in HD patient iPSC-derived neurons was only
394 partially replicated in the mouse model under our experimental conditions, possibly due to the
395 later and shorter treatment time frame. Whether the impact of PIAS1 reduction is consistent
396 across all cells or results in different effects in different cell types needs further investigation.



397

398

Fig. 8. Reduced PIAS1 rescues mitochondrial granule size and sheet aggregates in neurites of HD model neurons. Slices (~1.4 nm thick) through representative cryoET tomograms of neurites in **a** Q66, **b** *PIAS1* hetKO, and **c** Q18 iPSC-derived neurons show that *PIAS1* hetKO ameliorated the phenotypes of enlarged mitochondrial granules (first row) and sheet aggregates in putative autophagic organelles (second row) in HD iPSC-derived neurons (Q66). Slices (~1.4 nm thick) through representative cryoET tomograms of neurites in **d** BACHD, **e** BACHD *Pias1* KD, and **f** WT mouse neurons show that *Pias1* KD ameliorated the enlarged mitochondrial granules (third row) but not the sheet aggregates in putative autophagic organelles (fourth row) in BACHD-derived neurons.

406

407 **Artificial intelligence-based semi-automated 3D segmentation enabled quantification of**
408 **abnormally enlarged mitochondrial granules in neurites from HD patient iPSC-derived neurons**
409 **and mouse model primary neurons**

410 We observed both enlarged granules and disrupted cristae in the mitochondria of HD neuronal
411 processes (**Fig. 1-3**). The mitochondrial granules varied in size, seeming much larger in HD
412 mitochondria than in controls. To quantify their size distribution, we developed a semi-
413 supervised artificial intelligence method to automatically detect and segment mitochondria and
414 mitochondrial granules in the neurite tomograms (**Supplementary Fig. 6**). Among all tomograms
415 (**Supplementary Table 1**), the algorithm found that 139 contained mitochondria for the various
416 HD patient iPSC-derived neurons and 83 for mouse model neurons (**Supplementary Table 1**).

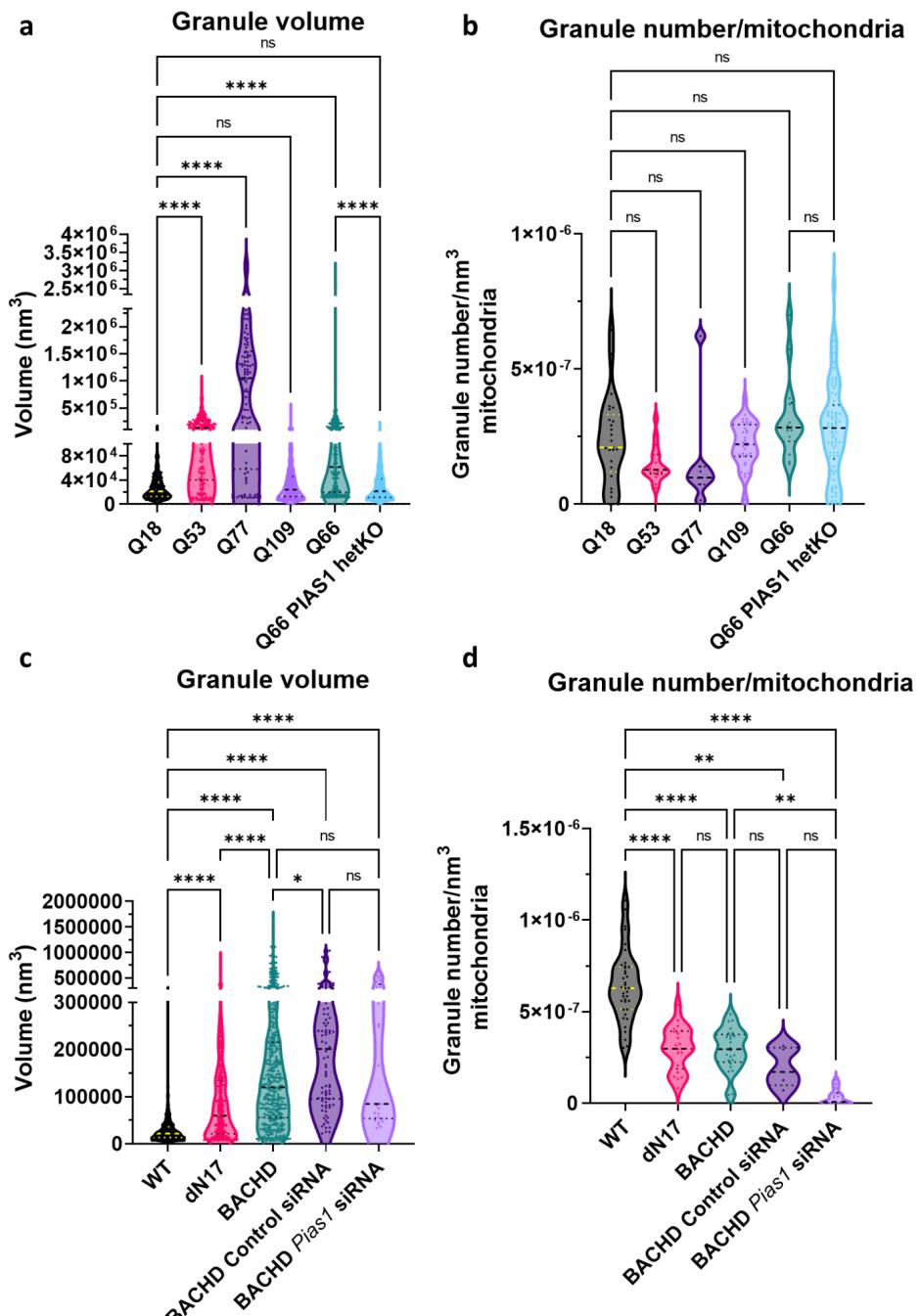
417 Using quantification of the granule volumes by segmentation estimation, our algorithm revealed
418 that the distribution of mitochondrial granule volumes was shifted towards larger sizes with
419 respect to controls for Q53, Q66, and Q77 (**Figure 9a**), as well as for BACHD, dN17-BACHD, BACHD
420 *Pias1* siRNA, and BACHD transfected with a control siRNA (**Figure 9c**). On the other hand, granule
421 volumes were not larger in Q109 compared to Q18 controls, and were significantly decreased in
422 the Q66 PIAS1 hetKO line when compared to Q66 (Dunn's multiple comparison Q66 vs. Q66 PIAS1
423 hetKO $p_{adj}<0.0001$), resembling results for the control Q18 line.

424 Surprisingly, granule volumes were not statistically reduced in BACHD treated with *Pias1* siRNA
425 in comparison to BACHD (Dunn's multiple comparisons BACHD vs. BACHD *Pias1* siRNA
426 $p_{adj}>0.9999$). On the other hand, control siRNA-treated BACHD neurons showed no difference
427 in granule size distribution compared to *Pias1* siRNA treatment, as expected, given that the latter
428 had no effect (Dunn's multiple comparisons BACHD Control siRNA vs. BACHD *Pias1* siRNA
429 $p_{adj}>0.9999$). Lastly, there was an unexpected modest increase in granule volumes in BACHD
430 upon control siRNA treatment.

431 While granule volumes were increased for all Qn iPSC-derived neurons except Q109, granule
432 numbers per nm^3 of mitochondrial volume were not different in any of them from those in

433 control Q18 cells. On the other hand, granule numbers per nm³ of mitochondrial volume were
434 decreased in all mouse cell lines with respect to WT, including in those treated with siRNAs.

435



436

437 **Fig. 9: Artificial Intelligence (AI)-based 3D quantification of mitochondrial granule volume and granule**
438 **number per nm³ of mitochondrial volume in cryoET tomograms of mitochondria in neurites**

439 **demonstrates a significant increase in granule size with higher polyQ in HD human and mouse model**
440 **neurons and this is rescued in the Q66 PIAS1 hetKO neurons.** Violin plots displaying AI measurements of
441 mitochondrial granule **a** volume (Kruskal Wallis statistic = 401.3, P value<0.0001) and **b** numbers per nm³
442 of mitochondrial volume (Kruskal Wallis statistic = 19.78 P= 0.0014) from cryoET tomograms of neurites
443 for five HD patient iPSC-derived neuronal cell lines (tomogram numbers: Q18=21, Q53=14, Q66=10,
444 Q77=5, Q109=37 and *PIAS1* hetKO=68; granule number:Q18=250, Q53=176, Q66=250, Q77=94,
445 Q109=539 and *PIAS1* hetKO=923) as well as mitochondrial granule **c** volume (Kruskal Wallis statistic =
446 750.8, P<0.0001) and **d** numbers per nm³ of mitochondrial volume (Kruskal Wallis statistic = 77.48,
447 P<0.0001) from cryoET tomograms of neurites for three mouse neuron models (tomogram numbers:
448 WT=31, BACHD=22, dN17 BACHD=15, BACHD control siRNA=5 and BACHD *Pias1* siRNA=12; granule
449 numbers: WT=1338, BACHD=380, dN17-BACHD=326, BACHD control siRNA=91 and BACHD *Pias1*
450 siRNA=27). The human neurons and mouse model neurons showed an increase in granule volumes in all
451 but Q109, a trend of reduced granule number in human neurons and a significant reduction in granule
452 number in mouse model neurons. ns=not significant, *** p<0.0001, ** p<0.01, * p<0.05 For full statistical
453 details refer to **Supplementary Table 2**.

454

455 **DISCUSSION**

456 In this study, we have defined Q-length dependent ultrastructural changes that occur in neuronal
457 processes (neurites) of human HD iPSC-derived neurons and BACHD primary cortical neurons.
458 Specifically, we found that HD neurons contain two types of double membrane-bound organelles
459 with abnormal densities inside, which are completely absent in healthy control neurons. First, we
460 observed ultrastructural changes in neuronal mitochondria, most notably enlarged granules in
461 all HD samples compared to controls (**Fig. 1-3**). Importantly, many HD samples also exhibited
462 severely disrupted cristae, similar to cryoET observations in other neurodegenerative disorders
463 such as Leigh syndrome⁶⁸. Second, we observed sheet aggregates within autophagic organelles
464 resembling mitochondria-derived vesicles, autophagosomes and/or amphisomes (**Fig. 4, 5,**
465 **Supplementary Fig. 3**). These findings are highly significant in demonstrating the disruption of
466 organellar structure in HD, possibly as very early events in pathogenesis that precede overt
467 neuronal dysfunction and the appearance of inclusions visible in neurons derived from HD
468 patient²¹ and mouse model^{20,69} brain tissues .

469 Cellular cryoET experiments ultimately yield 3D volumes (“tomograms”) that sample regions of
470 the crowded subcellular environment in intact cells. Generally, an experienced investigator

471 would use visualization graphics to inspect one tomogram at a time, a laborious discovery process
472 requiring expert knowledge. In the initial stages of this project, we visualized hundreds of neurite
473 tomograms, leading to the discovery of enlarged granules in mitochondria and sheet aggregates
474 in autophagic organelles within them. Using a newly developed semi-supervised artificial
475 intelligence-based method to segment and quantify the number and volume of mitochondrial
476 granules (**Supplementary Fig. 6**), we found that their enlargement is a structural signature of HD,
477 consistently present in both human iPSC- and mouse model-derived neurons (**Fig. 9**).

478 The aberrant accumulation of large mitochondrial granules and abnormal cristae are known
479 hallmarks of mitochondrial dysfunction as assayed by other methods in similar systems^{34,54}. As
480 members of the HD iPSC Consortium¹⁰, we previously showed using cell biology techniques that
481 striatal-like HD iPSC-neurons similar to those examined here have mitochondrial deficits
482 including altered mitochondrial oxidative phosphorylation. Indeed, we demonstrated impaired
483 oxygen consumption rate (OCR), altered spare glycolysis capacity (ECAR) and reduced ATP levels
484 in HD neurons¹³. Additional studies have also shown mitochondrial dysfunction, fragmentation
485 and disrupted cristae by traditional electron microscopy of chemically fixed cell lines expressing
486 mHTT⁷⁰.

487 The high scattering contrast of the enlarged granules we observed here in both human and
488 mouse HD model neurons could be attributable to RNA and/or calcium phosphate, which are
489 more electron dense and thus scatter the electron beam more strongly than most other
490 molecular components in mitochondria comprised of lighter elements such as carbon, nitrogen
491 and oxygen. Our mass spectroscopy data (**Figs. 6, 7 & Supplementary Fig. 4**) are consistent with
492 either interpretation. Indeed, assessing the proteome of mitochondria isolated from human HD
493 iPSC-derived neurons identified differentially enriched proteins related to protein import and
494 RNA binding (**Fig. 7**). RNA granules are normal features of the mitochondrial matrix⁶¹, and are
495 composed of newly synthesized RNA, RNA processing proteins and mitoribosome assembly
496 factors^{61,71–73}. Stressors such as dysregulation of RNA processing and RNA quality control defects
497 can cause aberrant accumulation of mitochondrial RNA granules, which comprise large
498 ribonucleoprotein complexes⁵⁵. RNA granules are components of mitochondrial post-

499 transcriptional pathways and are responsible for mitochondrial RNA translation⁷⁴. When these
500 granules aberrantly accumulate, the integrity of cristae is compromised to accommodate them⁷¹.
501 GRSF1, a nuclear encoded RNA-binding protein that regulates RNA processing in mitochondrial
502 RNA granules⁶² and is critical for maintaining mitochondrial function^{63,75}, was significantly
503 decreased in our mitochondrial proteomic dataset in HD. GRSF1 has also been implicated in
504 cellular senescence with levels declining in senescent cells and lowered GRSF1 levels causing
505 mitochondrial stress⁶³. Further evidence that these structures may represent mitochondrial RNA
506 granules is the enrichment of RNA binding proteins in the proteome of HD iPSC-derived neurons
507 versus controls.

508 Alternatively, mitochondria are known to normally contain calcium phosphate granules, which
509 store excess calcium, maintain mitochondrial calcium concentration, and contribute to
510 maintenance of mitochondrial function^{76,77}. Furthermore, calcium overload within cells is known
511 to cause ultrastructural remodeling of cristae^{63,78}, as in our experimental results described above,
512 which was particularly profound for Q109 neurons and dN17-BACHD neurons. Cellular calcium
513 dyshomeostasis is a well-established phenotype in HD^{79,80}, wherein an increase in store-operated
514 calcium entry (SOCE) can lead to increased calcium uptake by the mitochondria due to their
515 proximity to the ER⁸⁰, ultimately resulting in increased mitochondrial granule size due to
516 increased calcium uptake. Furthermore, mitochondrial calcium dysregulation was observed in
517 mitochondria isolated from transgenic YAC128 HD mice⁸¹. HD mitochondria are more susceptible
518 to calcium stress and form megapores more readily than control mitochondria⁸². Sequestration
519 of calcium into the mitochondria can protect neurons from glutamate excitotoxicity, a phenotype
520 observed in HD neurons^{83,84}. Thus, the enlarged mitochondrial granules we observed here across
521 multiple human and mouse HD model neurons may be composed at least in part of excess
522 calcium phosphate.

523 The two most likely possibilities for the chemical identity of enlarged mitochondrial granules (*i.e.*,
524 RNA granules versus calcium phosphate granules) need not be mutually exclusive. While future
525 studies such as elemental analysis with electron and/or x-ray microscopy⁸⁵ could help define the
526 chemical nature of these granules, our data provide clues as to their genesis and development.

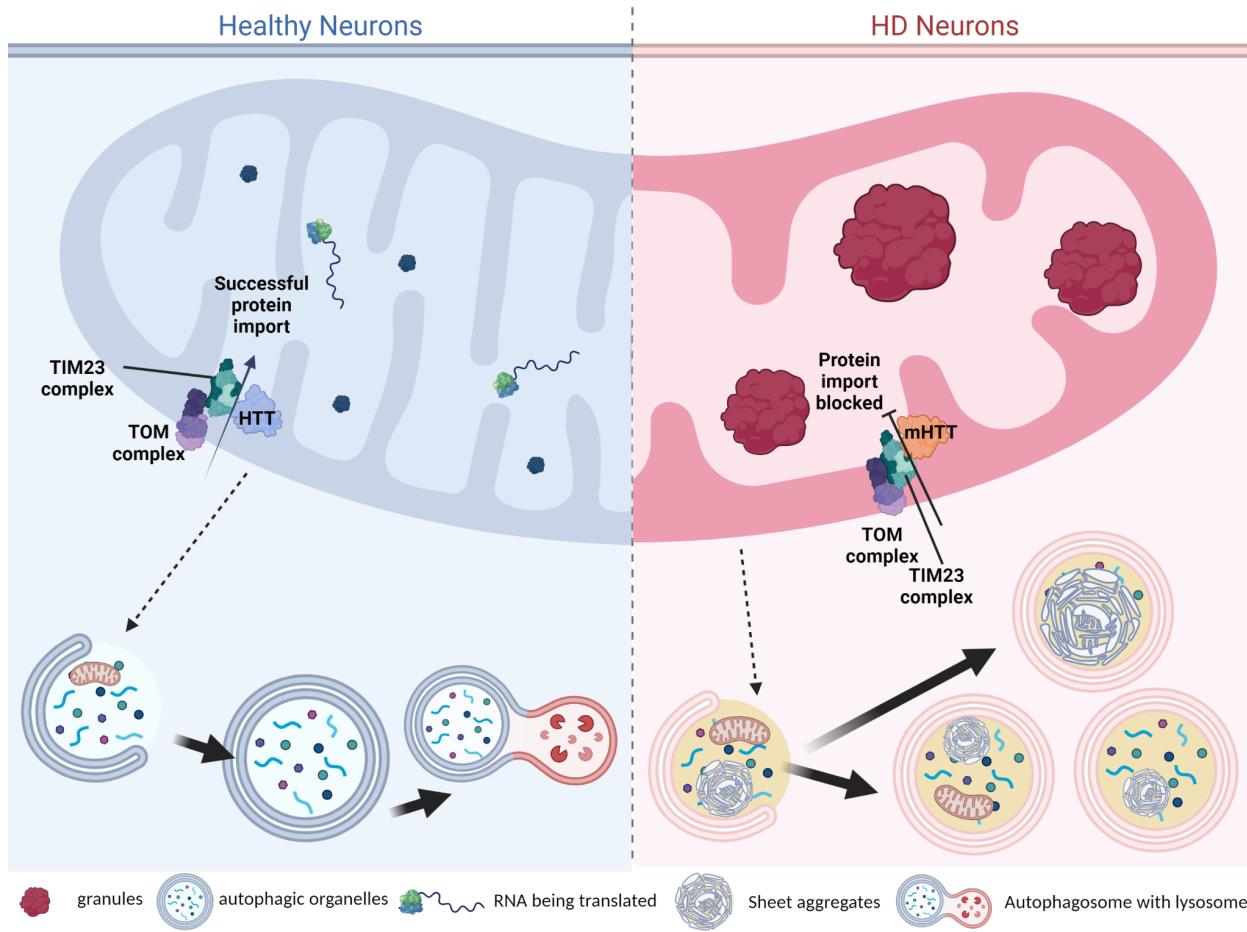
527 In iPSCs, granule volume is significantly increased in all cell lines examined herein, except Q109
528 (**Fig. 9a**), whereas the number of granules per nm³ of mitochondrial volume in all iPSC cell lines
529 is not significantly different from that in controls (**Fig. 9b**). This suggests that as new granules or
530 the materials that form them become available, they coalesce with each other and/or with
531 existing ones. On the contrary, for mitochondria in BACHD mice, the larger size of the granules
532 (**Fig. 9c**) is accompanied by a significant reduction in their number (**Fig. 9d**) with respect to
533 controls, suggesting that smaller granules may grow primarily by aggregation with other existing
534 granules rather than by addition of new materials. Further experiments are needed to elucidate
535 the chemical compositions of these granules, which will in turn allow probing for the production
536 of their component materials during neurodegeneration.

537 Interestingly, granule volumes are larger in iPSC Q66 and Q77 than in our HD mouse models,
538 despite BACHD containing mHTT with a longer polyQ tract (Q97). This may be attributable to
539 differences in the biology of the model systems. Another possible explanation for the statistical
540 discrepancy between cell lines and model systems is that Q53 through Q77 might predictably be
541 in a different (earlier) stage of neurodegeneration than Q109, the latter perhaps being in a state
542 more comparable to BACHD neurons. Importantly, recapitulation in multiple human iPSC lines of
543 the same structural phenotypes seen in mouse primary neurons helps to validate iPSCs as useful
544 HD models.

545 We also observed differences in the mitochondrial phenotype between the mouse models. While
546 both BACHD and dN17-BACHD mice showed enlarged mitochondrial granules with respect to
547 controls (albeit fewer in number), dN17-BACHD displayed smaller granules than BACHD and
548 severely distorted structures, such as seemingly enlarged mitochondria and swollen cristae. The
549 difference in granule size between BACHD neurons and those treated with control siRNA may be
550 attributable to off-target effects of the control siRNA, timing of treatment or Accell delivery in
551 general. Nonetheless, taken together, our data provide additional hints regarding potential
552 mechanistic underpinnings of normal HTT function. In HD, mitochondrial function appears to be
553 impacted by the altered import of mitochondrial precursor proteins in the presence of mutant
554 HTT⁴⁷. Indeed, the high affinity interaction of mHTT with the inner mitochondrial protein import

555 complex subunit TIM23 disrupts the HD mitochondrial proteome⁴⁸. Furthermore, protein
556 aggregation and alterations in RNA processing and quality control can disrupt protein import⁸⁶.
557 More recently, defects in mitochondrial protein import have also been connected to impaired
558 proteostasis⁸⁷. Mutant HTT can also disrupt mitochondrial trafficking and impair ATP production
559 prior to the appearance of detectable mHTT aggregates^{88,89}. The N17 domain is required for HTT
560 subcellular localization to mitochondria^{43,88,89} and the interaction of HTT with the protein import
561 complex TIM23 requires both the N17 and polyQ regions of HTT⁴⁷. Intriguingly, mHTT localizes
562 within the intermembrane space in mitochondria and is more strongly associated with TIM23
563 than WT HTT, potentially blocking import of nuclear-encoded mitochondrial proteins⁴⁸. This
564 suggests a potential mechanism (**Fig. 10**) underlying the aberrant structures we observe in HD
565 mitochondria (including enlarged granules), particularly for dN17-BACHD, where normal HTT
566 function is impaired by both the lack of the N17 domain and the expansion of the polyQ
567 tract^{34,47,54}.

568 The sheet aggregates we observed here within autophagic organelles appear to be another early
569 structural hallmark of HD since they were consistently present in the neurites of all HD model
570 systems we examined. In addition to cryoET tomograms, we also collected high-magnification
571 cryoEM images of sample areas with sheet aggregates; the lack of evidence for periodic
572 arrangement in them by Fourier analysis suggests that they are not classical amyloid filaments
573 with the known characteristic cross-beta sheet structures perpendicular to the filament axis⁹⁰;
574 however, this may not be surprising since it is possible that the sheet aggregates we observed
575 may not contain fibrillar mHTT, and HD does not strictly fit within the diseases known as
576 amyloidoses⁹¹. It is also possible that future purification of the sheet aggregates may render them
577 more amenable to increasingly clarifying high-resolution analyses without interference from the
578 cell membrane and surrounding milieu of the crowded cytosol. Whether the sheet aggregates
579 contain mHTT also awaits future biochemical analyses of purified autophagic organelles and/or
580 degenerated mitochondrial particles. Nevertheless, our data here suggest that these unusual
581 structural features, likely in components of autophagic pathways, are a consequence of the stress
582 and toxicity induced by the presence of full-length, endogenous mHTT or fragments derived from
583 it.



600 **Fig. 3g)**, it would be expected that instances of mitochondria that simultaneously exhibit some
601 recognizable cristate, cristae junctions, and granules, at the same time as some clear sheet
602 aggregate densities, might constitute extremely rare events that are hard to capture along a
603 dynamic degeneration process. That is, the sheet aggregates may develop from remodeled and
604 compromised mitochondrial membranes and/or granules through a previously unresolved
605 mechanism in HD cells endogenously expressing untagged mHTT, and may go through
606 intermediate states where mitochondrial features are extremely challenging to recognize or no
607 longer present due to degeneration, before the overt appearance of sheet aggregate densities
608 within them. Indeed, we observed many double membrane-bound compartments in most of our
609 specimens whose identities were not clearly assignable but that may actually correspond to
610 mitochondria in these hypothesized intermediate states, such as the organelle in the bottom left
611 of **Supplementary Fig. 3e**, and those in **Supplementary Fig. 3a-d**. In support of this hypothesis,
612 mitochondria have been recently demonstrated to interact directly with lysosomes via their
613 membranes⁵³. Indeed, the traditional paradigm for largely separate functions by these two
614 organelles has changed with emerging evidence that mitochondria and lysosomes are mutually
615 functional and interdependent, with profound implications to the underpinnings of aging⁹² and
616 neurodegeneration^{93,94}.

617 In spite of the challenges, this exciting observation provides an additional hint into resolving the
618 potential origin of the sheet aggregates. Mitochondrial autophagy (mitophagy) is a clearance
619 mechanism of defective mitochondria via autophagy³⁵ and is altered in many neurodegenerative
620 disorders such as Parkinson disease (PD) and Alzheimer disease (AD)⁹⁵. There is now emerging
621 evidence that mitophagy is also defective in HD. Altered mitophagy may also potentially
622 contribute to the bioenergetic deficits observed in various HD models^{13,96} and at least in part to
623 the excessive weight loss that is characteristic of late-stage human HD^{97,98}. Consistent with our
624 findings, recent work³¹ has identified non-fibrillar mHTT within single-membrane-bound
625 organelles in cortical and striatal tissue from zQ175 HD mice, including multivesicular bodies
626 (MVB) and amphisomes, using correlative light and conventional electron microscopy of samples
627 fixed by freeze substitution. The localization of non-fibrillar mHTT changed depending on the
628 disease stage, with presymptomatic stages showing localization within MVBs/amphisomes and

629 late stage disease showing localization to the autolysosomes or residual bodies³¹. Our findings
630 here in intact, cryo-preserved human patient and mouse model-derived neurons are suggestive
631 of even earlier events and thus are likely complementary.

632 Our previous studies showed improved synaptic-associated gene expression and mitochondrial
633 DNA integrity in HD iPSC-neurons after PIAS1 KD^{36,65}. Based on these data, we evaluated the
634 effect of reduced PIAS1 levels on the mitochondrial and autophagosome structural phenotypes
635 in HD patient iPSC- and mouse model-derived neurons and observed rescue of these phenotypes
636 in the former and partial rescue in the latter (Fig. 8 & 9). Interestingly, our findings on PIAS1 KD
637 also inform on the possible biochemical composition of mitochondrial granules. For example, we
638 previously identified MCUR1, CALM1, CALB1 and CABP1 as differentially expressed in iPSC-
639 derived neurons upon *PIAS1* knockdown⁶⁵, consistent with changes to calcium phosphate
640 granules. We also observed alterations in the levels of RNA binding and processing genes,
641 consistent with changes to mitochondrial RNA granules. Several mitochondrial proteins are
642 SUMO modified (identified in yeast) and require Siz1 or Siz2 for modification (*i.e.*, PIAS1/2)⁹⁹. For
643 instance, Fis1, involved in mitophagy, is SUMO modified and reduction of SUMO modification of
644 Fis1 restores appropriate mitophagy in Hela cells¹⁰⁰. Finally, a recent report identified PIAS1 as a
645 potential age-of-onset modifier. PIAS1 containing a S510G single nucleotide polymorphism,
646 which reduces SUMO modification of mHTT¹⁰¹, delays HD onset and produces milder disease
647 severity in HD mice, consistent with the concept that reduced PIAS1 levels may ameliorate
648 disease. Our studies thus represent a proof of concept that the synergistic combination of cryoET
649 and proteomics of iPSC- and mouse model-derived neurons or organelles within them can inform
650 on the impact of a given therapeutic strategy on structural features and ultimately function, and
651 may be applicable to other cellular systems and disease models.

652

653 **METHODS**

654 **Ethics Statement and mouse models**

655 All protocols involving the use of animals in the study, namely BACHD³² and deltaN17 BACHD³³,
656 were approved by the Institutional Animal Care and Use Committee at the University of California
657 in San Diego.

658

659 **iPSC culture, differentiation, and maintenance**

660 Neuronal differentiation was performed once iPSC colonies reached 60-70% confluence as
661 previously described⁴. Differentiation was initiated by washing iPSC colonies with phosphate
662 buffered saline pH 7.4 (PBS – Gibco) and switching to SLI medium (Advanced DMEM/F12 (1:1)
663 supplemented with 2 mM GlutamaxTM (Gibco), 2% B27 without vitamin A (Life technologies), 10
664 µM SB431542, 1 µM LDN 193189 (both Stem Cell Technologies), 1.5 µM IWR1 (Tocris)) with daily
665 medium changes. This was day 0 of differentiation; at day 4, cells were pretreated with 10 µM
666 Y27632 dihydrochloride (Tocris) and then washed with PBS and then passaged 1:2 with StemPro
667 Accutase (Invitrogen) for 5 minutes at 37 °C and replated onto plates which were coated with
668 hESC qualified matrigel (1 hour at 37 °C) in SLI medium containing 10 µM Y27632 dihydrochloride
669 for 1 day after plating and continued daily feeding with SLI medium until day 8. At day 8, cells
670 were passaged 1:2 as above and replated in LIA medium (Advanced DMEM/F12 (1:1)
671 supplemented with 2 mM GlutamaxTM, 2% B27 without vitamin A, 0.2 µM LDN 193189, 1.5 µM
672 IWR1, 20 ng/ml Activin A (Peprotech)) with 10 µM Y27632 dihydrochloride for 1 day after plating
673 and daily feeding was continued through day 16. At day 16, cells were plated on either the carbon
674 side of Quantifoil holey carbon film grids (Electron Microscopy Sciences; see below for grid
675 preparation for cryoET), or in 6 well Nunclon coated plates with PDL and hESC matrigel for
676 mitochondrial isolation. Cells were plated at 1x10⁶ for mitochondrial isolation and at half density
677 for cryoET of neurites in intact neurons at 500,000 per dish on the 35 mm Mat-tek glass bottomed
678 dish containing 3 holey-carbon grids in SCM1 medium (Advanced DMEM/F12 (1:1) supplemented
679 with 2 mM GlutamaxTM, 2% B27 (Invitrogen), 10 µM DAPT, 10 µM Forskolin, 300 µM GABA, 3 µM
680 CHIR99021, 2 µM PD 0332991 (all Tocris), to 1.8 mM CaCl₂, 200 µM ascorbic acid (Sigma-Aldrich),
681 10 ng/ml BDNF (Peprotech)). Cells on EM grids were topped up with an additional 1 ml of SCM1
682 (35 mm Mat-Tek dishes). We tried even lower densities to improve the probability of getting one
683 cell per grid square; however, the neurons did not survive or did not differentiate well. Medium

684 was 50% changed every 2-3 days. On day 23, medium was fully changed to SCM2 medium
685 (Advanced DMEM/F12 (1:1): Neurobasal A (Gibco) (50:50) supplemented with 2 mM
686 GlutamaxTM), 2% B27, to 1.8 mM CaCl₂, 3 µM CHIR99021, 2 µM PD 0332991, 200 µM ascorbic
687 acid, 10 ng/ml BDNF) and 50% medium changes were subsequently performed every 2-3 days.
688 Cells were considered mature and ready for subsequent analyses and experiments at day 37.

689 **Mouse model neuronal culture and maintenance**

690 Established protocols were followed to set up cortical neurons collected from mouse E18
691 embryos ^{102,103,104}. Briefly, cortical tissues were extracted from E18 mouse embryos and
692 extensively rinsed in HBSS with 1% Penicillin-Streptomycin, followed by dissociation in 0.25%
693 trypsin with 1 mg/ml DNase I. Cortical neurons were isolated and plated with plating media
694 (Neurobasal with 10% FBS, 1xB27,1xGlutaMAX) onto either glass coverslips for immunostaining
695 or into 12 well plates for biochemistry at appropriate density. Both the cover glasses and plates
696 were pre-coated with poly-L-lysine (Invitrogen). Plating medium was replaced with a
697 maintenance medium (Neurobasal, 1xB27, 1xGlutaMAX) the following day. Only 2/3 of the media
698 was replaced every other day until the conclusion of the experiments.

699 **Mitochondria isolation**

700 Day 37 neurons were harvested using the Miltenyi MACS human mitochondria isolation kit
701 (Miltenyi Biotec 130-094-532) with additional proteases added to the lysis buffer aprotinin (10
702 µg/ml), leupeptin (10 µg/ml), PMSF (1 mM), EDTA-free protease inhibitor cocktail III (1x Sigma-
703 Aldrich - 539134) at a concentration of 10 million/ml of lysis buffer and disruption was performed
704 using an 27 G needle using a syringe to triturate 10 times up and down and then proceeding to
705 labeling following the manufacturer's recommended protocol. Final mitochondrial pellet was
706 resuspended in 50 µl of storage buffer and flash frozen in liquid nitrogen, stored at -80 °C until
707 mass spec analysis.

708 **RNA extraction & concentration**

709 RNA extraction was performed using QIAGEN RNEasy kit following manufacturer's protocol. RNA
710 was eluted in 50 μ l of nuclease free H₂O and required further concentration for cDNA synthesis.
711 RNA concentration was performed using ZYMO RNA Clean and ConcentratorTM-5 kit.

712 **cDNA synthesis**

713 100 ng of RNA was used for cDNA synthesis using the Quantabio qScript[®] cDNA SuperMix kit
714 while 25 ng of the RNA was used for RT-reaction of just RNA and nuclease-free water. cDNA
715 synthesis was performed using the manufacturer's protocol at 25 °C for 5 minutes, then 42 °C for
716 30 minutes, and finally 85 °C for 5 minutes in a Bio-Rad T100 thermal cycler.

717 **qRT-PCR**

718 One μ l of cDNA at a concentration of 5 ng/ μ l was used per reaction, with 10 μ M primers for
719 mouse *Pias1* and *Eif4a2* for a housekeeping control using previously published primers⁶⁵.
720 Quantification was performed on a Quantstudio 5 using SYBR green reagent (Biorad) running
721 delta delta CT method and calculating fold change normalizing to non-transgenic primary
722 neurons control SMARTPool siRNA. Prism 9.0 was used to calculate statistical significance by two
723 way ANOVA.

724 **Western blot**

725 Protein was harvested from frozen cell pellets (for CRISPR validation of the clone) using RIPA lysis
726 buffer or from isolated mitochondria samples described above. Samples were subjected to SDS
727 (sodium dodecyl sulfate) polyacrylamide gel electrophoresis and Western blotting onto
728 nitrocellulose. Membranes were assessed using either infrared fluorescence on the Li-Cor.
729 Antibodies were as follows: LC3ab (Cell Signaling #12741), PIAS1 (Cell Signaling #3550), CTIP2
730 (abcam #ab18465), ATPB (abcam #ab14730), normalization of loading was calculated based on
731 REVERT total loading stain.

732 **Immunofluorescence**

733 Immunofluorescence staining on day 37 neurons was performed as previously described⁴

734 **Pias1 knockdown in neurons**

735 Knockdown of *Pias1* was performed for mouse model neurons using Accell SMARTPool siRNA
736 against *Pias1* (Horizon Discovery cat#E-059344-00-0005) and a non-targeting SMARTPool control
737 (Horizon Discovery cat#D-001910-10-05) for comparison. Treatment was performed at 3 days *in*
738 *vitro* (DIV3) at a concentration of 1 μ M in 1 ml of medium per 35 mm MatTek dish, medium was
739 topped up to 2 ml after 24 hours and then 2/3 of the media was replaced every other day until
740 the conclusion of the experiments.

741 **CRISPR-Cas9 heterozygous knockout of PIAS1 in iPSC**

742 Clones were selected from stem cell edited pools in a method that was previously described⁶⁵

743 **Grid preparation for cryoET of HD patient iPSC-derived neurons and mouse model neurons**

744 For iPSCs, Quantifoil®I R 2/2 Micromachined Holey Carbon grid: 200 mesh gold (SPI supplies
745 Cat#:4420G-XA) grids were prepared for cell plating by sterilizing using forceps to carefully
746 submerge them in 100% ethanol (Fisher Scientific) at an angle perpendicular to the liquid surface
747 and then passed quickly through a yellow flame. Grids were then placed into 1 ml of poly-D-lysine
748 (100 μ g/ml in borate buffer, pH 8.4) in a 35 mm Mat-Tek glass coverslip bottom dish (VWR P35G-
749 0-14-C) at an angle perpendicular to the liquid's surface, and coated on the bottom of the dish
750 for at least 1 hour at room temperature. When cells were ready for plating, the grids were
751 washed two times with sterile-filtered Milli-Q H₂O and a final wash with PBS before cell plating.

752 For mouse neurons, EM grids were briefly dipped into 70% ETOH to sterilize, followed by coating
753 with 0.1mg/ml PDL¹⁰⁵. The grids were rinsed with sterile dH₂O and loaded with isolated neurons.
754 The maintenance of these grids were exactly as described above. DIV14 neurons were used for
755 cryoEM/cryoET experiments .

756 For both iPSC-derived and mouse model neurons, cells grown on grids were vitrified using the
757 temperature- and humidity-controlled Leica GP2. Grids were retrieved from culture dishes using
758 the forceps for the Leica device, and 3 μ l of 15 nm BSA gold tracer (Fisher Scientific; Catalog

759 No.50-248-07) was pipetted onto the carbon- and cell-side of the grid. Grids were loaded and
760 blotted for 5 seconds in 95% humidity at 37 °C and immediately plunged into liquid propane. The
761 vitrified grids were transferred into grid storage boxes in clean liquid nitrogen and then stored in
762 clean liquid nitrogen prior to cryoET imaging.

763 Cells on the remainder of the dishes that were used for cryoET, were scraped using a cell scraper
764 in cold PBS and pelleted at 350 xg for 5 minutes, PBS was aspirated and the pellets were flash-
765 frozen in liquid nitrogen and stored at -80 °C for later analysis of knockdown.

766 **CryoEM/ET data collection**

767 For each specimen, namely vitrified HD patient iPSC-derived and mouse primary neurons, we
768 collected low magnification (6500X) cryogenic TEM images and assembled them into montages
769 to screen for regions of interest (ROI) before cryoET tilt series collection. All images were acquired
770 using a G3 Titan Krios™ cryo-electron microscope (ThermoFisher Scientific) operated at 300 kV,
771 energy filter at 30 eV, in low-dose mode using SerialEM software¹⁰⁶. At each tilt angle, we
772 recorded “movies” with 5-6 frames per movie using a Gatan K2™ or K3™ direct electron
773 detection camera with a BioQuantum™ Imaging Filter (Gatan, Inc). The tilt series were collected
774 at higher magnification (39000X, 3.47 Å/pixel sampling size) using a dose-symmetric tilting
775 scheme¹⁰⁷ from 0°, target defocus of -5 μm and a cumulative dose of ~120 e/Å².

776 **Tomographic reconstruction**

777 Upon data collection, all tilt series were automatically transferred to our computing and storage
778 data clusters and images were motion-corrected using MotionCor2¹⁰⁸ and reconstructed into full
779 tomograms automatically using EMAN2¹⁰⁹. This on-the-fly reconstruction facilitated the
780 screening of tomograms.

781 After screening the automated cryoET reconstructions, we used IMOD¹¹⁰ software for standard
782 weighted-back projection tomographic reconstruction of tomograms with interesting and
783 relevant features, using coarse cross-correlation-based alignment, gold fiducial-based
784 alignment, or patch tracking alignment. For each tilt series, unsuitable images with large drift,

785 excessive ice contamination, etc. were manually removed prior to tilt series alignment. For
786 cryoET tilt series containing prominent sheet aggregates and subjected to Fourier analysis and
787 subtomogram averaging attempts to determine whether they contained repeating features, we
788 corrected for the contrast transfer function (CTF) using IMOD's recent 3D-CTF correction
789 algorithm and reconstructed using a SIRT-like filter (16 iterations)(**Fig. 4** and **Supplementary Fig.**
790 **3**).

791 **Tomographic annotation and segmentation**

792 The tomograms containing mitochondrial granules were post-processed binning by 4x and
793 applying various filters, such as a low-pass Gaussian filter at a frequency=0.01, a Gaussian high-
794 pass filter to dampen the first 1-5 Fourier pixels, normalization, and thresholding at 3 standard
795 deviations away from the mean. For both types of tomograms (containing mitochondrial granules
796 or sheet aggregates), we carried out tomographic annotation of different features in binned-by-
797 4 tomograms using the EMAN2 semi-automated 2D neural network-based annotation⁴², and
798 performed manual clean-up of false positives in UCSF Chimera¹¹¹. The cleaned-up annotations
799 were thresholded, low-pass filtered, and turned into binary masks, which were multiplied by
800 contrast-reversed versions of the tomograms to segment out each corresponding feature.

801 **Visualization of tomograms containing sheet aggregates**

802 Initially, the sheet aggregates appeared filamentous in 2D z-slices through our cryoET
803 tomograms. When attempts at subtomogram averaging failed to yield averages with filamentous
804 morphology, and instead resulted in featureless sheets, we more carefully examined the
805 tomograms slice-by-slice to understand the causes for this apparent failure. This revealed that
806 the linear features in a section persisted through multiple sections above or below. While this
807 might be expected due to lower resolvability in z because of the missing wedge, we also noticed
808 that the linear features shifted their location from section to section (as if drifting sideways), and
809 in different directions. This anomaly suggested that the linear features seen in 2D slices
810 corresponded to sheets in 3D, oriented at various angles with respect to the x-y plane, and

811 explained the preliminary exploratory subtomogram averages that had also revealed a sheet
812 morphology.

813 The sheets seem to be composed of extremely electron dense materials yielding high contrast in
814 cryoEM/ET images, and are exceedingly thin (~20 Å, a bit more or less in some regions), as
815 measured from 3D-CTF corrected tomograms without any downsampling or low-pass filtration.

816 **Quantification and statistical analysis of mitochondria granules size.**

817
818 We developed a two-stage deep learning system for voxel level annotation of mitochondria and
819 granules in tomograms. In the first stage, our system is trained on a handful of annotated slices
820 on a subset of tomograms and learns to segment mitochondria and granules. In the second stage,
821 we use our model to make predictions on all the tomograms and use high confidence predictions
822 as pseudo-annotations to augment our training set. We then train a new model on this
823 augmented training set and use its predictions to quantify the number and sizes of mitochondria
824 and granules in the tomograms.

825 In the first stage, we train a 3D-UNet¹¹² model to perform segmentation of the 3D volumes
826 containing objects of interest. We train two separate models, one for segmenting mitochondria
827 and the other for segmenting granules. These models are trained in a semi-supervised fashion on
828 sparsely annotated 3D volumes - only 2% of the 2D slices are manually annotated with each pixel
829 being labeled as being part of the background or being part of the mitochondria / granule.

830 Since annotating each slice is a time-consuming process, we utilize pseudo-labelling to generate
831 more annotations. In the second stage, we run every tomogram through our model and add the
832 high confidence predictions to the training set. Next, we retrain our model on the expanded
833 training set which consists of both human- and machine (pseudo)-labeled slices. The new model
834 is then used to make the final voxel level predictions on all the tomograms.

835 We refine the 3D segmentations in the post-processing stage. We run a connected components
836 analysis to count the number of segmented objects. We filter out background noise in the
837 predictions by discarding objects which don't fall within the expected range of volumes. We

838 retain granule detections which are located within a detected mitochondrion. After post-
839 processing, we scale each voxel by a factor of 21.02 to get the volume in nm³.

840 Compilation of the data into graphs was performed in Prism 9.3
841 (<https://www.graphpad.com/scientific-software/prism/>) with statistics performed based on the
842 number of groups to compare and data normality. iPSC-derived neurons used Dunn's multiple
843 comparisons to compare control to the various HD lines, for the mouse primary neurons, Dunn's
844 multiple comparisons were used to compare WT vs all other groups and then BACHD vs dN17
845 and Control siRNA vs *Pias1* siRNA treatment.

846 **Sample preparation for proteomic analysis**

847 Isolated mitochondria were solubilized in a final concentration of 1% SDS and mitochondrial
848 proteome was extracted using methanol-chloroform precipitation. 400 µl methanol, 100 µl
849 chloroform and 350 µl water were added sequentially to each 100 µl sample, followed by
850 centrifugation at 14,000 x g for 5 min at room temperature. The top phase was removed and the
851 protein interphase was precipitated by addition of 400 µl methanol, followed by centrifugation
852 at 14,000 g for 5 min at room temperature. Pellet was air dried and resuspended in 8M urea, 25
853 mM ammonium bicarbonate (pH 7.5). Protein concentration was determined by BCA (Thermo
854 Fisher) and 2-4 µg total protein were subjected to reduction and alkylation by incubation with 5
855 mM DTT for 1 h at room temperature followed by 5 mM iodoacetamide for 45 min at room
856 temperature, in the dark. The samples were then incubated with 1:50 enzyme to protein ratio of
857 sequencing-grade trypsin (Promega) overnight at 37 °C. Peptides were acidified with
858 trifluoroacetic acid to a final concentration of 1%, desalted with µC18 Ziptips (Millipore Sigma),
859 dried and resuspended in 10 µL 0.1% formic acid in water.

860 **LC-MS/MS acquisition**

861 LC-MS/MS analyses were conducted using a QExactive Plus Orbitrap (QE) mass spectrometer
862 (Thermo Fisher) coupled online to a nanoAcquity UPLC system (Waters Corporation) through an
863 EASY-Spray nanoESI ion source (Thermo Fisher). Peptides were loaded onto an EASY-Spray

864 column (75 μ m x 15 cm column packed with 3 μ m, 100 \AA PepMap C18 resin) at 2% B (0.1% formic
865 acid in acetonitrile) for 20 min at a flow rate of 600nL/min. Peptides were separated at 400 nL/min
866 using a gradient from 2% to 25% B over 48 min (QE) followed by a second gradient from 25% to
867 37% B over 8 minutes and then a column wash at 75% B and reequilibration at 2% B. Precursor
868 scans were acquired in the Orbitrap analyzer (350-1500 m/z, resolution: 70,000@200 m/z, AGC
869 target: 3x10⁶). The top 10 most intense, doubly charged or higher ions were isolated (4 m/z
870 isolation window), subjected to high-energy collisional dissociation (25 NCE), and the product
871 ions measured in the Orbitrap analyzer (17,500@200 m/z, AGC target: 5e4).

872 **Mass spectrometry data processing**

873 Raw MS data were processed using MaxQuant version 1.6.7.0 (Cox and Mann, 2008). MS/MS
874 spectra searches were performed using the Andromeda search engine¹¹³ against the forward and
875 reverse human and mouse Uniprot databases (downloaded August 28, 2017 and November 25,
876 2020, respectively). Cysteine carbamidomethylation was chosen as fixed modification and
877 methionine oxidation and N-terminal acetylation as variable modifications. Parent peptides and
878 fragment ions were searched with maximal mass deviation of 6 and 20 ppm, respectively. Mass
879 recalibration was performed with a window of 20 ppm. Maximum allowed false discovery rate
880 (FDR) was <0.01 at both the peptide and protein levels, based on a standard target-decoy
881 database approach. The “calculate peak properties” and “match between runs” options were
882 enabled.

883 All statistical t tests were performed with Perseus version 1.6.7.0 using either ProteinGroups or
884 Peptides output tables from MaxQuant. Potential contaminants, proteins identified in the
885 reverse dataset and proteins only identified by site were filtered out. Intensity-based absolute
886 quantification (iBAQ) was used to estimate absolute protein abundance. Two-sided Student's *t*-
887 test with a permutation-based FDR of 0.01 and S0 of 0.1 with 250 randomizations was used to
888 determine statistically significant differences between grouped replicates. Categorical
889 annotation was based on Gene Ontology Biological Process (GOBP), Molecular Function (GOMF)
890 and Cellular Component (GOCC), as well as protein complex assembly by CORUM.

891 Additional analysis was performed on all potential identities of the differentially
892 enriched/depleted proteins that were significant by t-test, using Panther pathways and Panther
893 Overrepresentation algorithms for GO Molecular Function, GO Biological Processes and GO
894 Cellular Component at <http://www.pantherdb.org/>. Ingenuity Pathway Analysis was performed
895 using the significantly differential proteins to assess pathways, networks and upstream
896 regulators. For comparison of DEPs to PIAS1 knockdown DEGs, overlap statistics for
897 overrepresentation was performed at http://nemates.org/MA/progs/overlap_stats.html using
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899

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910

911 **AUTHOR CONTRIBUTIONS**

912

913 G-H.W., C.S-G., J.G.G-M., P.M., L.M.J., M.F.S, C.W., W.M., J.F., L.M.T., & W.C. were involved in
914 conception and design of the experiments related to differentiation and cryo electron
915 tomography.

916 G-H.W., J.G.G-M., M.F.S., &W.C. analyzed cryo-ET data.

917 C.S-G., optimized iPSC cell growth on EM grids, and performed all iPSC differentiations and cell
918 culture.

919 Y.G., performed all mouse model neuronal cultures.

920 G-H.W., L.M.J., & P.M. optimized cryoET grid preparation and screening and collected all cryoET
921 data.
922 J.G.G-M. & G-H.W. performed tilt series alignment and tomographic reconstruction.
923 J.G.G-M., G-H.W., and C.D. performed tomographic annotation.
924 J.G.G-M., G-H.W., & M.F.S. participated in cryoET data visualization.
925 C.S-G, R.A., J.F., and L.M.T. were involved in conception and design of the experiments for the
926 mitochondria mass spectrometry.
927 C.S-G, R.A., N.R.G., and L.M.T. were involved in the acquisition, the validation, the analysis and
928 interpretation of the mitochondrial mass spectrometry data.
929 C.S-G., Y.G., R.M., and K.Q.W. were involved in the differentiation and cell culture of the neurons
930 and validation of PIAS1 knockdown.
931 S.G., J.H. & S.Y were involved in creating an artificial intelligence-based algorithm for automated
932 segmentation of features in cryoET tomograms and quantitative analyses of mitochondrial
933 granules.
934 G-H.W., J.G.G-M., & C.D. were involved in extensive manual reference labeling to train artificial
935 intelligence algorithms for segmentation and quantification of features in cryoET tomograms.
936 G-H.W, C.S-G, J.G.G-M., R.A., C.W., S.G., S.Y., L.M.T. & W.C, wrote the manuscript.
937 G-H.W, C.S-G, J.G.G-M., M.F.S., L.M.T. & W.C., substantively revised the manuscript with input
938 from all authors.

939

940

941 **DATA AVAILABILITY**

942 We will deposit representative tomograms for each phenotype in EMDB
943 (<https://www.emdataresource.org/deposit.html>); and in Chorus, for proteomics.

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